AN ABSTRACT OF THE THESIS OF

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Title: Genetic Analysis of Catechol Siderophore Production by *Erwinia carotovora*

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Twenty-two strains of *Erwinia carotovora* produced catechol(s) and provided a functional siderophore to enterobactin mutants of *Escherichia coli* and *Salmonella typhimurium*. As levels of available iron decreased in media, the amount of catechol produced by *E. carotovora* W3C105 increased. A 4.3-kb genomic region from W3C105, designated *entB*$^+$, complemented *E. coli* AN192 (*entB*). A second distinct 12.8-kb region, designated *ent(DC)EA*$^+$, complemented *E. coli* strains AN90 (*entD*), MT147 (*entC147*), AN93 (*entE*), and AN193 (*entA*). Although the *entB*$^+$ and *ent(DC)EA*$^+$ regions from W3C105 were functionally indistinguishable from their counterparts in *E. coli*, only the *entE*$^+$ gene of *E. coli* hybridized to the catechol biosynthesis genes of W3C105. Catechol biosynthesis genes from W3C105 hybridized to genomic DNA of 21 diverse field strains of *E. carotovora*, but did not hybridize to the genomic DNA of *Erwinia chrysanthemi* PMV3937. Catechol mutants (Cat$^-$), aerobactin production (Iuc$^-$) and production and uptake mutants (Iuc$^-$ Iut$^-$), and mutants that did not produce any siderophore (Cat$^-$Iuc$^-$ or Cat$^-$Iuc$^-$Iut$^-$) of W3C105 (Cat$^+$Iuc$^+$Iut$^+$) were generated. Cat$^-$Iuc$^-$ strains grew only at relatively high levels of available iron. Cat$^+$Iuc$^-$ grew at slightly lower levels of available iron than did Cat$^-$Iuc$^-$ strains, thus demonstrating the role of catechol production in iron acquisition by W3C105. The Iuc$^+$ strains grew at the lowest levels of available iron tested, regardless of catechol production. Strains of *E. carotovora* and *E. coli* provided a functional siderophore to the Cat$^-$Iuc$^-$ mutants, whereas *E. chrysanthemi*

did not provide a siderophore to the mutants. Potato tubers inoculated with Cat^+Iuc^+, Cat^+Iuc^-, Cat^-Iuc^+, or Cat^-Iuc^- were indistinguishable with respect to weight of soft rot produced. This research demonstrated that siderophores are not pathogenicity factors in tuber soft rot or aerial stem rot of potato caused by *E. carotovora*. Siderophores play a role, however in virulence in relation to aerial stem rot but the relative roles of the siderophores have not been determined.

Genetic Analysis of Catechol Siderophore Production by *Erwinia carotovora*

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DEDICATION

I dedicate this dissertation to Ms. Aria Dawn Freedline, Mr. David Jay Freedline and Ms. Kristi Lynn Freedline, so that they will remember that they are what our family may become. They are our future.

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PREFACE

"There are enough examples where the introduction of desirable organisms into the rhizosphere has increased plant production to be optimistic that these successes can be extended. Nevertheless, we must recognize that the extreme complexity of plant root-microbe interactions requires the development of multi-disciplinary research and a sound understanding of the interactions between the biology of the system and physical and chemical properties of soil."

A. D. Rovira (1985)

GENETIC ANALYSIS OF CATECHOL SIDEROPHORE PRODUCTION BY *ERWINIA CAROTOVORA*.

INTRODUCTION

Erwinia carotovora Jones is a phytopathogenic bacterium that causes soft rot diseases of potato and other vegetable and ornamental crops (Pérombelon and Kelman, 1980). *Erwinia carotovora* is a member of the pectolytic group (carotovora group or soft-rot group) of the genus *Erwinia*, which is characterized by strong pectolytic activities (Dye, 1969a, Lelliot, 1974). Pectolytic strains of *Erwinia* produce a plethora of pectic enzymes including pectic lyases (PL), pectin lyase (PNL), exopolygalacturonate lyases (exoPL), polygalacturonase (PG), and exo-poly-α-D galacturonasidase (exoPG), which result in cleavage of α, 1,4-linkages of pectic glycosidic polymers. Breakdown of the pectic polymers results in the loss of structural integrity of the host tissue due to maceration of parenchymatous tissue (Collmer and Keen, 1986).

Erwinia carotovora causes damage to potatoes at economically-significant levels. An estimated 50-100 million dollars in potato crop loss is attributed world wide due to diseases caused by *E. carotovora* (Pérombelon and Kelman, 1980). Losses in the United States alone are estimated at 14 million dollars (Kennedy and Alcorn, 1980). In addition, *E. carotovora* is rated second among 43 bacterial pathogens with respect to significance and economic loss in the US (Kennedy and Alcorn, 1980).

A factor contributing to the economic significance of *E. carotovora* is the importance of potatoes (*Solanum tuberosum* L.) as a food crop world wide. The

Columbia Basin in the Pcific Northwest has the longest growing season in the US (~ 150 days) and the largest yields in North America (45 Tons/acre have been reported) (IPMI Bulletin). The western United States produces two thirds of the potatoes grown in the US on half of the total potato acreage (IPMI Bulletin).

Classification of pectolytic strains of *Erwinia.*

Soft rot was first described to be caused by *Bacterium carotovorum* on carrot (Jones, 1900). At that time, all members of the Enterobacteriaceae were placed in the genus *Bacterium.* Later, plant pathogens were moved from this genus solely on the basis of pathogenicity. In 1917, the genus *Erwinia* was constructed for phytopathogenic, Gram-negative rods that have peratrichous flagellae and do not form spores (Winslow *et al.*, 1917). These phytobacteria were named for the esteemed American phytobacteriologist Erwin Frink Smith, who some consider to be the father of phytobacteriology (Dowson, 1949). The plant pathogens were given species status based on the host from which they were isolated. This classification was confusing due to the lack of host specificity as well as the wide range of phenotypic characteristics represented by species of *Erwinia* (Brenner, 1981; Starr, 1983).

Due to the heterogeneity within the genus, *Erwinia* strains are grouped into four types: the amylovora group (true erwininiae, *Erwinia sensu stricto*) (Dye, 1968a), herbicola group (yellows) (Dye, 1969b), carotovora group (pectolytic group or soft-rot erwinia) (Dye, 1969a) and an atypical *Erwinia* group (Dye, 1969c). The pectolytic *Erwinia* are different from the other groups of *Erwinia* based on phenotypic, ecological and genetic evaluations (Waldee, 1945; Brenner *et al.*, 1973). It has been proposed that the pectolytic group be placed into a separate genus *Pectobacterium* (Waldee, 1945; Brenner *et al.*, 1973), although this taxon has

not been widely accepted by plant pathologists or bacteriologists (Starr, 1983; Graham, 1972).

The pectolytic *Erwinia* are divided into two species, *Erwinia carotovora* and *Erwinia chrysanthemi*, based on biochemical characteristics and DNA/DNA hybridization studies (Brenner, *et al.*, 1973; Graham, 1972; Dickey, 1979). *Erwinia carotovora* is further split into the intraspecific designations, *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *Erwinia carotovora* subsp. *betavasculorum*, based on DNA/DNA relatedness studies (Brenner *et al.*, 1973; Thomson *et al.*, 1977) and phenotypic characteristics such as optimal temperatures for growth, host range and bacteriological characteristics (Cowan *et al.*, 1974; Dye, 1969a; 1969b; Thomson *et al.*, 1977).

Diseases of potato caused by pectolytic *Erwinia*.

Disease of field-grown plants and postharvest decay of tubers are caused by pectolytic *Erwinia* spp.. The general characteristic of diseases caused by pectolytic *Erwinia* is the maceration of parenchymatous tissue. Symptoms vary with environmental factors, the specific pathogen(s), cultivar, and host tissue infected, (Pérombelon and Kelman, 1980; 1978; Sivasithamparam, 1982). Five field diseases and two postharvest problems are now recognized to be caused by pectolytic *Erwinia* spp. (TABLE I-1.) (Pérombelon and Kelman, 1987).

Symptoms resulting from infections of the seed piece include non-emergence, blackleg and stolon end rot. Non-emergence or blanking is the result of decay that causes below-ground death of the sprout (Cother, 1979; Lund, 1979; Pérombelon and Kelman, 1980; 1987). Blackleg is a disease problem wherever tubers are grown (Powelson and Apple, 1984). The dark brown basal stem rot of blackleg can result in wilting leaves and or plant desiccation. Stolon end rot sometimes follows

TABLE I-1. Diseases caused by pectolytic strains of *Erwinia*

Field Diseases	Storage and Transit Diseases
Non-emergence (blanking or missing hills): below ground death of sprout.	Tuber Soft Rot: soft rot of tubers after harvest.
Blackleg: dark brown basal stem rot can result in wilting leaves or plant desiccation.	Lenticular Hard Rot: hard rot developing when soft rot is arrested at an early stage.
Stolon End Rot: central tuber decay of progeny tubers from plants with blackleg symptoms.	
Aerial Stem Rot: black-tan lesion forming on stems.	

From Pérombelon and Kelman, 1987.

blackleg symptoms when progeny tubers from blackleg plants have central tuber decay. The source of inoculum is often the seed tuber (Pérombelon and Kelman, 1987).

Aerial stem rot and soft rot of progeny tubers have sites of initial infection other than the seed piece, however a decaying seed piece may be the source of inoculum for these diseases. Aerial stem rot results from infection of the exposed stem, which forms a black to tan lesion. Progeny tubers may become infected by non-plant derived inoculum, such as that present in irrigation water or soil (Pérombelon and Kelman, 1987).

Significant yield reduction in potatoes results from rotting of tubers in storage or in transit (Pérombelon and Kelman, 1980). Tuber soft rot or lenticular hard rot results from tubers contaminated in the field or during processing and storage (Logan, 1964; Campos *et al.*, 1982). Lenticular hard rot will form after infection when disease has been arrested at an early stage, such as when tubers are moved to an environment nonconducive to soft rot (Logan, 1964). Though rotting of the seed piece or daughter tubers in the field can occur, the term soft rot is restricted to the rot of daughter tubers in storage and transit (Pérombelon and Kelman, 1987).

All of the soft rot erwiniae can infect potato, although only *E. chrysanthemi*, *E. c.* subsp. *atroseptica* and *E. c.* subsp. *carotovora* are generally associated with potatoes (Pérombelon and Kelman, 1980; 1987). *Erwinia carotovora* subsp. *betavasculorum*, the sugarbeet pathogen, causes typical blackleg symptoms when stems are inoculated in greenhouse conditions. *Erwinia carotovora* subsp. *betavasculorum* rarely is isolated from blackleg plants in potato fields (Thomson *et al.*, 1977).

Symptoms caused by different potato-associated pectolytic *Erwinia* are

indistinguishable under certain environmental conditions. For example, blackleg of potatoes is a soft rot disease characterized by the rotting of the basal portion of the stem. Historically, *E. c.* subsp. *atroseptica* was described as the blackleg pathogen and was thought to be the only causal agent of this disease (Pérombelon and Kelman, 1980; 1987). Subsequently, *E. c.* subsp. *carotovora* and *E. chrysanthemi* also have been shown to cause blackleg under favorable environmental conditions (Powelson, 1980; De Lindo *et al.*, 1978; Lumb *et al.*, 1986). Temperature is the most critical factor determining which organism will be responsible for a given disease manifestation in a given region. This is probably directly related to the temperature optima and maxima for growth of the different soft-rot *Erwinia* (Pérombelon and Kelman, 1980).

Control measures for diseases of potatoes caused by pectolytic *Erwinia.*

Control measures for diseases caused by soft rot erwinias are presently limited to sanitation and cultural practices. The use of certified seed is a common practice to avoid seed piece contamination (Graham, 1962; Pérombelon, 1973). Resistance also has been employed although complete resistance has not been found. Several potato cultivars, such as the popular Russet Burbank, show moderate resistance to the soft rot pathogens (IPMI Bulletin; Hooker, 1986).

Cultural practices for soft rot control include optimizing the timing and environmental conditions of planting. For example, warming tubers before cutting and planting in well drained areas of the field aids in the rapid suberization of the seed piece, which enhances resistance. To reduce the spread of the bacteria by insect vectors, insecticides are recommended. In storage, warehouse disinfestation and maintenance of dry cool (4 C) conditions reduce decay. Removal of diseased tubers prior to storage also is recommended (IPMI Bulletin, 1986; Hooker, 1986).

Certified seed may reduce the incidence of blackleg, although other important sources of inoculum often are present. Populations of *Erwinia* in fields in the spring after winter fallow generally are small and not detectable with standard techniques. Nevertheless, non-detectable populations of *E. carotovora* may be important sources of inoculum for field infections. Enrichment with pectate and fluorescent antibody staining techniques, however, are useful for detection of *E. c.* subsp. *carotovora* in such fields (De Boer and Kelman, 1979). Using enrichment techniques, *Erwinia* strains can be isolated from soil, irrigation water, river water and weeds (McCarter-Zorner *et al.*, 1982; Powelson and Apple, 1984; De Boer and Kelman, 1979). Strains of *Erwinia* detected in soils prior to planting may differ serologically from those contaminating potato tubers. Strains isolated from aerial stem rot lesions have the same serotypes as soil isolates, indicating that the soil is an important source of inoculum for aerial soft rot (Powelson and Apple, 1984). Thus, inoculum from sources other than tubers are important in disease development and must be considered in disease control practices.

An alternative method of control that can protect seeds and daughter tubers from infection by soilborne *Erwinia* would complement certified seed programs and other cultural practices currently used to control this pathogen. Non-traditional control methods must therefore be evaluated for this system. One potential method is biological control.

Biological control of *Erwinia carotovora.*

Biological control of soilborne pathogens involves the introduction of microbes or manipulation of indigenous microflora to control disease. Bacterization of planting material with fluorescent pseudomonads provides protection from some diseases. Fluorescent pseudomonads have potential as biological control agents because they are soil and rhizosphere colonists (Burr and Ceasar, 1984; Loper and

Schroth, 1986; Suslow, 1982; Weller, 1988) and produce a wide variety of secondary metabolites (Fravel, 1988; Défago and Haas, 1990; Gutterson, 1990).

The application of control agents to planting material or roots places the agents at the infection court where protection can occur.

Numerous reports describe the use of biological agents to improve yield and control diseases of potato (TABLE I-2). Many biological agents are selected for their antibiosis against *E. carotovora* in culture. The use of these biological control agents to control diseases caused by *E. carotovora* in the field is being explored.

Biological control organisms often are isolated from the location where they will be used; such agents presumably are well adapted to the environment in which they eventually must operate. Potential antagonists are screened for antagonism against a target pathogen(s) in culture and for disease control in greenhouse experiments and eventually in the field. There is little correlation between the capacity to inhibit a pathogen in culture and the capacity to control the pathogen in the field (Kommendahl and Windels, 1986; Burr *et al.*, 1978; Cook and Baker, 1983; Weller, 1988). Because screening all potential antagonists in field trials is unwieldy, most researchers select antagonists in greenhouse studies or by other secondary screening procedures.

Treatment of seed pieces with fluorescent pseudomonads reduces disease caused by *E. carotovora* as assessed by several criteria: blackleg symptoms, percent emergence, plant growth, soft rot potential of daughter tubers, and tuber size and weight. The application of fluorescent pseudomonads to potato seed pieces increases yield and plant growth, in part by reducing populations of *E. carotovora* (Kloepper, 1983; Xu and Gross, 1986a; Rhodes and Logan, 1986). Because population size of *E. carotovora* is correlated to disease incidence (Aleck and

TABLE I-2. Reports of application of biological agents to potatoes to improve plant growth, increase yield, and control diseases

Biological agent(s) applied	Criterion for strain selection	Response tested	Citation
Fluorescent pseudomonads strains BK-1, TL-3, TL-10, *Bacillus* spp. S-1-B, *E. quercina* Ac-1	Antibiosis against *E. carotovora*	Plant growth, yield	Burr *et al.*, 1978
Fluorescent pseudomonads B10, E6, A1, BK1, T13B2	Antibiosis against *E. carotovora*	Plant growth, yield, antagonist colonization	Kloepper *et al.*, 1980a
Fluorescent pseudomonads and siderophore isolated from antagonist	Antibiosis against *E. carotovora*	Plant growth, yield, effect on rhizosphere microflora	Kloepper *et al.*, 1980b
Fluorescent pseudomonads	Antibiosis against 40 bacterial isolates including *E. c.* subsp. *carotovora*	Bacterial and fungal populations, plant growth	Kloepper and Schroth, 1981a
Fluorescent pseudomonads	Isolated from potato fields for antibiosis against *E. c.* subsp. *carotovora*	Plant weight, yield, field variability	Howie and Echandi, 1983
Pseudomonas fluorescens, *P. s.* pv. *syringae*, *P. s.* pv. *glycinea*, *P. s.* pv. *lachramans*	Induced resistance against *P. solanacearum*	Disease severity	Kempe and Sequeira, 1983

TABLE I-2. (Continued)

Fluorescent pseudomonads	Antibiosis against *E. c.* subsp. *carotovora*	Populations of *E. carotovora*, % tubers naturally infested with *E. carotovora*	Kloepper, 1983
Fluorescent pseudomonads	Antibiosis against fungi and bacteria including *E. carotovora*	Plant weight, yield, # tubers	Geels and Schippers, 1983a
Fluorescent pseudomonads	Antibiosis against fungi and bacteria including *E. carotovora*	Plant weight, yield, # tubers	Geels and Schippers, 1983b
P. putida (M17)	Antibiosis against *E. carotovora* sp.	Soft rot potential of progeny tubers, post soft rot severity	Colyer and Mount, 1984
Fluorescent pseudomonads		Plant growth and yield	Vrany and Fiker, 1984
Fluorescent pseudomonads B10, I13	Antibiosis against *E. carotovora*	Blackleg severity, plant emergence, and growth, soft rot potential of progeny tubers, seed piece breakdown	Rhodes and Logan, 1986
Fluorescent pseudomonads	Antibiosis against *E. c.* subsp. *carotovora*	Plant emergence	Xu and Gross, 1986a

TABLE I-2. (Continued)

Fluorescent pseudomonads	Antibiosis against *E. c.* subsp. *carotovora*	Plant emergence, yield, and populations of *E. c.* subsp. *carotovora*	Xu and Gross, 1986b
P. fluorescens (M-4)	Antibiosis against *Verticillium dahliae*	Plant weight and yield and size of propagules of *V. dahliae*	Leben *et al.*, 1987
E. c. subsp. *betavasculorum*	*Antibiosis against* *E. c.* subsp. *carotovora*	Populations of *E. carotovora* in tuber infections	Axelrood *et al.*, 1988
Fluorescent pseudomonads	Antibiosis against *E. c.* subsp. *carotovora*	Populations of *E. carotovora*, plant yield	Gross, 1988

Harrison, 1978), reduction in population size of *E. carotovora* results in a reductionin disease incidence. Increases in yield correlate to reductions in pathogen populations (Kloepper, 1983; Kloepper *et al.*, 1980a; Kloepper and Schroth, 1981a). Populations of *Erwinia* spp. on seed tubers, roots and daughter tubers are reduced on field-grown plants when seed pieces are treated with biological control agents (Kloepper, 1983). Mixtures of fluorescent pseudomonads also delay seed piece decay (Rhodes and Logan, 1986). Fluorescent pseudomonads control blackleg caused by *E. c.* subsp. *atroseptica* (Rhodes and Logan, 1986). In one study, however, fluorescent pseudomonads decreased the population size of *E. carotovora* but did not reduce incidence of blackleg (Xu and Gross, 1986b).

Non-emergence, caused by infection of seed pieces with *E. carotovora*, is reduced by treating planting material with fluorescent pseudomonads (Xu and Gross, 1986a; Rhodes and Logan, 1986). In the greenhouse, emergence of plants from seed pieces treated with fluorescent pseudomonads is significantly greater than that from seed pieces inoculated only with *E. c.* subsp. *atroseptica* (Xu and Gross, 1986a). Biological control of non-emergence also is obtained in the field (Rhodes and Logan, 1986).

Bacterization of planting material with fluorescent pseudomonads reduces postharvest soft rot (Colyer and Mount, 1984; Rhodes and Logan 1986; Xu and Gross, 1986b). Soft rot potential is the proportion of lenticels per tuber that rot under favorable conditions (Lund and Kelman, 1977; De Boer and Kelman, 1975). In field trials, tubers from plants treated with *P. putida* strain W4P63 have lower soft rot potential than tubers from nontreated plants (Xu and Gross, 1986b). Treatment of planting material with other strains of rhizosphere bacteria also results in significantly fewer number of infections developing per tuber compared to non-treated tubers (Rhodes and Logan, 1986). Biological control of postharvest soft rot

also is achieved by bacterization of the tubers after harvest (Colyer and Mount, 1984).

The success of fluorescent pseudomonads in the biological control of *E. carotovora* is promising but biological control is not yet commercially feasible. One reason that biological control systems are not available for control of *E. carotovora* or many other pathogens is the lack of consistent control of disease in field plots. Regardless of the response measured, biological control of *E. carotovora* is inconsistent. Treatment of seed pieces with strains of *P. putida* increases yield of field grown potatoes in some but not all field trails (Burr *et al.*, 1978; Kloepper, 1983; Xu and Gross, 1986b). Biological control of blackleg (Rhodes and Logan, 1986), soft rot of daughter tubers (Rhodes and Logan, 1986), and non-emergence (Xu and Gross, 1986a; Rhodes and Logan, 1986) also have been inconsistent. Variability in biological control occurs from year to year in the same field sites. Biological control was achieved by fluorescent pseudomonads in 1984 but not in 1985 (Rhodes and Logan, 1986). The success of biological control also appears to be site dependent (Burr *et al.*, 1978; Rhodes and Logan, 1986). It has been suggested that there is a need to develop strains which are adapted to a known set of site-dependant factors (Rhodes and Logan, 1986).

Mechanisms of biological control.

In order to predict the success of a biological control agent in disease control, it is important to first understand by what mechanism biological control is operating. Detailed reviews of the hypothesized mechanisms and approaches to studying these mechanisms are available (Handelsman and Parke, 1989; Baker, 1968; Vidaver 1976; 1983; Moore and Warren, 1979; Leong, 1986; Blakemann and Fokkema, 1982). Originally, three primary mechanisms by which biological control is

achieved were proposed: antibiosis, parasitism, and competition (Baker, 1968). The present discussion is limited to these three mechanisms.

A. Antibiosis.

Jackson (1965) and other authors (Fravel, 1988; Baker, 1968) define antibiosis in a broad sense, to include antagonism mediated by metabolic agents produced by microorganisms that have deleterious effects on other organisms. Antibiotics and bacteriocins are the most well-studied metabolic products involved in antibiosis of plant pathogens. Phage, volatiles, and enzymes also have received attention. Although the latter metabolites are implicated in biological control, definitive studies demonstrating their importance have been lacking. Fravel (1988) states that developments in microbial genetics and recombinant DNA technology provide new methodologies for studying these mechanisms and will provide rigorous evidence for their importance in biological control.

1. Antibiotics.

Antibiotics mediate in part the activity of many biological control agents. Antibiotics are low molecular weight compounds that are produced by microbes and have deleterious effects on growth or metabolic activities of other microorganisms (Fravel, 1988). Many antibiotics are produced and purified from cultures of biological control agents. In addition, purified antibiotics such as streptomycin inhibit plant pathogens and are used as pesticides (PNW Plant Disease Control Handbook). Thus, microbially-produced antibiotics, if present in adequate concentrations on plant surfaces, control certain plant diseases.

In order to demonstrate that antibiotics are involved in biological control, demonstration of *in situ* production and consistent correlation between antibiotic production and susceptibility of the pathogen to control must be evaluated.

Antibiotics produced by biocontrol agents in rich culture media may not be produced in the soil or plant rhizospheres, where nutrients are limited. Although direct isolation of antibiotics from the soil has been difficult, two antibiotics involved in biological control, phenazine-1-carboxylate (Thomashow and Weller, 1988) and 2,4-diacetylphloroglucinol (Keel *et al.*, 1992) have been isolated from rhizosphere soil.

Comparison of antibiotic-biosynthesis mutants and parental strains for disease suppression provides evidence correlating antibiotic production and biocontrol activity. Well-characterized mutants that do not produce antibiotics are valuable tools for assessing the potential role of such compounds in biological control. For example, *P. putida* strain M17 controls soft rot of potato tubers and produces an antibiotic that is toxic to *E. carotovora* (Colyer and Mount, 1984). Strain M74, a mutant of strain M17 that does not produce an antibiotic, does not inhibit the growth of *E. carotovora* in culture and does not control soft rot of tubers. Mutagenesis by chemical treatment or ultra violet irradiation, although useful, may cause multiple and non-detectable mutations in a bacterial cell. Thus, transposon mutagenesis, which introduces single, marked insertions into a bacterial genome, often is used preferentially to evaluate the role of metabolites in biological control activity of bacterial antagonists (Kraus and Loper, 1992; Thomashow and Weller, 1990; Voisard *et al.*, 1989).

2. Bacteriocins.

Bacteriocins are antibiotics with bactericidal effects only on strains closely related to the producing bacterium (Vidaver, 1976; 1983). Strains of antagonistic bacteria that produce bacteriocins may be promising biocontrol agents for suppression of closely-related phytopathogens because related bacteria may occupy similar niches on plant surfaces. An antagonist that inhabits the infection court of a

target pathogen may be an optimal biological control agent (Cook and Baker, 1983). Although biological control of bacterial pathogens by closely-related antagonists that produce bacteriocins seems promising, examples of effective biological control operating by this mechanism are few. Nevertheless, *Agrobacterium radiobacter* strain K84, one of the most successful biological control agents and one of the few that is commercially-available, effects biocontrol in part through the production of a bacteriocin. Production of the bacteriocin agrocin 84 by *A. radiobacter* is responsible largely for the biological control of *A. tumefaciens* (Cooksey and Moore, 1982a; 1982b).

The production of bacteriocins has not been implicated as a mechanism mediating antagonism among strains of *E. carotovora*. Nevertheless, strains of *E. carotovora* produce bacteriocins that are toxic to other strains of the species (Crowley and De Boer, 1980). Bacteriocin-producing strains of *E. carotovora* are phytopathogens and are not useful in their natural state as biocontrol agents. The potential for using related phytopathogens as biological control agents is being examined for one system in which antibiotics are implicated as a mechanism by which antagonism is operating. Strains of *E. c.* subsp. *betavasculorum* produce antibiotics that inhibit the growth of *E. c.* subsp. *carotovora* in wounds of potato tubers (Axelrood *et al.*, 1989). *Erwinia carotovora* subsp. *betavasculorum* also causes tuber soft rot and is not useful as a biological control agent in its natural state. Non-pathogenic mutants of *E. c.* subsp. *betavasculorum*, which produce antibiotics, are being derived as potential biological control agents (José Costa, personal communication). Similar nonpathogenic mutants of bacteriocin-producing *E. carotovora* may be useful as biocontrol agents.

B. Parasitism.

Parasitism has been implicated in biological control by *Sporidiesmium*

sclerotivorum, *Gliocladium* spp., and *Trichoderma harzianum* and other biological control agents (Ayers and Adams, 1981; Baker, K., 1987; Chet, 1987; Cook and Baker, 1986; Papavizas, 1985). Mycoparasitism (parasitism of fungi) is one type of parasitism operative in biological control. Parasitism is the direct contact between organisms that results in the death of one organism and the utilization of nutrients by the other. Parasitism generally reduces the inoculum density of the phytopathogenic fungus. Genetic tools were used to determine the role of mycoparasitism in biological control activity of *Gliocladium virens*. A mutant of *G. virens* that no longer is mycoparasitic but continues to produce the antibiotic gliovirin is as efficient as the parental strain for control and destruction of *Rhizoctonia solani* (Howell, 1987). In addition, mutants that no longer produce gliovirin do not control seedling emergence diseases caused by *R. solani* (Howell and Stipanovic, 1983). Although these studies suggest that antibiotic production was more significant than mycoparasitism as the mechanism of biological control, these studies were the first using molecular genetics to study the role of mycoparasitism in biological control.

Parasitism is rarely implicated as a mechanism by which the control of bacterial diseases is achieved, although examples exist. *Bdellovibrio bacteriovorus* is a parasite of bacteria. Bacterial blight of soybean, caused by *Pseudomonas syringae pv. glycinea* is reduced by inoculation with *B. bacteriovorus* (Scherff, 1973). Parasitism has not been implicated in the biological control of *E. carotovora*.

C. Nutrient competition.

Nutrient competition results when a particular substance is limiting to the growth of one or more organisms. A nutrient may be unavailable and limiting to the growth of one organism while simultaneously being available to another organism that can utilize the limited supply of the nutrient. Biological control via

nutrient competition is hypothesized to operate because the biocontrol agent is better than the target pathogen at acquiring the limiting nutrient or serves as a nutrient sink, making the nutrient even more unavailable to the pathogen (Paulitz, 1990). In this way, growth and activity of the pathogen are limited but those of the control agent are not limited. Iron, carbon, oxygen, and many micronutrients may limit growth of a pathogen; the limiting component varies among soil, pathogen, and host systems (Cook and Baker, 1983; Handelsman and Parke, 1989; Paulitz, 1990).

Traditionally, a mutagenesis approach is not used to study nutrient competition because phenotypes that affect nutrient acquisition are mostly unidentified. In many cases, mutations for auxotrophy of a given nutrient generally are not meaningful for studying nutrient competition, due to complex regulation and the number of genes involved in amino acid biosynthesis. Other mutations affecting nutrient acquisition may be lethal. A general approach to studying nutrient competition is to add the nutrient that is thought to be limiting to the soil or plant surface. If biological control is not observed when the nutrient is no longer limiting, nutrient competition is considered a likely mechanism. Results from nutrient addition studies often can be misleading because addition of a nutrient affects all components of the microflora, not just the biological control agent and pathogen (Handelsman and Parke, 1989).

More sophisticated methods can be used to study competition for nutrients for which specific nutrient-scavenging molecules are known. Such is the case with competition for iron. Iron is an essential element to most microorganisms. Iron has two stable valences that are employed in oxidation-reduction reactions in energy transfer (Neilands, 1981b). Although iron is the fourth most abundant element in the earth's crust, it is not readily available to microorganisms. In oxygenated environments, iron is insoluble and limiting to microbial growth (Lindsay, 1979).

Siderophores are low molecular weight, virtually ferric-specific, iron-chelating

compounds that are produced by bacteria and other microorganisms under iron-limiting conditions (Neilands, 1981a; 1981b; 1982). When iron is limiting, siderophores are produced, are excreted into the environment, and act to transport iron into the producing organism. Specific outer membrane proteins are required for the uptake of the ferric-siderophore complex (Neilands, 1982). Siderophores sequester available iron from the environment, thus making iron limiting to microbes that can not utilize a prevalent ferric-siderophore (Neilands, 1982). Microbes lacking an outer-membrane receptor that recognizes the predominant ferric-siderophore complex in a habitat may be starved for iron. It is hypothesized that biological control agents and pathogens compete for limiting iron in the rhizosphere. Competition for iron is believed to be mediated by microbial siderophores (Loper and Buyer, 1991; Loper and Ishimaru, 1991; Buyer and Leong, 1986; Neilands, 1986). Microorganisms that do not produce siderophores use cofactors other than iron in the electron transport chain and therefore are not dependant on the presence of iron (Neilands, 1981b).

Siderophores differ greatly in their overall structure, thus allowing for specificity of the outer-membrane receptors (Leong, 1986). Nevertheless, the functional groups (ligands) that form a complex with the ferric ion do not vary to a great extent among bacteria. Siderophores that form complexes with the ferric ion through catechol residues, such as 2,3- dihydroxy benzoic acid (DHBA), are termed catechol siderophores (Neilands, 1981a). The most well studied catechol siderophore is enterobactin. Siderophores that form a complex with the ferric ion through hydroxamate groups are also common (Neilands, 1981). Aerobactin is a hydroxamate siderophore produced by enteric bacteria. Most hydroxamate siderophores, however, are produced by fungi. Genetics of aerobactin- and enterobactin-mediated iron uptake are well understood and serve as models for other siderophore-mediated iron uptake systems.

The fluorescent pseudomonads, including those that have been used as biological

control agents, produce siderophores, that are structurally distinct from catechol and hydroxamate siderophores. Pyoverdines (also called pseudobactins) are fluorescent siderophores produced by fluorescent pseudomonads. The first pseudobactin to be characterized was that produced by *P. fluorescens* B10. This siderophore consists of a hexa-peptide bound to hydroxyquinoline, hydroxy- carboxylate and hydroxamate moieties, which act as ligands (Teintz *et al.*, 1981). Pseudobactins produced by many other fluorescent pseudomonads have been characterized (Buyer and Leong, 1986; Yang and Leong, 1984; Abdallah, 1991). Number and configuration of amino acids within the peptide chain varies among the pseudobactins. Variability in peptide composition allows for specificity of receptors for these molecules (Neilands and Leong, 1986).

The ability of an antagonist to produce a siderophore is not proof that iron competition mediated through siderophores is the mechanism by which biological control occurs. Handelsman and Parke (1989) suggest that several criteria must be met in order to show that a particular mechanism is responsible for biological control. Here, they are summarized into two criteria: 1) There must be a strict correlation between production of molecules or activities needed for the proposed mechanism and control of the target pathogen; 2) There must be strict correlation between sensitivity to the mechanism (or molecules) by the pathogen and sensitivity to biological control.

Research dealing with iron competition has shown that there is a correlation between the production of siderophores by biological control agents and biological control in some systems, thus meeting the first criterion. When iron is not limiting, siderophores are not produced and biological control does not occur. For example, under iron-limiting conditions, *P. fluorescens* B10 or purified pseudobactin inhibits *E. carotovora* (Kloepper *et al.*, 1980a, 1980b). Also, the addition of iron to media

reverses the inhibition of *E. carotovora* by *P. fluorescens* B10 (Kloepper *et al.*, 1980a, 1980b).

In greenhouse studies, siderophore production by fluorescent pseudomonads is correlated to the control of non-emergence of potato caused by *E. carotovora*. Fluorescent pseudomonads producing siderophores with high iron-acquisition activity are better control agents than are those with moderate or low activity (Xu and Gross, 1986a). These examples indicate that iron competition mediated through siderophore production is involved in biological control of *E. carotovora*.

Genetic approaches provide another means to study the role of siderophores in biological control. Bacterization of cotton seeds with *P. fluorescens* strain 3551 results in decreased colonization of cotton seed by *Pythium ultimum* and increased seedling emergence over nontreated seeds. Nonfluorescent mutants of strain 3551 do not increase seedling emergence by *P. ultimum* (Loper, 1988). Thus, a correlation exists between pyoverdine production and biological control activity of strain 3551.

Iron competition implies that both the pathogen and the control agent are trying to utilize the available iron (FIGURE I-1). Although siderophores produced by biological control agents have been implicated as a mediating factor in biological control, the importance of siderophores produced by target pathogens in iron-competition has not been studied adequately. The research discussed in this dissertation identifies a general class of siderophores produced by *E. carotovora* and begins to elucidate the role of siderophores produced by *E. carotovora* in iron-competition with *P. fluorescens*.

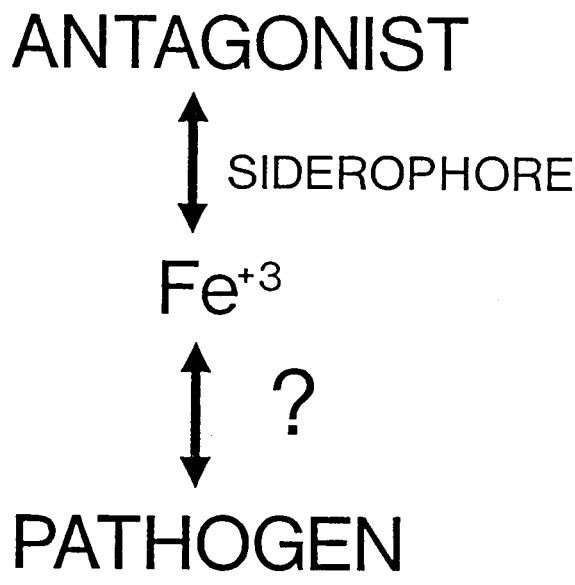


Figure I-1. Siderophore mediated iron competition between biological control agents and phytopathogens.

Factors potentially influencing variability of biological control.

Field variability has been a major obstacle to the application of biological control to commercial systems. Biological control is a complex interaction involving and varying with environmental factors, pathogen, host and the antagonist. Many reasons for the failure of biological control have been discussed by other authors (Handelsman and Parke, 1989; Weller, 1988; Cook and Baker, 1983; Gutterson, 1990). Three possible reasons for the failure of biological control are discussed here.

A. Colonization of the antagonist as a factor in the success of biological control.

Bacterization of planting material is the most common method by which biological agents are applied for the control of soilborne plant pathogens. Control of pathogens that infect seed does not require movement of the biological control agent because the agent is directly applied to the infection court. Control of root diseases, however, relies on colonization of the root system by the biological control agent. The relative importance of colonization in the control of root diseases varies among systems. Many factors, such as those discussed in the following sections, influence the extent to which colonization is important.

The perceived importance of colonization in biological control can be seen by the number of publications that describe the population dynamics of biocontrol agents in the rhizosphere of plants to be protected. For many years it was assumed that a large rhizosphere population size of the antagonist would optimize biological control. This hypothesis was tested and confirmed for one system. As populations of *P. fluorescens* 2-79 increase on individual sections of seminal wheat roots, number of lesions formed by *Gaeumannomyces graminus* var. *tritici* decreases on those root sections (Bull *et al.*, 1991). This inverse correlation on individual sections of roots is important because there is a lognormal distribution of introduced bacteria on roots indicating that some sections of root will be virtually unprotected

while others are well colonized (Loper *et al.*, 1984a; Bahme and Schroth, 1987). Variability in populations of introduced bacteria on individual roots or among roots of wheat plants is great. Approximately 60% of individual seminal roots of wheat are colonized to some extent while 40% are left unprotected (Bull, 1987). This variability may lead to significant variation in control of pathogens.

Colonization of potato roots and daughter tubers occurs when seed pieces are inoculated with fluorescent pseudomonads used as biological control agents. Sizes of antagonist populations that develop on daughter tubers and roots are variable (Loper *et al.*, 1984; 1985; Bahme and Schroth, 1987; Gross, 1988). Successful biological control of soft rot and blackleg caused by *E. carotovora* is assumed to be dependant upon colonization of potato roots and daughter tubers by the biological control agents (Gross, 1988), but this hypothesis has not been tested. It is important to know if there is a direct relationship between population of the antagonist and biological control and to identify the size of the antagonist population that is required for biological control.

B. Timing and production of key biocontrol metabolites as a factor in the success of biological control.

Metabolites, such as antibiotics or siderophores, must be produced by biological control agents in adequate concentrations and at the appropriate time to be effective in disease control. Some authors question the extent to which the rhizosphere and bulk soil are rich enough in nutrients to support production of antibiotics or other secondary metabolites by rhizosphere bacteria (Williams, 1982; Williams and Vickers, 1986). It is not known what effect concentration and timing of production of key phenotypes play in biological control. Proof that these compounds are produced in the soil or rhizosphere is lacking in most cases and information concerning the timing of production is almost non-existent. Because of this lack of information, I will present information on the few cases in which *in situ* production

has been demonstrated and will describe a novel approach to study *in situ* production.

B-1. Direct isolation of metabolites.

Direct isolation of metabolites from the rhizosphere and bulk soil would be definitive evidence that those compounds are produced *in situ*. Phenazine-1-carboxylate (phenazine), an antibiotic responsible in part for the biological control of *G. g.* var. *tritici*, is produced by *P. fluorescens* strain 2-79 in the rhizosphere of wheat roots inoculated with the phenazine producing strains 2-79. Phenazine is not detected from wheat rhizospheres that are not treated with 2-79 or from those treated with a mutant of 2-79 that does not produce phenazine (Thomashow and Weller, 1988). A second compound implicated in biological control, 2,4-diacetylphloroglucinol, also is detected in the rhizosphere (Keel *et al.*, 1992). Although these studies provide critical evidence that antibiotics are produced in the rhizosphere, the methods used to detect phenazine and 2,4-diacetlylphloroglucinol are not universally applicable for detection of antibiotics in rhizosphere soil. The detection of phenazine may be easier than the detection of other antibiotics because it is pigmented. High Pressure Liquid Chromatography (HPLC) analysis can detect phenazine within the complex environment of the rhizosphere soil.

Only one siderophore, schizokinen, has been isolated from the soil (Akers, 1983). Schizokinen is produced by *Bacillus megaterium* and is not hypothesized to be involved in biological control. Monoclonal antibodies being developed for the detection of ferric-pseudobactin may be useful in monitoring pseudobactin production in the soil and rhizosphere (Buyer *et al.*, 1990).

B-2. Use of reporter gene systems to detect production of phenotypes important in biological control.

Reporter gene systems have readily quantifiable phenotypes that can be fused to the regulatory elements of a native gene with a phenotype that is difficult to quantify and/or detect. Because the reporter gene is expressed from the promoter of the native gene of interest, quantification of the product of the reporter gene is a measure of the expression of the native gene.

For the study of plant-microbe interactions, reporter genes with phenotypes distinguishable from those present in plant and soil samples are needed and are available. Several reporter gene systems are currently used in biological control studies; *inaZ* is being used successfully in our laboratory to study *in situ* expression of genes important in biological control (Loper and Lindow, 1991; Lindow and Loper, 1990; Loper *et al.*, 1991). The *inaZ* reporter gene system is based on ice nucleation activity expressed due to the presence of an outer membrane protein (InaZ) that orients water in a structure mimicking the crystalline structure of ice. Ice nucleation activity is quantified by a droplet freezing assay (Lindow, 1990). Ice nucleation activity [$\log_{10}$(nuclei/cell)] increases with InaZ protein content of bacterial cells (Lindgren *et al.*, 1989). The ice nucleation reporter gene system functions in many Gram-negative plant pathogenic bacteria (Lindgren *et al.*, 1989). The *inaZ* gene is available independently or cloned into the transposon Tn*3*. The presence of the *inaZ* gene within a transposon is a powerful tool because the transposon can be used as a mutagen as well as a reporter gene system. The transposon Tn*3*-Spice, which preferentially inserts into plasmid rather than chromosomal DNA, is particularly useful for generation of fusions with genes that are cloned into plasmids.

In situ expression of genes determining biosynthesis of pyoverdine, a siderophore produced by *Pseudomonas* spp., is being studied using the *inaZ* reporter

gene system (Loper *et al.*, 1991; Loper and Lindow, 1991; Lindow and Loper, 1990). The promoterless *inaZ* gene is cloned downstream of an iron-regulated promoter of a pyoverdine-biosynthesis gene from *P. syringae*. In this construct, the *inaZ* gene is expressed when the promoter for pyoverdine biosynthesis gene is active. By monitoring the expression of ice nucleation activity, transcription of the pyoverdine-biosynthesis gene by *Pseudomonas* spp. *in situ* is demonstrated (Loper *et al.*, 1991). Reporter gene systems also will be useful in demonstrating that genes for the biosynthesis of other metabolites involved in biological control are transcribed in the rhizosphere.

C. Resistance of a pathogen to key biocontrol metabolites as a factor in the success of biological control.

Biological control may not be achieved in fields with pathogen populations that are insensitive to a biological control agent. In the disease control literature, the importance of pathogen diversity in sensitivity or resistance to control methods is accepted. Populations of pathogens that are resistant to agrichemicals cause disease even when the agrichemicals are employed (Dekker and Georgopoulos, 1982). For example, populations of *E. amylovora* that are resistant to streptomycin may cause fire blight even when streptomycin is applied to pear as a control measure (Loper *et al.*, 1991). The importance of pathogen diversity in sensitivity to biological control agents needs to be further examined. In biological control research, few studies have sought to understand the role of pathogen diversity in the failure of biological control in field trials.

Differences in sensitivity of pathogen populations to biological control agents have been reported. For example, a strain of *P. putida* produces an antibiotic that inhibits *E. carotovora* and controls potato soft rot. Within pockets of macerated potato tissue however, cells of *E. carotovora* that are resistant to the antibiotic

produced by *P. putida* are found (Colyer and Mount, 1984). Colyer and Mount (1984) suggest that cells of *E. carotovora* that are resistant to the antibiotic cause the pockets of macerated tissue that develop in the presence of the biocontrol agent.

Xu and Gross (1986a) demonstrated the variable sensitivity of strains of *E. carotovora* to biological control by *Pseudomonas* spp.. Fluorescent pseudomonads significantly suppressed non-emergence of potato caused by *E. c. subsp. atroseptica* W3C37 but had little effect on that caused by *E. c.* subsp. carotovora W3C105. In a field dominated with strains similar to W3C37, one would expect biological control to be obtainable but in fields dominated by strains similar to W3C105, biological control probably will not be achieved. Through the study of pathogen diversity, information leading to recommendations for the successful use of biological control may be obtained.

Differential sensitivities of W3C105 and W3C37 to suppression by *Pseudomonas* spp. has been correlated to siderophore production by these pathogens (Loper and Ishimaru, 1991). The biological control agents used by Xu and Gross (1986a) are hypothesized to control non-emergence due to siderophore-mediated iron-competition. Strains W3C105 and W3C37 both produce catechols but W3C105 is one of a few strains of *E. carotovora* that produces the hydroxamate siderophore aerobactin (Ishimaru and Loper, 1992; Ishimaru and Van Buren, 1991). Aerobactin production by W3C105 is hypothesized to be important in the reduced sensitivity of this pathogen to siderophore-mediated biological control by *Pseudomonas* spp. (Loper and Ishimaru, 1991).

The biocontrol-virulence connection: Role of siderophores in virulence of pathogens.

A. Role of siderophore production by animal pathogens in virulence.

Siderophores produced by certain bacterial pathogens of mammals are known virulence determinants (Weinberg, 1978; 1986). Iron competition occurs between pathogenic microbes and their mammalian hosts. Specific iron-binding proteins such as lactoferrin in the liver and transferrin in the blood have bacteriostatic properties that are reversed by the addition of iron (Schade and Caroline, 1946). Bacterial pathogens will not multiply unless they develop a method to overcome the iron limitation imposed by the presence of transferrin and lactoferrin (Weinberg, 1986; Payne, 1988). The siderophore aerobactin, which was originally isolated from *Aerobacter aerogenes* (Gibson and McGrath, 1969), has a very high affinity for iron in blood serum and can overcome iron starvation by sequestering iron from transferrin and transporting the iron into the bacterial cell via an outer-membrane receptor. Enterobactin, a siderophore that is also produced by many enteric pathogens, has a higher affinity for iron than does aerobactin but enterobactin is inactivated in serum. Thus, aerobactin is a virulence determinant of enteric pathogens of mammals whereas enterobactin is not required for virulence (Weinberg, 1978; 1986).

B. Siderophore production by plant pathogenic bacteria.

Siderophores are produced by plant pathogenic bacteria (Leong and Expert, 1990; Loper and Buyer, 1991). Strains of *A. radiobacter*, *A. tumefaciens*, *E. carotovora* subsp. *carotovora*, *E. amylovora* and *Pseudomonas syringae* pv. *phaseolicola* produce molecules with siderophore-like properties (Leong and Neilands, 1982). Nevertheless, siderophores produced by several phytopathogenic bacteria are not required for pathogenicity nor are they virulence factors. Mutants of *P. syringae* pv. *syringae* that are unable to export or produce pyoverdin are no less virulent than parental strains on cherry (Cody and Gross 1987a, 1987b) or bean

(Loper and Lindow, 1991). Agrobactin, a catechol siderophore produced by *A. tumefaciens* is not a virulence factor for crown gall disease (Ong *et al.*, 1979; Leong and Neilands, 1981). In contrast, *E. chrysanthemi* produces the catechol siderophore chrysobactin, which is a virulence determinant (Persmark *et al.*, 1989; Enard *et al.*, 1988). Chrysobactin-biosynthesis mutants of *E. chrysanthemi* do not cause systemic soft rot on anexically grown *Saintpaulia* plants whereas parental strains cause systemic infection. Strains with mutations in ferric-chrysobactin receptor genes also do not produce systemic rot (Enard *et al.*, 1988).

Erwinia carotovora subsp. *carotovora* strain 78 produces three major substances that react with ferric chloride; one of these substances is DHBA, a precursor in the biosynthesis of the catechol siderophore enterobactin. The two other substances are highly unstable but are possibly novel catechol siderophores. Production of these catechols is repressed at iron levels greater than 10μM (Leong and Neilands, 1982), as is common among microbial siderophores. In addition, uncharacterized catechols are produced by other strains of *E. carotovora* (Ishimaru and Loper, 1992). Nevertheless, the role of catechols and other iron-reactive compounds in iron acquisition by *E. carotovora* has not been established.

Some strains of *E. carotovora* produce aerobactin (Ishimaru and Loper, 1992; Ishimaru and Van Buren, 1991). Aerobactin is not essential for virulence of *E. carotovora* because many pathogenic strains do not produce aerobactin (Ishimaru and Loper, 1992; Ishimaru and Van Buren, 1991). Because every strain of *E. carotovora* produces catechol, however, the production of catechol but not aerobactin may have a role in virulence of this organism.

An important goal of the present study is to analyze the relative roles of catechol and aerobactin production by *E. carotovora* in virulence and sensitivity to biological control. Aerobactin production by *E. carotovora* has been chemically and

genetically analyzed (Ishimaru and Loper, 1992). In contrast, catechol production is a common trait among *E. carotovora* spp. but has not been characterized chemically or genetically. The molecular genetic analysis of catechol siderophore production by *E. carotovora* to be described here began with an understanding of the genetics of siderophore production in *E. coli*.

Aerobactin- and enterobactin-mediated iron uptake as model systems for studying siderophores produced by phytopathogenic members of the Enterobacteriaceae.

Siderophores produced by *E. coli* provide model systems to which siderophores produced by other microorganisms are compared (Enard *et al.*, 1989; Greenwood and Luke, 1980; Payne *et al.*, 1983; Pollack *et al.*, 1970; Young and Gibson, 1979; Schmidt and Payne, 1988; Ishimaru and Loper, 1992). In some studies, genes determining siderophore biosynthesis or uptake of *E. coli* are useful in isolating analogous genes of other organisms (Ishimaru and Loper, 1992). A general review of the genetics of siderophore production by *E. coli* and other members of the Enterobacteriaceae is presented below.

A. Biosynthesis of aerobactin.

Aerobactin is a well-studied member of the group of siderophores that have hydroxamic groups [R-CO-N(OH)R'] as ligands of Fe^{3+}. Aerobactin is produced by a wide range of bacteria including *A. aerogenes*, from which it was first isolated (Gibson and McGrath, 1969), *E. coli* (Williams, 1979), *S. flexneri* (Payne, 1980), *S. typhimurium* (McDougall and Neilands, 1984), *E. cloacae (Crosa* et al,., 1988), and *E. carotovora* (Ishimaru and Loper, 1992).

Invasive strains of *E. coli* produce aerobactin, which is a virulence factor in mammalian hosts (Payne, 1988). Aerobactin-biosynthesis and uptake genes may be

present on the chromosome or on plasmids, such as pColV-K30 (Bindereif and Neilands 1985a; Williams, 1979; Warner *et al.*, 1981). Four genes are involved in the production of aerobactin from citrate, N^{ε}-hydroxylysine, and acetate (TABLE I-3). *I*ron *u*ptake *c*helate (*iucA-iucD*) genes are responsible for the generation of side chains of N^{ε}-acetyl-N^{ε}-hydroxylserine and the addition of the side chains to citric acid (De Lorenzo and Neilands, 1986a; 1986b) (FIGURE I-2). An additional gene *iutA* (*i*ron *u*ptake *t*ransport) codes for the outer-membrane receptor for ferric-aerobactin (Van Tiel-Menkvel *et al.*, 1982). The *iuc* genes and *iutA* are commonly flanked by IS elements (McDougall and Neilands, 1984). The DNA cluster including the IS elements is referred to as the aerobactin operon (FIGURE I-3). Cloned aerobactin biosynthesis and uptake genes of *E. coli* have been useful in the analysis of genes involved in aerobactin production and uptake by *E. carotovora* (Ishimaru and Loper, 1992).

Genes for aerobactin biosynthesis and uptake by *E. c.* subsp. *carotovora* W3C105 have been cloned and are expressed in *E. coli* (Ishimaru and Loper, 1992). Aerobactin biosynthesis genes of W3C105 are similar but not identical to those of *E. coli*. Derivatives of W3C105 with mutations in aerobactin biosynthesis and uptake genes have been obtained and will be useful in studying the role of aerobactin in the ecology and virulence of *E. c.* subsp. *carotovora*.

B. Biosynthesis of enterobactin.

Enterobactin is a catechol siderophore synthesized by several enteric bacteria including *E. coli* (Cox et al 1970), *S. typhimurium* (Pollack *et al.*, 1970), *S. flexneri* (Payne, 1980), and *K. pneumonia* (O'Brien and Gibson, 1970), and *Aeromonas hydrophila* (Barghouthi *et al.*, 1989a; 1989b). The biosynthesis of enterobactin

TABLE I-3. Genes involved in uptake, biosynthesis and regulation of aerobactin in *E. coli*

Mutation	Product	Function	Reference
iutA (*i*ron *u*ptake *t*ransport)	74-kDa protein	Receptor for ferric-aerobactin complex and cloacin DF13	Van Tiel-Menkveld, *et al.*, 1982
iucA (*i*ron *u*ptake *c*helate)	63-kDa protein	Synthetase: Addition of first N^{ϵ}-acetyl-N^{ϵ}-hydroxylserine side chain to citric acid	de Lorenzo and Neilands, 1986; de Lorenzo *et al.*, 1986
iucB	33-kDa protein	Acetylase: Acetylation of N^{ϵ}-hydroxylyserine to N^{ϵ}-acetyl-N^{ϵ}-hydroxylserine	Coy *et al.*, 1986
iucC	62-kDa protein	Synthetase: Synthesis of aerobactin from N^{ϵ}-acetyl-N^{ϵ}-hydroxylserine and the citric acid N^{ϵ}-acetyl-N^{ϵ}-hydroxylserine molecule	de Lorenzo and Neilands 1986
iucD	53-kDa protein	Oxygenase: N^{ϵ} hydroxylation of L-lysine	de Lorenzo *et al.*, 1986
fur (*f*erric *u*ptake *r*egulation)		Repressor of transcription of many iron-regulated genes	Ernst *et al.*, 1978; Hantke, 1981

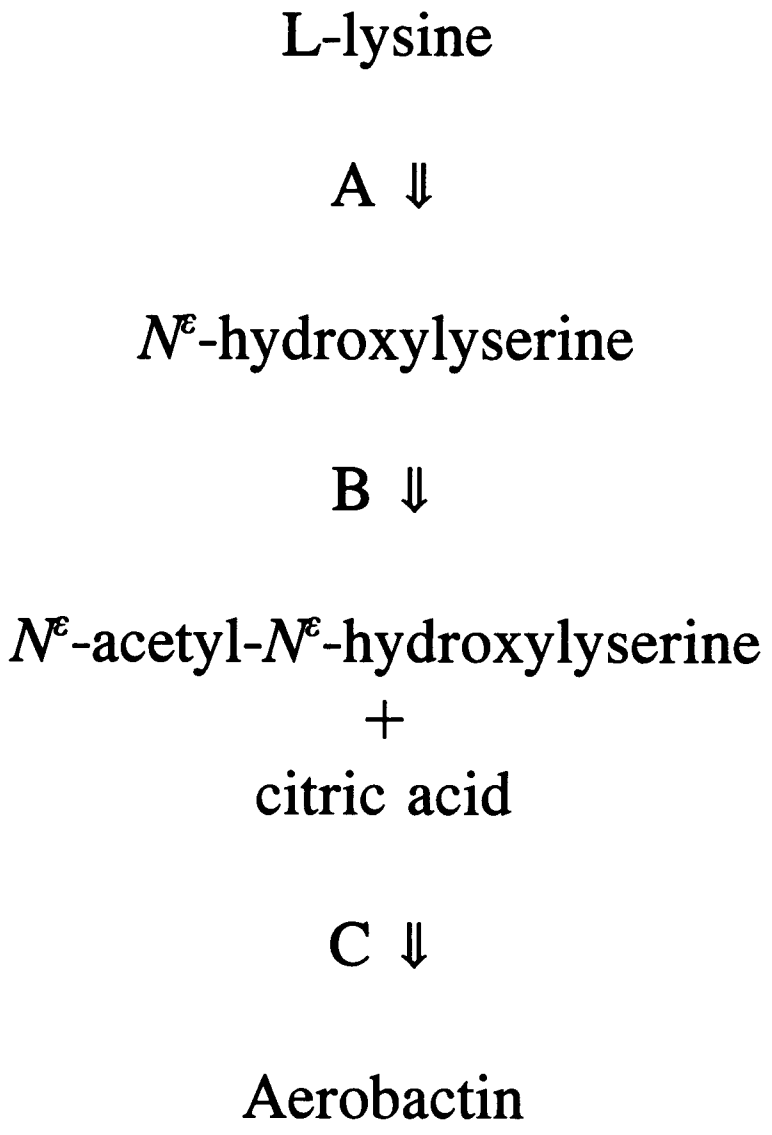


FIGURE I-2. Aerobactin biosynthesis pathway.
Biosynthesis of aerobactin from L-lysine, acetic acid, and citric acid. (A) Oxygenase, (B) acetylase, (C) synthetase. (Neilands, 1989)
(Neilands, 1989).

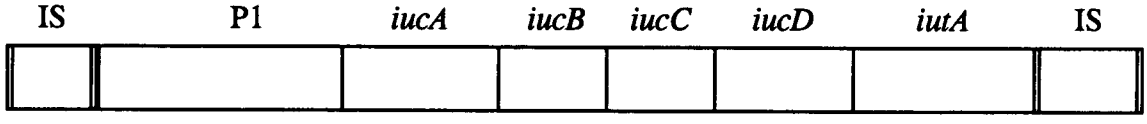


FIGURE I-3. Aerobactin operon.
IS=insertion sequence; P1=promoter of aerobactin operon; *iuc*=*i*ron *u*ptake *c*helate; *iut*=*i*ron *u*ptake *t*ransport.

involves the synthesis of DHBA from chorismate followed by the condensation of DHBA and L-serine into enterobactin (FIGURE I-4). *Ent*erobactin-biosynthesis genes (*ent*), *entC*, *entB*, and *entA*, are responsible for the generation of 2,3-DHBA from chorismate whereas *entE* is involved in the activation of DHBA. The order of these genes is similar among several strains for which DHBA is an intermediate to catechol siderophore production. The genes encoding enzymes that convert chorismate to DHBA are linked and termed the DHBA operon (Buyer and Massad, personal communication). The enterobactin-synthetase (ent-synthetase) complex including *entE*, *entD*, and *entF*, encodes an enzyme complex that catalyzes the production of enterobactin from L-serine and chorismate (TABLE I-4) (Earhart, 1987).

Chorismate is a common precursor of enterobactin, anthranilate, and p-aminobenzoate (PABA). A family of genes encoding chorismate-utilizing enzymes have similar nucleotide sequences. Due to the nucleotide sequence similarity of *entC* to *trpE* and *pabB*, *entC* is included in this gene family (Nahilik *et al.*, 1989). The *entC* gene codes for isochorismate synthetase, a 44-kDa protein that catalyzes the isomerization of chorismate to isochorismate (Young *et al.*, 1969; Nahilik *et al.*, 1989) (TABLE I-4; FIGURE I-4). This isomerization is the first reaction in a series converting chorismate to DHBA.

Hydrolysis of the enolpyruvyl side chain of isochorismate results in the formation of 2,3-dihydro-2,3 dihydroxy benzoic acid (Greenwood and Luke, 1980). 2,3-dihydro-2,3 dihydroxybenzoic acid synthetase is coded for by *entB*. In addition, a mutation in the 3' terminus of the *entB* gene results in the loss of enterobactin-synthetase activity, which is encoded by *entD*, *entF*, *entE*, and *entG*. EntB may be a bifunctional protein; in addition to the hydrolysis of isochorismate, its function in the enterobactin-synthetase complex was formerly designated as *entG* (Staab and Earhart, 1990).

Chorismate

entC ⇓

Isochorismate

entB ⇓

2,3-dihydro-2,3-dihydroxy benzoic acid

entA ⇓

2,3-dihydroxybenzoic acid

entD

entE ⇓

entF

entG

Enterobactin

FIGURE I-4. Enterobactin biosynthesis pathway.
From Ozenberger *et al.*, 1989.

TABLE I-4. Genes involved in enterobactin production, uptake and utilization

Mutation	Gene Product	Function or enzymatic activity	Citation
entC	Isochorismate synthetase, 44-kDa	Isomerization of chorismate to isochorismate	Ozenberger et al., 1989
entB	2,3-dihydro-2,3-dihydroxy benzoic acid synthetase,	Hydrolysis of enolypyruvyl side chain of 2,3-dihydro- 2,3-dihydroxy benzoic acid	Greenwood and Luke, 1980; Staab and Earhart, 1990
entA	2,3-dihydro-2,3-dihydroxy benzoic acid dehydrogenase, 26-kDa	Oxidation of 2,3-dihydro-2,3-dihydroxybenzoate to DHBA	Liu *et al.*, 1989; Nahilik *et al.*, 1989
entE	Enterobactin synthetase complex, 58-kDa	2,3 dihydroxybenzoate amp-ligase, DHBA activation	Greenwood and Luke, 1976
entF	Enterobactin synthetase complex	L-serine activation	Greenwood and Luke, 1976
entD	Enterobactin synthetase complex	Physically associated with EntF and may attach complex to outer-membrane	Greenwood and Luke, 1976
entG	Enterobactin synthetase complex	Maps to 3' terminus of *entB*; no separate protein identified	Staab and Earhart, 1990

TABLE I-4. (Continued)

fepA	Outer-membrane receptor protein, 84-kDa	Outer-membrane receptor for ferric-enterobactin	Hancock *et al.*, 1976; Pugsley and Reeves, 1976; Lundrigan and Kadner, 1986
fepB	Periplasmic shuttle protein	Inner membrane transport function	Pierce and Earhart, 1986
fepC	Cytoplasmic receptor protein	May be part of a cytoplasmic receptor complex	Pierce and Earhart, 1986
fes (*f*erric *e*nterobactin *s*terase	Enzymatic release of iron from ferric-enterobactin	Esterase activity or reductase activity, responsible for the removal of iron from the ferric enterobactin complex in the cell.	Pierce *et al.*, 1983; Hollifield and Neilands, 1978
fur	Ferric uptake regulation	Repressor of transcription of many iron-regulated genes.	Ernst *et al.*, 1978; Hantke, 1981
tonB	Responsible for energized energy state needed for membrane transport	Pleiotropic	Wookey and Rosenberg, 1978; Frost and Rosenberg, 1975
exeB	Involved in energy dependant steps in membrane transport systems	Pleiotropic	Wookey and Rosenberg, 1978

Oxidation of 2,3-dihydro-2,3 dihydroxy benzoic acid results in the formation of DHBA (Liu *et al.*, 1989). A 26-kDa polypeptide, 2,3-dihydro-2,3 dihydroxy benzoic acid dehydrogenase, encoded by *entA*, is responsible for the oxidation. The *entA* gene product also may be a secondary factor in isochorismate synthetase reactions because the *entC401* lesion maps within the *entA* gene (Nahilik *et al.*, 1989).

DHBA is condensed with L-serine to form enterobactin. The enterobactin-synthetase complex responsible for the condensation reaction is composed of gene products of *entE*, *entD*, and *entF* (Greenwood and Luke, 1976; Luke and Gibson, 1971; Woodrow *et al.*, 1975). A fourth locus, *entG*, is involved but does not code for a unique gene product (Staab and Earhart, 1990). The *entE* and *entF* gene products catalyze the activation of 2,3-DHBA and L-serine (Greenwood and Luke, 1976). The remaining gene, *entD*, may attach the complex to the membrane through which enterobactin is excreted (Woodrow *et al.*, 1979).

C. Siderophore specific uptake and utilization.

Three components are described for transport systems for essential metabolites. These transport systems consist of an outer-membrane receptor, periplasmic shuttle and a cytoplasmic membrane permease complex (Ames, 1986). The enterobactin iron transport system contains these three components (Ozenberger *et al.*, 1987; Earhart, 1987).

In the Enterobacteriaceae, specific receptors are present for each high affinity iron-uptake system (FIGURE I-5). Although the ligands of siderophores are similar and belong to only a few groups, the siderophores vary in structure. The variation in siderophore structure contributes to specificity between outer-membrane receptors and uptake of a ferric-siderophore complex (Neilands, 1982).

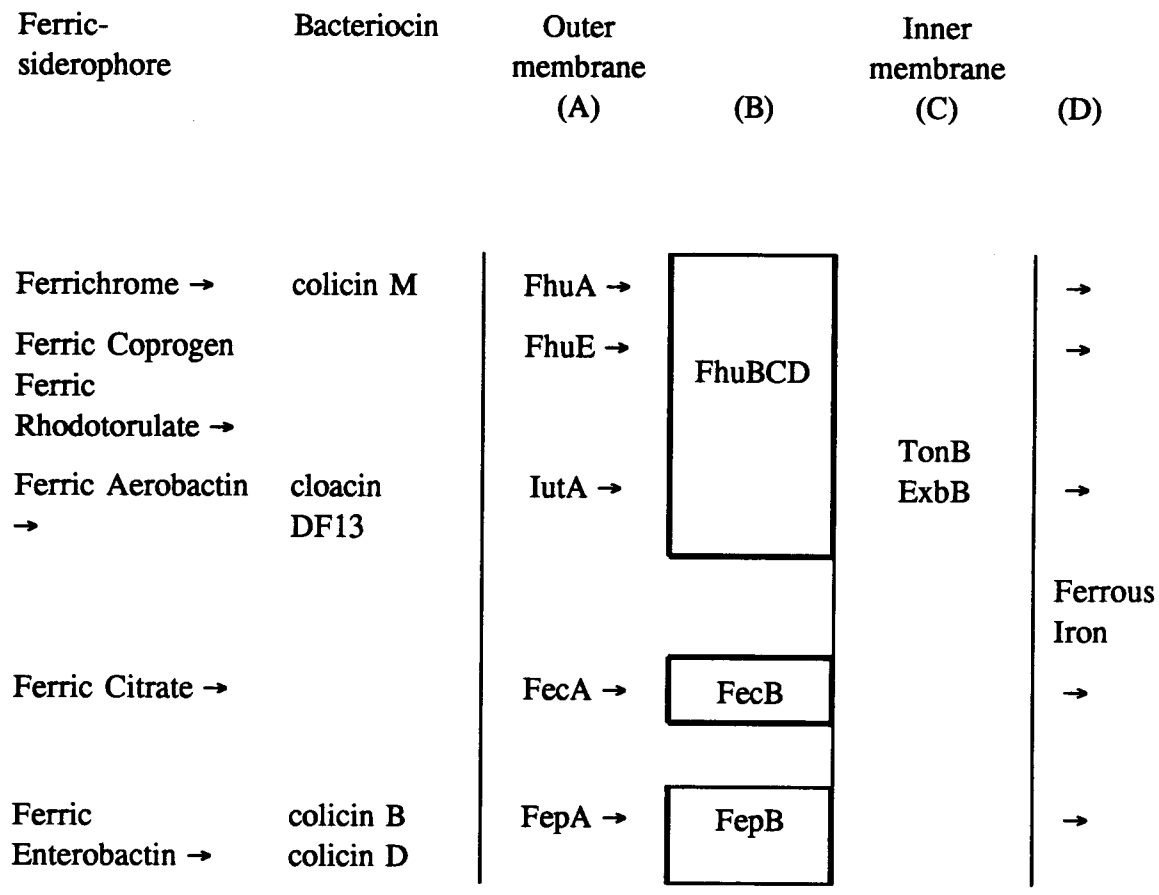


FIGURE I-5. Well characterized envelope components of high-affinity iron transport systems and bacteriocin uptake systems of *Escherichia coli* K-12. From Braun *et al.*, 1987; Bagg and Neilands, 1987. (A) Outer-membrane receptors. (B) Inner membrane receptors (periplasmic shuttle proteins). (C) Inner membrane permeability factors (D) Cytoplasm.

Enterobactin is recognized by the 84-kDa outer-membrane protein, FepA (Hancock *et al.*, 1976; Pugsley and Reeves, 1976; Lundrigan and Kadner, 1986; McIntosh *et al.*, 1979). FepA, like other outer-membrane proteins, is originally translated with a signal peptide attached and is cleaved to generate the mature protein (Lundrigan and Kadner, 1986; Fleming *et al.*, 1985). A second locus *fepB* is responsible for internalization of ferric enterobactin. FepB serves as the periplasmic shuttle for this system (Pierce and Earhart, 1986). The cytoplasmic receptor for ferric-enterobactin is FepC (Wookey and Rosenberg, 1978) (TABLE I-4). Two additional products are needed for the energized state of the membrane associated with transport (Frost and Rosenberg, 1975). TonB and ExbB are involved in the energy dependant steps of many transport systems (Wookey and Rosenberg, 1975). The FepA receptor has homology to other proteins that interact with TonB (Lundrigan and Kadner, 1986).

In the cytoplasm of the cell, *f*erric *e*nterobactin esterase encoded by *fes* is required to release iron from the ferric-enterobactin complex (Langman *et al.*, 1972; O'Brien *et al.*, 1971). Fes consists of two components, A (140-kDa) and B (22-kDa) which are inactive separately (O'Brien *et al.*, 1971; Langman *et al.*, 1972). Originally, Fes was thought to operate by hydrolysis of the ester bond of ferric enterobactin to release the ferric ion and N-2,3-dihydroxy benzoylserine (DBS). The mechanism of release is now in question, however, and Fes may be acting as a reductase (Earhart, 1987; Hollifield and Neilands, 1978).

The genes involved in enterobactin biosynthesis and uptake are linked in a clockwise orientation on 24-kb of DNA at the 13-minute map site of the *E. coli* chromosome (Cox *et al.*, 1970). The genes are arranged in the following order: *entD*, *fepA*, *fes*, *entF*, *fepC*, *fepB*, *entCEBA* (Fleming *et al.*, 1983; Pierce and Earhart, 1986; Nahilik *et al.*, 1989). These genes have been cloned into convenient

vectors and can be used to identify and compare similar genes from other organisms.

D. Regulation of siderophore mediated iron uptake.

The transcription of enterobactin- and aerobactin-mediated iron uptake is under the control of *fur* (*f*erric *u*ptake *r*egulation), as is that of all high-affinity iron-transport systems (Neilands, 1989) (TABLE I-4). The *fur* mutation is identified by the constitutive expression of iron-regulated proteins in enteric bacteria (Ernst *et al.*, 1978; Hantke, 1981). The *fur* gene codes for a 17-kDa protein which is hypothesized to act as repressor with iron as a corepressor (Neilands, 1989).

Fur binds to the aerobactin promotor region just upstream of the -10 box and at a second site two bases from this site. The region of DNA to which Fur binds is similar among many iron regulated proteins involved in high-affinity iron uptake and iron-regulated toxins (De Grandis, *et al.*, 1987; de Lorenzo *et al.*, 1987). A consensus sequence defined as the "iron box" is the preferred site for Fur binding (Neilands, 1989; de Lorenzo *et al.*, 1987).

E. Relationship between siderophore uptake and bacteriocin sensitivity.

The outer-membrane ferric-siderophore receptors are often receptors for bacteriocins (FIGURE I-5). FepA, the receptor for ferric enterobactin is also a receptor for colicin B and colicin D (Pugsley and Reeves, 1976; Hancock *et al.*, 1976). A mutant deficient in FepA is not sensitive to colicin B or D and cannot use ferric-enterobactin as a source of iron (McIntosh *et al.*, 1979). Because FepA is a colicin receptor, *fepA* mutants can be selected by resistance to colicin B or D. The relationship between bacteriocin sensitivity and siderophore receptors was used to

TABLE I-5. Genes involved in chrysobactin biosynthesis uptake and regulation

Mutation	Gene product size (kDa)	Enzyme production or activity	Corresponding gene from *E. coli*	reference
cbsC	38	*C*hrysob*a*ctin *synthesis*	*entC*	Enard *et al.*, 1991
cbsE		Chrysobactin synthesis	*entE*	Enard *et al.*, 1991
cbsB	34	Chrysobactin synthesis	*entB*	Enard *et al.*, 1991
cbsA	32	Chrysobactin synthesis	*entA*	Enard *et al.*, 1991
fct	82	*f*erric *c*hrysobactin *t*ransport	*fepA*	Enard *et al.*, 1988
cbu		*c*hrysob*a*ctin *u*ptake		Enard *et al.*, 1988
cbr		*c*hrysob*a*ctin *r*egulation	*fur*	Enard *et al.*, 1991

identify genes encoding the ferric-chrysobactin receptor of *E. chrysanthemi* (Expert and Toussaint, 1985).

F. Genetic characterization of biosynthesis uptake and regulation of chrysobactin.

Erwinia chrysanthemi is closely related to *E. carotovora*. The catechol siderophore chrysobactin produced by *E. chrysanthemi* may be similar to the catechol(s) produced by *E. carotovora*. Thus, it is useful to compare catechol siderophore production of these two pathogens.

The trivial name chrysobactin is given to a unique siderophore, N-(N^{β}-(2,3-dihydroxybenzoyl)-D-lysyl)-L-serine, produced by *E. chrysanthemi* (Persmark *et al.*, 1989). Several structural and stoichiometric characteristics, including the D chirality of the lysine residue and a 2:1 stoichiometry of ferric-chrysobactin, make chrysobactin unique among siderophores (Enard *et al.*, 1991; Persmark *et al.*, 1989). Although chrysobactin is structurally distinct, the biosynthetic pathway producing chrysobactin appears to be similar to that of other catechol siderophores.

Chorismate is a common precursor to the biosynthesis of both chrysobactin and enterobactin (Persmark *et al.*, 1989; Enard *et al.*, 1988). Several chryso*b*actin *s*ynthesis (*cbs*) genes are involved in the production of DHBA from chorismate (TABLE I-5). Plasmid R'4, which contains cloned genomic DNA of *E. chrysanthemi*, complements *cbsC*, *cbsE*, *cbsB*, and *cbsA* (Enard *et al.*, 1991) mutants of *E. chrysanthemi*. In addition, R'4 complements *entC*, *entE*, *entB*, and *entA* mutants of *E. coli* (Enard *et al.*, 1991) and therefore contains a DHBA operon. The *cbsC*, *cbsE*, *cbsB and cbsA* genes are functionally similar to the *entCEBA* genes of *E. coli* (Enard *et al.*, 1991). The arrangement of genes within the chrysobactin DHBA operon is similar to the arrangement of analogous genes in *E. coli*, with the gene order being *cbsCEBA* in *E. chrysanthemi* and *entCEBA* in *E. coli* (Franza and

Expert, 1991). Although these genes from *E. coli* and *E. chrysanthemi* appear to be functionally similar, they share only partial homology and the DNA restriction maps differ (Enard et al., 1991). Other genes that may be involved in the conversion of DHBA to chrysobactin, including those involved in the addition of lysine and serine to DHBA, have not yet been identified.

Chrysobactin uptake is mediated via an 82-kDa outer-membrane protein, Fct (*f*erric-*c*hrysobactin up*t*ake) (Enard *et al.*, 1988). The *fct* locus is located just upstream from the *cbs* genes on R'4. Antiserum prepared against FepA and IutA, the outer-membrane receptors for enterobactin and aerobactin, respectively, cross-reacted with Fct. It may be possible to identify outer-membrane receptors for other catechol siderophores by cross-reactivity with antiserum prepared against FepA.

In addition to biosynthesis and uptake genes, a regulatory locus, *c*hryso*b*actin *r*egulation (*cbr*) is identified. The *cbr* locus is thought to encode a transcriptional repressor because there is constitutive expression of chrysobactin in *cbr* mutants whereas the wildtype strain produces chrysobactin only in an iron-limited medium. Although it is clear that a trans-acting factor conferred by *cbr* influences the iron-mediated production of chrysobactin in *E. chrysanthemi*, further work is needed to understand the relationship of this (Cbr) factor to the Fur protein of *E. coli* (Enard *et al.*, 1991).

G. Use of siderophore model systems in studying catechol production in *Erwinia carotovora*.

In *E. coli*, the genes involved in enterobactin production, uptake, and regulation were identified by complementation of specific mutants in each step of the enterobactin biosynthesis pathway (Pickett *et al.*, 1984). Mutants of *E. coli* also have been used to identify genes involved in the catechol siderophore production in

other genera within the Enterobacteriaceae (Enard *et al.*, 1988; Enard *et al.*, 1991; Schmitt and Payne, 1988). Because DHBA is a precursor common to the biosynthesis of enterobactin and chrysobactin, genes encoding early enzymatic steps in the chrysobactin biosynthesis pathway complement *entA*, *entB*, and *entC* mutants of *E. coli*. The enterobactin mutants of *E. coli* also may be useful in identifying genes involved in catechol siderophore production by *E. carotovora*. Because aerobactin biosynthesis genes from *E. c.* subsp. *carotovora* W3C105 are expressed in *E. coli* (Ishimaru and Loper, 1992), it is likely that catechol biosynthesis genes from W3C105 will also be expressed in *E. coli*. Thus, it is likely that catechol biosynthesis genes from *E. carotovora* can be identified by complementation of *E. coli* biosynthesis mutants.

Rationale and research approach.

Erwinia carotovora subsp *carotovora* W3C105 produces two molecules with siderophore-like properties, a catechol and aerobactin (Ishimaru and Loper, 1992; Bull *et al.*, 1989; Bull and Loper, 1991). A catechol(s) is produced by all strains of *E. carotovora* tested, but aerobactin is produced by only a few strains (Ishimaru and Loper, 1992; Ishimaru and Van Buren, 1991). Because *E. carotovora* is a member of the Enterobacteriaceae and closely related to *E. coli* and *E. chrysanthemi*, it is possible that *E. carotovora* produces siderophores similar to enterobactin or chrysobactin. In addition, it is possible that the catechol siderophore is involved in virulence of *E. carotovora* because every strain tested produces a catechol and catechol production is a virulence factor of *E. chrysanthemi*. Although the catechol produced by *E. carotovora* may be important in virulence and/or disease control, the structure and function of the catechol(s) has not been determined.

Aerobactin is not necessary for virulence in *E. carotovora* because only a few pathogenic strains produce this siderophore. Strain W3C105, which produces

aerobactin, is less sensitive to biological control mediated via iron competition with *Pseudomonas* spp. than are strains that do not produce aerobactin (Xu and Gross, 1986a; Ishimaru and Loper, 1992). Genes involved in aerobactin production by *E. carotovora* are characterized. Nevertheless, the importance of aerobactin in iron competition between *E. carotovora* and fluorescent pseudomonads has not been determined. Derivatives of strain W3C105 with mutations in aerobactin biosynthesis or uptake genes will be useful in determining the role of aerobactin in iron competition between *E. carotovora* and *Pseudomonas* spp.

The goal of this research is to genetically analyze catechol-siderophore production by *E.* c. subsp. *carotovora* W3C105 and to begin to elucidate the relative role of the catechol siderophore and aerobactin in virulence and sensitivity to biological control. My approach will be to 1) analyze siderophore production by strains of *E. carotovora* by identifying and cloning genes involved in catechol siderophore production, 2) generate mutants deficient in catechol siderophore production using a transposon carrying a reporter gene system, 3) compare virulence and sensitivity to biological control of mutant and parental strains.

CHAPTER 1. CHARACTERIZATION OF CATECHOL PRODUCTION BY *ERWINIA CAROTOVORA*

INTRODUCTION

Iron is an essential element needed in trace amounts by most microorganisms for many oxidation-reduction reactions. Although iron is abundant in the soil, at neutral pH it is present as insoluble colloidal hydroxides that are not biologically available (Lindsay, 1979). Bacteria overcome the problem of iron limitation by producing siderophores. Siderophores are low molecular-weight molecules that form tight, soluble, coordination complexes with iron (Neilands, 1981; 1982). Siderophores are produced by microorganisms under iron-limiting conditions, excreted into the soil where they chelate iron, and transported back into the cell via specific outer-membrane receptors (Neilands, 1982). Iron released from the ferric-siderophore complex inside the cell is available for cellular functions (Earhart, 1987).

Catechols and hydroxamates are two types of siderophores produced by members of the Enterobacteriaceae (Crosa, 1989; Neilands, 1981). Enterobactin is a well-characterized catechol siderophore produced by *Escherichia coli* and other enteric bacteria (Neilands, 1986). Phytopathogenic members of the Enterobacteriaceae also produce catechol (Bull *et al.*, 1989; Bull and Loper, 1991; Leong and Neilands, 1982; Persmark *et al.*, 1989) and hydroxamate siderophores (Ishimaru and Loper, 1988; 1992; Ishimaru and Van Buren, 1991). *Erwinia carotovora* subsp. *carotovora* and *E. c.* subsp. *atroseptica* are phytopathogens that

cause soft rot diseases of potato (*Solanum tuberosum* L.) (Pérombelon and Kelman, 1980, 1987). Of twenty-two diverse strains of *Erwinia carotovora* that were surveyed for production of siderophores, only *E. c.* subsp. *carotovora* strain W3C105 produced a hydroxamate although all of the strains produced a catechol(s) with siderophore-like properties (Ishimaru and Loper, 1992). In a separate report, *E. c.* subsp. *carotovora* strain 78 produced an uncharacterized catechol siderophore (Leong and Neilands, 1982). A related pathogen, *Erwinia chrysanthemi* 3937, produces a catechol siderophore, chrysobactin (Persmark *et al.*, 1989). Mutants of strain 3937 that no longer produce nor utilize chrysobactin are altered in systemic virulence on *Saintpaulia ianotha* (Enard, *et al.*, 1988; Persmark, *et al.*, 1989). Although chrysobactin production is a virulence factor of *E. chrysanthemi* and catechol siderophore production is a common trait of *E. carotovora*, the role of siderophores in the virulence or ecology of *E. carotovora* has not been identified.

Characterization of the iron-uptake systems of *E. carotovora* is an important first step in understanding the role of these systems in pathogenicity and ecology of this microorganism. In this report, I present evidence that a catechol siderophore produced by a strain of *Erwinia carotovora* was similar functionally to enterobactin and distinct from chrysobactin. This is the first genetic characterization of a catechol siderophore produced by *E. carotovora*.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains used in these studies are listed in TABLE 1-1 and TABLE 1-2. Plasmids are listed in TABLE 1-3 and TABLE 1-4. *Escherichia coli*, *Erwinia chrysanthemi* and *Salmonella typhimurium* strains were cultured routinely on Luria Bertani medium (LB) (Gerhardt *et al.*, 1981) at 37 C and *Erwinia carotovora* on LB at 27 C. Soft agar consisted of nutrient broth (Difco, Detroit, MI) containing 0.7% (w/v) agar. LB agar was supplemented with 5-bromo-4-chloro-3 indolyl-β-d-galactopyranoside (X-Gal) (40 μg/ml) (International Biotechnologies, Inc.) and isopropyl-β-d-thiogalactopyranoside (IPTG) (100 μg/ml) in some cloning experiments. CVP (Cuppels and Kelman, 1974) and pectate agar (Beraha, 1968) were prepared as described. Catechol production by *E. carotovora* and other bacteria was determined from cultures grown in tris minimal salts medium (TMS) (Simon and Tessman, 1963) supplemented with tryptophan (0.003% w/v), thiamine (0.0002% w/v), and deferrated casamino acids (0.3% w/v). The iron availability of TMS medium was varied by adding $FeCl_3$ (100 μM or 0.1 μM) or 2,2'-dipyridyl (75 μM, 150 μM, or 225 μM). Antibiotics were used at the following concentrations: ampicillin (Ap, 100 μg/ml), tetracycline (Tc, 20 μg/ml), spectinomycin (Sp, 50 μg/ml), streptomycin (Sm, 100 μg/ml), kanamycin (Km, 50 μg/ml). All chemicals were purchased through Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Spontaneous rifampicin-resistant colonies were isolated and purified as described (Weller and Saettler, 1978). Generation times of the rifampicin-resistant mutants and parental strains were determined by the change in OD_{600nm} of cultures grown with shaking in LB broth. Only rifampicin-resistant mutants with generation times

TABLE 1-1. Field isolates of *Erwinia* species

					Cross-feeding of enterobactin biosynthesis mutants[s]			
					Escherichia coli		*Salmonella typhimurium*	
Strain	Origin	Source	CAS[u]	Catechol[v]	AN93[w]	AN192[x]	*enb-1*[y]	*enb-7*[z]
Erwinia carotovora subsp. *carotovora*								
W3C105	potato, North Dakota	D. Gross	+	+	+	+	+	+
CC101	potato, Oregon	M. Powelson	+	+	+	+	+	+
CC102	potato, Montana	M. Powelson	+	+	+	+	+	+
CC103	potato, North Dakota	M. Powelson	+	+	+	+	+	+
CC104	potato, Montana	M. Powelson	+	+	+	+	+	+
CC106	potato, Montana	M. Powelson	+	+	+	+	+	+
CC108	potato, Montana	M. Powelson	+	+	+	+	+	+
CC110	potato, Oregon	M. Powelson	+	+	+	+	+	+
CC501	potato, Oregon	M. Powelson	+	+	+	+	+	+
CC505	potato, Oregon	M. Powelson	+	+	+	+	+	+
SCRI193	potato, Scotland	J. Hinton	+	+	+	+	+	+
SR319	soil, Wisconsin	A. Kelman	+	+	+	+	+	+
CC303	soil, Oregon	M. Powelson	+	+	+	+	+	+
CC306	soil, Oregon	M. Powelson	+	+	+	+	+	+

TABLE 1-1. (Continued)

274-1-2	water, Colorado	M. Powelson	+	+	+	+	+	+
JL1128	artichoke, California	M. Powelson	+	+	+	+	+	+
JL1132	broccoli, Oregon	M. Powelson	+	+	+	+	+	+
JL1133	lettuce, Oregon	M. Powelson	+	+	+	+	+	+
JL1134	broccoli, Oregon	M. Powelson	+	+	+	+	+	+
Erwinia carotovora subsp. *atroseptica*								
W3C37	potato, Washington	D. Gross	+	+	+	+	+	+
SCRI1043	potato, Scotland	J. Hinton	+	+	+	+	+	+
Erwinia chrysanthemi								
3937	african violet, France	D. Expert	+	+	-	+	-	+

[s]Molten TMS containing 2,2'-dipyridyl (150 μM) was seeded with 10^6 cfu/ml of an indicator strain. Strains to be tested were spotted onto the surface of solidified, seeded TMS medium and incubated at 26 C. After 24-48 h, plates were observed for growth of the indicator strain surrounding the test colonies.

[t]Serogroup determinations (Powelson, personal communication).

[u]Siderophore production was detected by observation of orange halos surrounding organisms grown on CAS agar. CAS agar, a universal siderophore detection medium, was prepared as described (Schwyn and Neilands, 1987).

[v]Catechol production was detected from supernatants of cultures grown for 24-48 h in TMS as described by Arnow (1937) and Rioux *et al.* (1983).

[w]*Escherichia coli* AN93, an *entE* mutant, produces DHBA and utilizes only ferric-enterobactin.

[x]*Escherichia coli* AN192,an *entB* mutant utilizes either DHBA or ferric-enterobactin.

[y]*Salmonella typhimurium enb-1*, produces DHBA and utilizes only ferric-enterobactin.

[z]*Salmonella typhimurium enb-7*, utilizes either DHBA or ferric-enterobactin.

TABLE 1-2. Strains of *Escherichia coli* and *Salmonella typhimurium*

Strain	Description	Relevant characteristics	Source or reference
Escherichia coli:			
DH5a	F$^-$, *endA1*, *hsd17* (r_k^-, m_k^+), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, ø80*dlacZ*Δ, m15, λ$^-$	Ent$^+$	Bethesda Research Laboratories
AN194	F$^-$, *tonA23*, *proC14*, *leu16*, *trpE38*, *thi-1*, Sm^r	Ent$^+$	Langman *et al.*, 1972
AN193	*entA403* derivative of AN194, Sm^r	EntA403$^-$, Sm^r	J. B . Neilands
AN192	*entB402* derivative of AN194, Sm^r	Ent402$^-$, Sm^r	J. B. Neilands
AN191	*entC401* derivative of AN194, Sm^r	EntC401$^-$, Sm^r	J. B. Neilands
AN93	*entE405* derivative of AN194, Sm^r	EntE405$^-$, Sm^r	J. B. Neilands
AN90	*entD* derivative of AN194, Sm^r	EntD$^-$, Sm^r	Cox *et al.*, 1970
AN117	*entF* derivative of AN194, Sm^r	EntF$^-$, Sm^r	Pettis and McIntosh, 1987
MT147	*entC147*::Km derivative of AB1515	EntC147$^-$, Km^r	Ozenberger *et al.*, 1989
ID2	*fes* derivative of AB1515	Fes$^-$	Pierce *et al.*, 1983
UT6600	*fepA* derivative of AB1515	FepA$^-$	McIntosh *et al.*, 1979
RWB18	*iuc*$^-$, *iut*$^-$, *entA*, *fepA*	EntA$^-$, FepA$^-$	J. Crosa
LG1522	*iuc*$^-$, *iut*$^-$, *entA*	EntA$^-$	J. Crosa
JL1628	Spontaneous Rif^r mutant of AN193	EntA403$^-$, Rif^r	Ishimaru, unpublished
JL1753	Spontaneous Rif^r mutant of AN93	EntE405$^-$, Rif^r	This study
JL1754	Spontaneous Rif^r mutant of AN191	EntC401$^-$, Rif^r	This study
JL1773	Spontaneous Rif^r mutant of AN90	EntD$^-$, Rif^r	This study

TABLE 1-2. (Continued)

JL1774	Spontaneous Rif^r mutant of AN117	EntF$^-$, Rif^r	This study
JL1775	Spontaneous Rif^r mutant of UT6600	FepA$^-$, Rif^r	This study
JL1776	Spontaneous Rif^r mutant of ID2	Fes$^-$, Rif^r	This study
JL1794	Spontaneous Rif^r mutant of AN192	EntB402$^-$, Rif^r	This study
JL1886	Spontaneous Rif^r mutant of MT147	EntC147$^-$, Rif^r	This study
ψ	Universal colicin indicator		R. R. Brubaker
CA.7	Col V$^+$, Col M$^+$	Col V$^+$, Col M$^+$	R. R. Brubaker
CA.18	Col B$^+$, Col M$^+$	Col B$^+$, Col M$^+$	R. R. Brubaker
CA.23	Col D$^+$	Col D$^+$	R. R. Brubaker
CA.53	Col Ia$^+$	Col Ia$^+$	R. R. Brubaker
AG097	Col B$^+$	Col B$^+$	R. R. Brubaker
Salmonella typhimurium:			
enb-1	Derivative of LT-2, *asc-1*, Sm^r	Ent$^-$, Sm^r	Pollack *et al.*, 1970
enb-7	Derivative of LT-2, *asc-1*, Sm^r	Ent$^-$, DHBA$^-$, Sm^r	Pollack *et al.*, 1970
JL1882	Spontaneous Rif^r mutant of *enb-1*	Ent$^-$, Sm^r, Rif^r	This study
JL1893	Spontaneous Rif^r mutant of *enb-7*	Ent$^-$, DHBA$^-$, Sm^r, Rif^r	This study

TABLE 1-3. Plasmids used for Chapter 1

Plasmid	Relevant Characteristics	Vector	Source
pRK2013	Km^r, Tra^+, pRK2-$ColE1_{rep}$		Figurski and Helinski, 1979
pUC8	Ap^r, *lacZ*$^+$		Vieira and Messing, 1982
pUC19	Ap^r, *lacZ*$^+$		Yanisch-Perron *et al.*, 1985
pMS101	10.5-kb *Hin*dIII fragment, *entD*$^+$, *fes*$^+$, *entF*$^+$, Ap^r, Cr^r	pBR322	Laird *et al.*, 1980
pCP410	6.7-kb *Eco*RI fragment, *entCEBA*$^+$	pACYC184	Pickett *et al.*, 1984
pCP1492	2.4-kb *Eco*RI-*Pvu*II fragment, *entE*$^+$, Tc^r	pACYC184	Pickett *et al.*, 1984
pJS151	0.85-kb *Acc*I-*Eco*RV fragment + 0.2-kb of pBR322, *entA*$^+$, Ap^r	pGEMblue	C. F. Earhart
pITS47	*entC147*$^+$, Ap^r	pBR322	Ozenberger *et al.*, 1987
pCP104	*entD*$^+$, *fepA*$^+$, *fes*$^+$, Ap^r	pBR322	Coderre and Earhart, 1984

TABLE 1-4. Plasmids derived in Chapter 1

Plasmid	Relevant Characteristics	Vector
pJEL1594	*entB*$^+$, Tc^r,	pLAFR3
pJEL1595	*entB*$^+$, Tc^r	pLAFR3
pJEL1596	*entB*$^+$, Tc^r	pLAFR3
pJEL1892	*Eco*RI deletion of pJEL1596, *entB*$^+$	pLAFR3
pJEL1893	*Bam*HI deletion of pJEL1596, *entB*	pLAFR3
pJEL1894	*Hin*dIII deletion of pJEL1594, *entB*	pLAFR3
pJEL1895	*Eco*RI deletion of pJEL1594, *entB*$^+$	pLAFR3
pJEL1896	*Bam*HI deletion of pJEL1594, *entB*$^+$	pLAFR3
pJEL1597	*ent(DC)EA*$^+$, Tc^r	pLAFR3
pJEL1598	*ent(DC)EA*$^+$, Tc^r	pLAFR3
pJEL1599	*ent(DC)EA*$^+$, Tc^r	pLAFR3
pJEL1600	*entA*$^+$, Tc^r	pLAFR3
pJEL1601	*ent(DC)EA*$^+$, Tc^r	pLAFR3
pJEL1602	*ent(DC)EA*$^+$, Tc^r	pLAFR3
pJEL1604	*ent(DC)EA*$^+$, Tc^r	pLAFR3
pJEL1804	2.0-kb *Eco*RI fragment from pJEL1602 subclone, *ent(DC)EA*, Ap^r	pUC8
pJEL1818	5.8-kb *Bam*HI fragment from pJEL1602 subclone, *ent(DC)EA*, Ap^r	pUC8
pJEL1819	1.3-kb *Bam*HI fragment from pJEL1602 subclone, *ent(DC)EA*, Ap^r	pUC8
pJEL1868	5.6-kb *Kpn*I fragment from pJEL1602 subclone, *ent(DC)*, *entEA*$^+$, Ap^r	pUC19
pJEL1874	*Bam*HI deletion of pJEL1599, *ent(DC)*$^+$, *entEA*, Tc^r	pLAFR3

that did not differ significantly from those of parental strains were used (TABLE 1-2).

Detection of siderophore production.

Siderophore production by bacterial strains was detected by observation of orange halos surrounding bacterial colonies grown on CAS agar. CAS agar, a universal siderophore detection medium, was prepared as described (Schwyn and Neilands, 1987). Catechol was detected from supernatants of bacterial cultures grown for 24-48 h in TMS as described by Arnow (1937) or Rioux *et al.* (1983).

Enterobactin bioassay.

Enterobactin production was detected by a cross-feeding bioassay that relied upon the inability of indicator strains of *E. coli* and *S. typhimurium*, which had mutations in the enterobactin-biosynthesis pathway, to grow unless enterobactin or DHBA, an intermediate in the biosynthesis of enterobactin, was provided from an exogenous source (Pollack *et al.*, 1970). Molten TMS containing 2,2'-dipyridyl (150 μM) was seeded with an indicator strain (10^6 cfu/ml) that had been grown overnight with shaking in TMS broth. Strains to be tested for enterobactin production were also grown overnight with shaking in TMS broth. Ten microlitres of an overnight test culture was spotted onto the surface of solidified, seeded TMS medium and incubated at 27 C. After 24-48 h, plates were observed for the growth of an indicator strain surrounding DHBA and enterobactin-producing colonies. *E. coli* AN194, which produces enterobactin, and AN192, which produces neither enterobactin nor DHBA, were test strains included as controls in all experiments.

Colicin sensitivity assay.

In *E. coli*, the outer membrane receptor (FepA) for the ferric-enterobactin complex is also a receptor for colicins B and D (Gutterman and Dann, 1973). Colicin-producing strains of *E. coli* were CA.7 (colicins M and V), CA.18 (colicins B and M), CA.23 (colicin D), CA.53 (colicin Ia), and AG097 (colicin B). Producing strains were grown with shaking in LB broth overnight. Twenty microliters of the culture was spotted onto the surface of LB agar and plates were incubated for 18-24 h at 37 C. Producer cells were killed by inverting the agar plates over chloroform for 30 min. Strains to be evaluated for colicin sensitivity (indicator strains) and *E. coli* strain ψ, which is sensitive to all colicins tested, were grown with shaking in LB broth overnight. Cells were collected by centrifugation, suspended in 0.1 M $MgSO_4$, adjusted to a uniform density of 0.1 OD_{600nm}, and 10 μl of the suspension was mixed in molten soft agar. The seeded soft agar was poured over the LB agar surface supporting the dead producer cells and the plates were incubated at 27 C for 24-48 h.

Recombinant DNA techniques.

An existing library of W3C105 genomic DNA, constructed in the cosmid vector pLAFR3, was used in this study (Ishimaru and Loper, 1992). Individual clones of the library were stored in a pooled sample. Recombinant plasmids from the library were mobilized in mass by mating into individual enterobactin-biosynthesis mutants of *E. coli*. Transconjugants were screened for production of zones on CAS agar.

Cosmids from approximately 1500 members of the genomic library of W3C105 were mobilized into AN192 (*entB402*) and transconjugants were screened for production of zones on CAS agar. Screening of the W3C105 genomic library was repeated, in order to identify cosmids complementing the *entA403* and *entE405* mutations of *E.* coli. Cosmids that complemented *entB402*, *entA403* or *entE405*

mutations were mobilized into other enterobactin-biosynthesis mutants and the transconjugants were screened for zones on CAS agar. Cosmids from approximately 2500 members of the W3C105 genomic library were mobilized into AN117 in an attempt to identify a cosmid complementing the *entF* mutation.

Mobilization of recombinant cosmids into *E. coli* strains was accomplished by tri-parental matings using the helper plasmid pRK2013 (Figurski and Helinski, 1979). Transformation of *E. coli* and *S. typhimurium* strains were performed according to a procedure described for *E. coli* DH5α by Bethesda Research Laboratories (BRL, Bethesda, MD).

Plasmids and cosmids were isolated from *E. coli* and *S. typhimurium* strains by an alkaline-lysis extraction procedure and were further purified by cesium chloride density gradient centrifugation for Southern hybridization analyses and cloning experiments (Sambrook *et al.*, 1989). Genomic DNA was isolated according to published methods (Sambrook, *et al.*, 1989; Ausubel, *et al.*, 1987). T4 ligase and restriction endonucleases were used according to recommendations of the supplier (BRL). DNA was separated by gel electrophoresis at 50 V for 3 h in 0.7% agarose gels (SeaKem LE, FMC BioProducts, Rockland, ME).

Southern hybridizations.

DNA was transferred from agarose gels to nylon membranes (Nytran, Schleicher & Schuell [S&S], Keene, NH) by standard methods (Sambrook *et al.*, 1989). Probes used in hybridization studies are listed in TABLE 1-5. Fragments used as probes were purified from agarose gels (SeaKem GTG, FMC BioProducts) by adsorption and elution from NA-45 DEAE membranes, according to the manufacturer's recommendations (S&S). Probes were labeled by nick translation of the fragments with ^{32}P-dCTP (New England Nuclear, Boston, MA) and used at

TABLE 1-5. Probes used in Southern hybridizations for Chapter 1

Probe	Fragment used as probe	Vector	Source or reference
entD$^+$, *entF*$^+$, *fepA*$^+$, *fes*$^+$ of *E. coli*	pMS101, 10.5-kb *Hin*dIII fragment, *entD*$^+$, *entF*$^+$, *fepA*$^+$, *fes*$^+$	pBR322	Laird *et al.*, 1980
entCEBA$^+$ of *E. coli*	pCP410, 6.7-kb *Eco*RI fragment, *entCEBA*$^+$	pACYC184	Pickett *et al.*, 1984
entE$^+$ of *E. coli*	pCP1492, 2.4-kb *Eco*RI-*Pvu*II fragment, *entE*$^+$	pACYC184	Pickett et al., 1984
entA$^+$ of *E. coli*	pJS151, 0.85-kb *Acc*I-*Eco*RV, *entA*$^+$	pGEMblue	C. Earhart
entF$^+$ of *E. coli*	pITS32, 3.6-kb *Hin*dIII-*Eco*RI fragment *entF*$^+$	pBR322	Pettis and McIntosh, 1987
entC147$^+$ of *E. coli*	pITS47, 0.65-kb *Eco*RI-*Hin*dIII fragment, *entC147*$^+$	pBR322	Ozenberger *et al.*, 1987
entEA$^+$ of *E. carotovora*	pJEL1868, 5.6-kb *Kpn*I fragment from pJEL1602, *entA*$^+$, *entE*$^+$	pUC19	This study
*Eco*RI fragment from *E. carotovora* that hybridizes to *entE*$^+$ of *E. coli*	pJEL1804, 2.0-kb *Eco*RI fragment from pJEL1602	pUC8	This study
entB$^+$ of *E. carotovora*	pJEL1895, 9.1-kb *Hin*dIII fragment, *entB*$^+$	pLAFR3	This study

concentrations of 0.025 μg/ml. Hybridization conditions were of moderate stringency (55-65 C, 50% formamide and 0.16 X SSC) or low stringency (44-55 C, 50% formamide and 0.2 X SSC) (Sambrook *et al.*, 1989).

RESULTS

Catechol production by *E. carotovora.*

Twenty strains of *E. carotovora* subsp. *carotovora*, two strains of *E. carotovora* subsp. *atroseptica* and one strain of *E. chrysanthemi* produced halos on CAS agar, indicating siderophore production (TABLE 1-1). All strains produced catechol in TMS broth (TABLE 1-1).

In cross-feeding bioassays, *E. coli* AN93 (*entE*) and *S. typhimurium enb-1* grew on an iron-limited medium only when provided with enterobactin. *E. coli* AN194 (Ent^+) and strains of *E. carotovora* provided a functional siderophore to AN93 and *enb-1* (TABLE 1-1), indicating that these strains of *E. carotovora* produced a siderophore(s) that was functionally similar to enterobactin. Strains of *E. carotovora* also provided a functional siderophore(s) to DHBA-utilizing strains AN193 (*entA*) and *enb-7.*

Erwinia chrysanthemi strain 3937, which produces the catechol siderophore chrysobactin (Persmark, *et al.* 1989), did not provide a functional siderophore to *E. coli* AN93 or *S. typhimurium enb-1.* In contrast, strain 3937 provided a functional siderophore to DHBA-utilizing strains AN193 (*entA*), and *enb-7.* It is likely that DHBA, an intermediate in the production of chrysobactin (Enard *et al.*, 1988), was the compound provided to the enterobactin-biosynthesis mutants by 3937.

The influence of iron concentration on catechol production by one strain of *E. c.* subsp. *carotovora* was evaluated. Catechol production by strain W3C105 and by *E. coli* strain AN194 was inversely related to the levels of available iron in TMS medium (FIGURE 1-1). Thus, catechol production by W3C105 and enterobactin

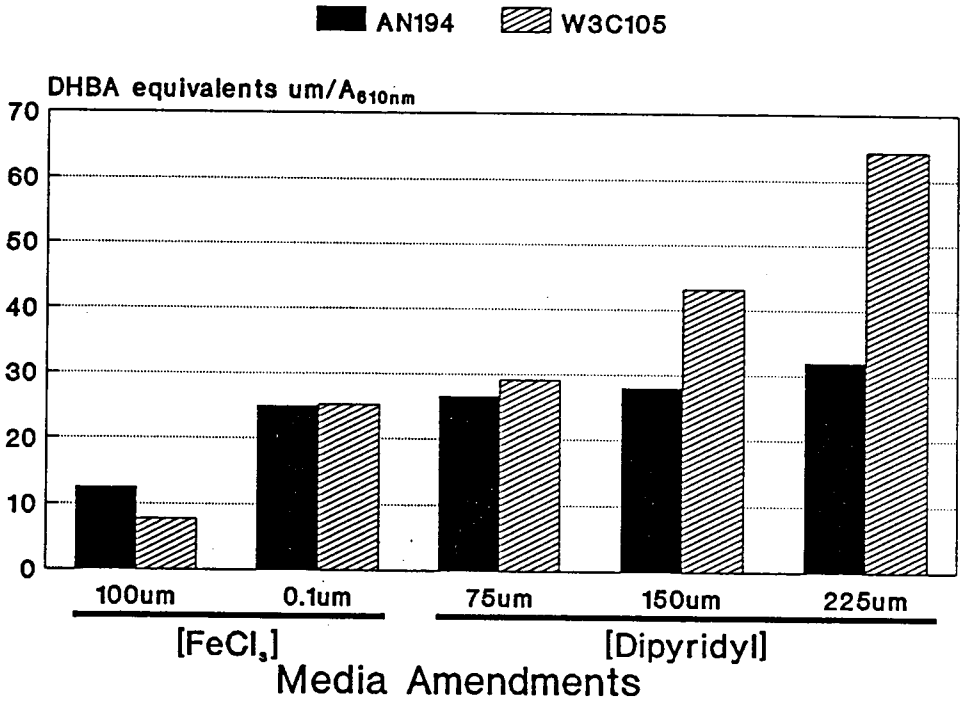


FIGURE 1-1. Iron regulation of catechol production by *Escherichia coli* AN194 and *Erwinia carotovora* subsp. *carotovora* W3C105.
Catechol was detected from supernatants of bacterial cultures grown for 24-48 h in TMS as described by Rioux *et al.* (1983). Catechol production by *E. carotovora* and *E. coli* was determined from cultures grown in tris minimal salts medium (TMS) (Simon and Tessman, 1963), supplemented with tryptophan (0.003% w/v), thiamine (0.0002% w/v), and deferrated casamino acids (0.3% w/v). The iron availability of TMS medium was varied by adding $FeCl_3$ (100 μM or 0.1 μM) or 2,2'-dipyridyl (75 μM, 150 μM, or 225 μM). Means were from three replications per treatment. The experiment was repeated.

production by *E. coli* AN194 were regulated by iron availability of the growth medium.

Complementation of enterobactin-biosynthesis mutants.

Of the 1500 cosmids mobilized into *E. coli* AN192 (*entB*) from a genomic library of W3C105, three cosmids (pJEL1594, pJEL1595 and pJEL1596) complemented the *entB* mutation. These cosmids did not complement other mutations in the enterobactin biosynthesis pathway (TABLE 1-6). These cosmids were designated *entB*$^+$ based on complementation of AN192.

Of the 1500 recombinant cosmids mobilized into AN193 (*entA403*), five cosmids (pJEL1597, pJEL1598, pJEL1599, pJEL1600, and pJEL1601) complemented *entA403* (TABLE 1-6). Of the 1500 recombinant cosmids mobilized into AN93 (*entE405*), two cosmids (pJEL1602, and pJEL1604) complemented *entE405*. These two cosmids also complemented *entA403*. Cosmids pJEL1597 and pJEL1598 were identical and pJEL1602 and pJEL1604 were identical. All seven cosmids complemented also complemented *entC401*, a mutation thought to be identical to *entA403* (Ozenberger *et al.*, 1989). and were designated *entA*$^+$. Six cosmids of the seven cosmids (pJEL1597, pJEL1598, pJEL1599, pJEL1601, pJEL1602, and pJEL1604) complemented *entD*, *entC147*, *entE*, *enb-7*, and *enb-1* in addition to *entA* (TABLE 1-6) and was designated *entA*$^+$. These cosmids were designated *ent(DC)EA*$^+$ based on complementation of enterobactin-biosynthesis mutants of *E. coli*. Cosmid pJEL1600 complemented only *entA* and *enb-7* (TABLE 1-6). None of the cosmids identified as *entB*$^+$ or *ent(DC)EA*$^+$ complemented *entF*. In addition, none of the 2500 recombinant cosmids from the W3C105 genomic library that were mobilized into *E. coli* AN117 (*entF*) complemented the *entF* mutation.

TABLE 1-6. Complementation of enterobactin biosynthesis mutants with genomic regions cloned from W3C105

	Catechol siderophore production[x]							
	Mutants of *Escherichia coli*						Mutants of *Salmonella typhimurium*	
Plasmid[y]	*entB*	*entD*	*entC147*	*entA*	*entE*	*entF*	*enb-1*	*enb-7*
pLAFR3	-	-	-	-	-	-	-	-
pJEL1594[z]	+	-	-	-	-	-	-	-
pJEL1595	+	-	-	-	-	-	-	-
pJEL1596	+	-	-	-	-	-	-	-
pJEL1597	-	+	+	+	+	-	+	+
pJEL1598	-	+	+	+	+	-	+	+
pJEL1599	-	+	+	+	+	-	+	+
pJEL1600	-	-	-	+	-	-	-	+
pJEL1601	-	+	+	+	+	-	+	+
pJEL1602	-	+	+	+	+	-	+	+
pJEL1604	-	+	+	+	+	-	+	+

[x]Siderophore production was detected by observation of a halo surrounding a colony grown on CAS agar, cross-feeding of strain AN93 (*entE*), and by catechol assays (Arnow, 1937; Rioux *et al.*, 1983). + = zone observed; - = no zone observed. [y]Presence of plasmids in enterobactin mutants was confirmed by extraction.

[z]Plasmids were mobilized into *E. coli* (except MT147, *entC147*) by conjugal transfer and into *S. typhimurium* strains and *E. coli* MT147 by transformation.

Characterization of cosmids complementing *entB*.

The *entB*$^+$ cosmids had a 6.2-kb region in common (FIGURE 1-2). In pJEL1596, the common region spanned an 8.1-kb *Bam*HI fragment and a 2.2-kb *Bam*HI-*Hin*dIII fragment. The *Hin*dIII site of the 2.2-kb fragment was contributed by the vector, pLAFR3. In pJEL1594 and pJEL1595, the common region spanned a 4.3-kb *Bam*HI-*Hin*dIII fragment and a 4.8-kb *Bam*HI-*Hin*dIII fragment. The *Hin*dIII site of the 4.3-kb *Bam*HI-*Hin*dIII fragment of pJEL1594 and pJEL1595 was contributed by the vector. Of a series of plasmids derived from the original three *entB*$^+$ cosmids by deletion of restriction fragments, only those that contained a 4.3-kb *Bam*HI-*Hin*dIII fragment complemented the *entB* mutation. The 4.3-kb *Bam*HI-*Hin*dIII fragment was sufficient for complementation of *entB*.

Characterization of cosmids complementing *ent(DC)EA*.

The seven *entA*$^+$ cosmids had similar restriction patterns (FIGURE 1-3) and contained overlapping sequences of DNA from W3C105. A 2.0-kb *Eco*RI fragment (pJEL1804), 5.8-kb *Bam*HI fragment (pJEL1818), 1.3-kb *Bam*HI fragment (pJEL1819) and 5.6-kb *Kpn*I fragment (pJEL1868) were subcloned into pUC8 or pUC19 (FIGURE 1-4). Of these four plasmids, only pJEL1868, which contained the 5.6-kb *Kpn*I fragment from pJEL1602, complemented any of the enterobactin-biosynthesis mutants. This plasmid complemented *entE* and *entA* mutations, as did the cosmid (pJEL1602) from which it was derived. Plasmid pJEL1868 did not complement *entC147* and *entD* mutants of *E. coli*. The 5.6-kb *Kpn*I fragment, subcloned on plasmid pJEL1868, was sufficient for complementation of *entA* and *entE* and was designated *entEA*$^+$ (FIGURE 1-4). Cosmid pJEL1600, which did not complement the *entE* mutation, lacked 0.5-kb of the 5.6-kb *Kpn*I fragment located to the left, as viewed in FIGURE 1-4. Because pJEL1600 complemented the *entA* mutation, the *entA*-complementing region was located to the right of the *entE*-complementing region, as viewed in FIGURE 1-4.

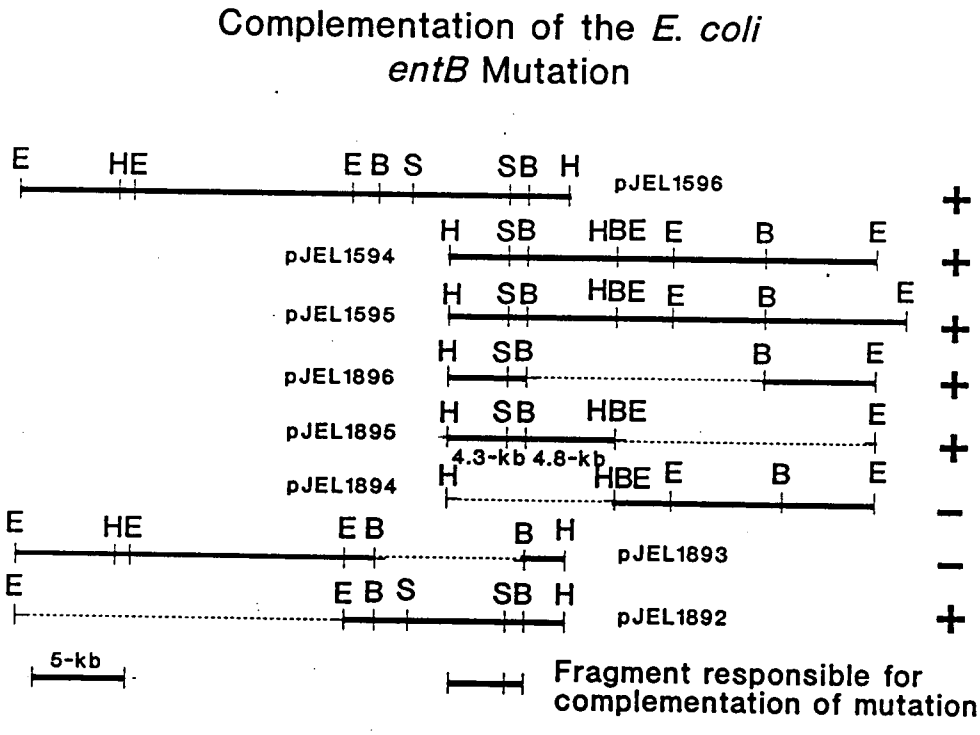


FIGURE 1-2. Physical maps of *entB*$^+$ cosmids derived from *Erwinia carotovora* subsp. *carotovora* W3C105 genomic DNA.
Complementation (+) and lack of complementation (-) was assayed by siderophore production assayed on CAS agar and by cross-feeding bioassays. Horizontal lines refer to genomic DNA derived from W3C105. Cosmid vectors are not shown. The *Eco*RI and *Hin*dIII sites at the extreme right and left of each inset were contributed by the cosmid vector, pLAFR3. Restriction-endonuclease cut sites are abbreviated: *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Sal*I (S).

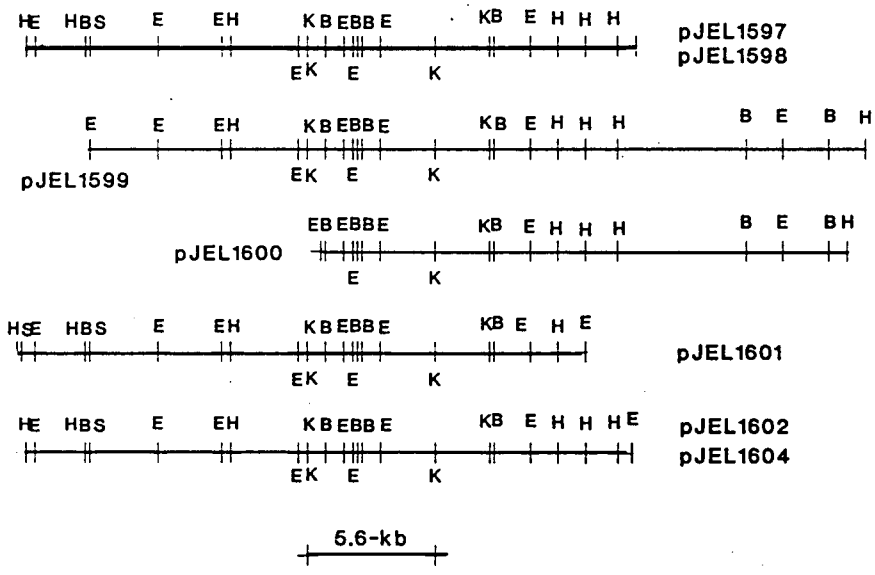


FIGURE 1-3. Physical map of *ent(DC)EA*$^+$ and *entA*$^+$ cosmids derived from *Erwinia carotovora* subsp. *carotovora* W3C105 genomic DNA.
Horizontal lines refer to genomic DNA derived from W3C105. Cosmid vectors are not shown. The *Eco*RI and *Hin*dIII sites at the extreme right and left of each inset were contributed by the cosmid vector, pLAFR3. Restriction-endonuclease cut sites are abbreviated: *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Sal*I (S), *Kpn*I (K).

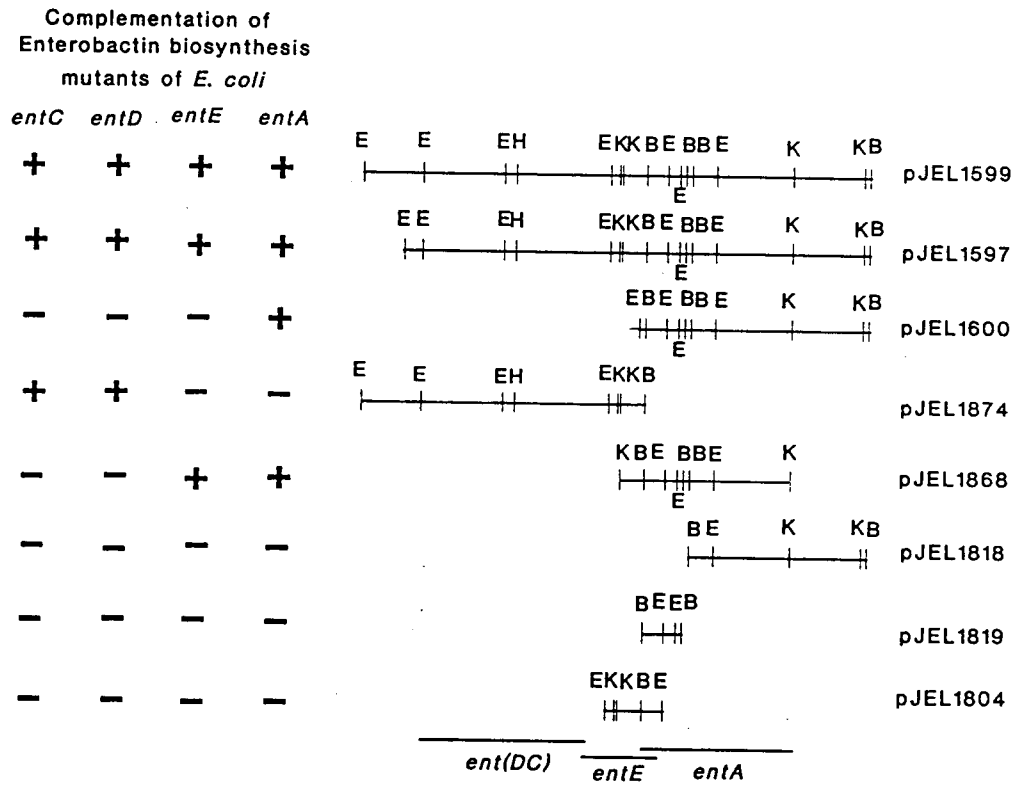


FIGURE 1-4. Physical maps of *ent(DC)EA*$^+$ cosmids and subclones derived from *Erwinia carotovora* subsp. *carotovora* W3C105 genomic DNA.
Complementation (+) and lack of complementation (-) of siderophore production was determined on CAS agar, by catechol production assayed by methods of Arnow (1937) and Rioux *et al.* (1983) and by cross-feeding bioassays. Horizontal lines refer to genomic DNA derived from W3C105. Vectors are not shown. Restriction-endonuclease cut sites are abbreviated: *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Sal*I (S), *Kpn*I (K). Cosmids pJEL1599, pJEL1597 and pJEL1600 are not shown in their entirety. pJEL1874 was made by deleting *Bam*HI fragments from pJEL1599. Fragments in subclones are regions from pJEL1602 cloned into pUC19(pJEL1868), and pUC8(pJEL1818, pJEL1804 and pJEL1819).

Cosmid pJEL1874, generated by deleting the *Bam*HI fragments from pJEL1599, complemented *entC147*, *entD* and *enb-7* but did not complement *entA* nor *entE*. Because pJEL1874 contained only a portion of the 5.6-kb *Kpn*I fragment, these results confirm the role of the 5.6-kb *Kpn*I fragment in complementation of *entE* and *entA*. The region cloned in pJEL1874 was designated *ent(DC)*$^+$. Cosmids pJEL1597 and pJEL1598 also complemented *entC147* and *entD* even though these cosmids were truncated on their left border. Cosmid pJEL1600, which was further truncated than pJEL1598 on the left border, did not complement *entC147* and *entD*. Thus, the DNA responsible for the complementation of *entD* and *entC147* was localized within a 8.0-kb region of DNA to the left of the 5.6-kb *Kpn*I fragment (FIGURE 1-4). The relative arrangement of *entD*$^+$ and *entC147*$^+$ regions was not determined.

Complementation of *fepA* mutants of *E. coli*.

Escherichia coli strain AN194, which produces enterobactin and utilizes ferric-enterobactin as an iron source, grew on an iron-limited medium (TMS amended with 225 μM 2,2'-dipyridyl), whereas three *fepA* strains, which were deficient in ferric-enterobactin uptake, did not grow on the iron-limited medium. The plasmid, pPC104, which contains the wildtype *fepA*$^+$ gene of *E. coli*, conferred iron-limited growth to RWB18, a *fepA*, *entA* strain. In contrast, the FepA$^-$ strain LG1522 and derivative FepA$^+$ strain LG1522(pPC104) did not differ in iron-limited growth; both strains grew on unamended TMS and on TMS amended with 75 μM or 150 μM 2,2'-dipyridyl whereas neither strain grew on TMS amended with 225 μM 2,2'-dipyridyl. Neither the *fepA* strain JL1775 nor the derivative strain JL1775(pCP410) grew on TMS; iron-limited growth of these strains could not be evaluated on this medium.

Because a cloned *fepA* gene could be detected by complementation of strain

RWB18 for iron-limited growth, cosmids from *E. carotovora* that complemented the enterobactin-biosynthesis mutants of *E. coli* were introduced into RWB18 and transformants were evaluated for growth on TMS containing 2,2'-dipyridyl. The *ent(DC)EA*$^+$ cosmids pJEL1597 and pJEL1598 did not confer iron-limited growth on the strain RWB18. In contrast, the *entB*$^+$ cosmids pJEL1595 and pJEL1596 conferred iron-limited growth on strain RWB18. Thus, pJEL1595 and pJEL1596 may contain a *fepA*-like gene from *E. carotovora*.

Because FepA is a receptor for colicins B and D (Guterman and Dann, 1973), colicin sensitivity assays were used for detecting complementation of *fepA*. All colicin producers tested except CA.7 inhibited growth of the universal colicin indicator indicating that all but CA.7 produced colicins under the assay conditions (TABLE 1-7). No differences in the zones of inhibition of strains AN194 (*fepA*$^+$) and JL1775 (*fepA*) were detected when colicins D and Ia were produced by CA.23 and CA.53 respectively. When colicin M-colicin B producer, CA.18, was used, the zone of inhibition for the *fepA*$^+$ strain was 5 mm whereas that of the *fepA* strain JL1775 was 1 mm. This difference was expected because FepA is the colicin B receptor. Colicins did not inhibit JL1148, the rifampicin resistant mutant of *E. c.* subsp. *carotovora* W3C105.

The usefulness of sensitivity to colicin B as a phenotype for detecting *fepA* complementation was further examined. All FepA$^+$ and FepA$^-$ strains had a 2-mm zone surrounding colonies of CA.18. The FepA$^+$ strains, AN194, JL1775 (pMS101) and JL1775 (pPC104), also had turbid inhibition zones of greater than 6 mm surrounding colonies of CA.18. Strain JL1775 had only the 2 mm clear zone (TABLE 1-8). Differences in sensitivity to CA.18 by JL1775 was useful in determining complementation of *fepA*. Transconjugants of JL1775 containing *entB*$^+$ or *ent(DC)EA*$^+$ cosmids from W3C105 had only 2-3 mm zones of no growth.

TABLE 1-7. Sensitivity of *Escherichia coli* and *Erwinia carotovora* strains to strains of *E. coli* that produce colicins

		Zone of inhibition (mm)[w]				
		Producing strains (Colicin produced)[x]				
Indicator[y]	Strain Characteristics	CA.23 (Col D)	CA.53 (Col Ia)	CA.18 (Col B, Col M)	CA.7 (Col V, Col M)	AG097 (Col B)
E. coli ψ	Universal indicator	4	5	8	0	1
E. coli JL1775	FepA$^-$, Rif^r	2	2.5	1	0	0
E. coli AN194	FepA$^+$, Ent$^+$	2	2	5	0	0
E. carotovora JL1148[z]	Cat$^+$	0	0	0	0	0

[w]Zones of inhibition were measured from the edge of the producing strain colony to the boundary of confluent growth of the indicator strain. Data was collected 24-48 h after inoculation of indicator strains.

[x]Overnight LB cultures (20 μl) of colicin producers were spotted onto LB agar. Cultures were grown overnight at 37 C. Cells were killed by inverting the agar plate over chloroform for 30 minutes.

[y]Soft agar (10 ml) was melted and seeded with 10 μl of a 0.1 OD_{600nm} LB-bacterial suspension of an indicator strain. The seeded soft agar was overlayed onto LB agar with killed producer cells and plates were incubated at 37 C.

[z]JL1148 is a Rif^r derivative of W3C105 (Ishimaru, unpublished).

TABLE 1-8. Sensitivity of *Escherichia coli* to colicin B and M producing strain *E. coli* CA.18

Indicator[y]	Phenotype	Zone of inhibition (mm)[z]
AN194	$FepA^+$, Ent^+	5
JL1775	$FepA^-$, Ent^+	2
JL1775(pMS101)	$FepA^+$, Ent^+	7
JL1775(pPC104)	$FepA^+$, Ent^+	7
JL1775(pJEL1594)	Ent^+	3
JL1775(pJEL1596)	Ent^+	2
JL1775(pJEL1597)	Ent^+	2.5
JL1775(pJEL1598)	Ent^+	2
JL1775(pJEL1599)	Ent^+	3
RWB18	$FepA^-$, $EntA^-$	7
RWB18(pPC104)	$FepA^+$, $EntA^-$	7
RWB18(pCP410)	$FepA^-$, Ent^+	6
RWB18(pJEL1595)	Ent^-	5
RWB18(pJEL1596)	Ent^-	4
RWB18(pJEL1597)	Ent^+	6
RWB18(pJEL1599)	Ent^+	6

[y]Overnight LB cultures (20 μl) of colicin producers were spotted onto LB agar. Cultures were grown overnight at 37 C. Cells were killed by inverting the agar plate over chloroform for 30 minutes.

[z]Zones of inhibition were measured from the edge of the colony of the producing strain to the boundary of confluent growth of the indicator strain. Data was collected 24-48 hrs after inoculation of indicator strains. Soft agar (10 ml) was melted and seeded with 10 μl of a 0.1 OD_{600nm} bacterial suspension of an indicator strain in LB broth medium. The seeded soft agar was overlayed onto LB agar with killed producer cells and plates were incubated at 37 C.

Thus, the *entB*$^+$ and *ent(DC)EA*$^+$ cosmids did not complement *fepA*, as determined by this method.

RWB18 and transconjugants RWB18 (pMS101) and RWB18 (pPC104) had both 2 mm clear zones and 6-7 mm turbid zones surrounding colonies of CA.18 (TABLE 1-8). Thus, sensitivity to CA.18 was not a useful indicator of the FepA$^+$ phenotype in strain RWB18. Transformants of RWB18 containing *entB*$^+$ or *ent(DC)EA*$^+$ cosmids from W3C105 also had both zones surrounding colonies of CA.18. Nevertheless, the presence of a *fepA*-like gene on the *entB*$^+$ or *ent(DC)EA*$^+$ cosmids was not shown by sensitivity to CA.18.

In cross-feeding bioassays, AN194 cross-fed RWB18 (pPC104), which is FepA$^+$, but not RWB18, which is FepA$^-$. Cross-feeding of RWB18 transconjugants, which contain *entB*$^+$ or *ent(DC)EA*$^+$ cosmids, may be a useful approach for identification of a *fepA*-like gene in *E. carotovora*.

Lack of Complementation of *fes* mutants of *E. coli*.

Complementation of *fes* was detected by differences in growth of *fes* mutants and complemented mutants on TMS amended with 2,2'-dipyridyl. Strain JL1776, a *fes* mutant of *E. coli*, did not grow on TMS agar. Transconjugant JL1776 (pPC104), which was complemented for the *fes* mutation, grew on TMS containing 225 μM 2,2'-dipyridyl. Plasmid pPC104 restored iron-limited growth of the *fes* mutant to levels equivalent to that of AN194. Transconjugants of JL1776 containing the *entB*$^+$ or *ent(DC)EA*$^+$ cosmids from the W3C105 library did not grow on TMS agar. The *entB*$^+$ and *ent(DC)EA*$^+$ cosmids from W3C105 did not complement the *fes* mutation.

Following incubation of either *fepA* or *fes* mutants on LB agar for longer than 5

days, the media turned dark brown presumably due to overproduction of enterobactin. The ferric-enterobactin complex is brown and forms upon chelation of iron in the medium. Complemented Fes$^+$ or FepA$^+$ strains do not turn the media dark brown, presumably because enterobactin is not overproduced. Observation of media color may be an alternate method of screening the W3C105 genomic library for clones that complement the *fepA* or *fes* mutations of *E. coli*.

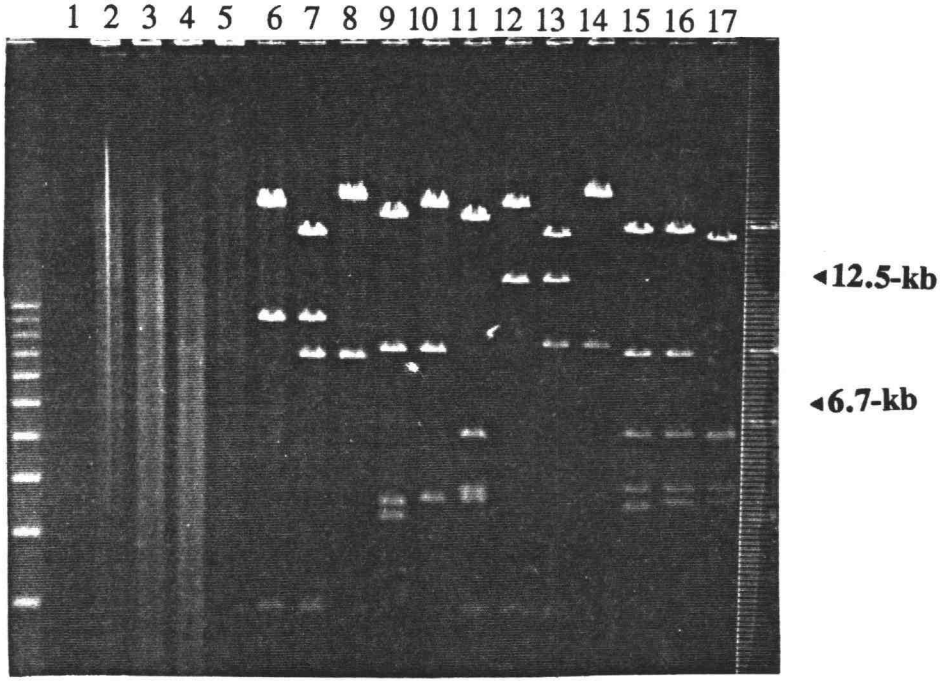
Hybridization of enterobactin genes from *E. coli* to *entB*$^+$ cosmids from W3C105.

Under low stringency conditions, the 6.7-kb *Eco*RI-*Hin*dIII fragment from pCP410, which contains *E. coli* enterobactin biosynthesis genes *entCEBA*, did not hybridize to pJEL1594 nor pJEL1595 (FIGURE 1-5). The probe hybridized strongly to the 6.7-kb *Eco*RI fragments of *E. coli* AN194 and pCP410. Hybridization to fragments in lanes containing cloned DNA from *E. carotovora* corresponded to chromosomal fragments in the *E. coli* host and not to restriction fragments of cloned DNA. For example in the pJEL1594 and pJEL1595 samples digested with *Eco*RI, *Eco*RI-*Hin*dIII, *Bam*HI or *Bam*HI-*Eco*RI, the probe hybridized to the 6.7-kb *Eco*RI chromosomal fragment of *E. coli* from which the probe DNA was derived. Although a 4.8-kb *Bam*HI-*Hin*dIII fragment of pJEL1594 and pJEL1595 was responsible for complementation of AN192 (*entB*), it did not hybridize to enterobactin-biosynthesis genes of *E. coli* including *entB*$^+$.

Hybridization of *entB*$^+$ region of W3C105 to *ent(DC)EA*$^+$ cosmids from W3C105 and to enterobactin biosynthesis genes of *E. coli*.

The 9.1-kb *Hin*dIII fragment of pJEL1895, containing the 4.3-kb region sufficient for *entB* complementation, did not hybridize to genomic DNA of AN194 nor to pCP410, which contained *entCEBA*$^+$ genes of *E. coli* (data not shown). This

FIGURE 1-5. Southern blot analysis of *entB*$^+$ cosmids derived from genomic DNA of *Erwinia carotovora* subsp. *carotovora* W3C105.
An ethidium-bromide-stained gel is above and an autoradiogram below on the following page. The blot was hybridized with the 6.7-kb *Eco*RI fragment from pCP410 containing *entCEBA*$^+$ from *E. coli*. Lanes: 1) pCP410, *Eco*RI; 2) AN194 genomic DNA, *Eco*RI; 3) W3C105 genomic DNA, *Eco*RI; 4) W3C105 genomic DNA, *Eco*RI-*Hin*dIII; 5) W3C105 genomic DNA, *Hin*dIII; 6) pJEL1594, *Eco*RI; 7) pJEL1594, *Eco*RI-*Hin*dIII; 8) pJEL1594, *Hin*dIII; 9) pJEL1594, *Bam*HI-*Hin*dIII; 10) pJEL1594, *Bam*HI; 11) pJEL1594, *Bam*HI-*Eco*RI; 12) pJEL1595, *Eco*RI; 13) pJEL1595, *Eco*RI-*Hin*dIII; 14) pJEL1595, *Hin*dIII; 15) pJEL1595, *Bam*HI-*Eco*RI; 16) pJEL1595, *Bam*HI-*Hin*dIII; 17) pJEL1595, *Bam*HI; 18) pJEL1595, *Bam*HI-*Eco*RI. The lane on the far left has the 1-kb ladder (BRL); that on the right is λ, *Hin*dIII, included as size markers.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◂12.5-kb
◂6.7-kb

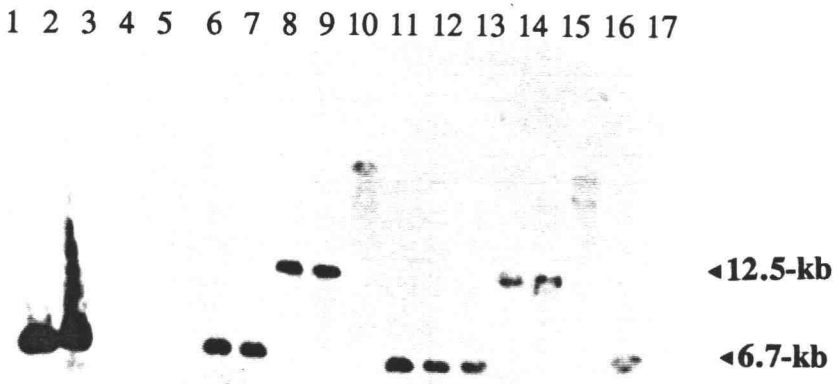

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
◂12.5-kb
◂6.7-kb

FIGURE 1-5.

result further indicated that there were differences between structural genes of *entB*$^+$ of *E. coli* and the functionally similar genes of W3C105.

The *entB*$^+$ gene probe from *E. carotovora*, which was comprised of the 9.1-kb *Hind*III fragment of pJEL1895, hybridized to an 8.1-kb *Bam*HI fragment and a 4.8-kb *Bam*HI-*Hin*dIII fragment of chromosomal DNA from W3C105. The 8.1-kb and 4.8-kb fragments were of the sizes predicted from maps of the *entB*-complementing region of W3C105 (FIGURE 1-2).

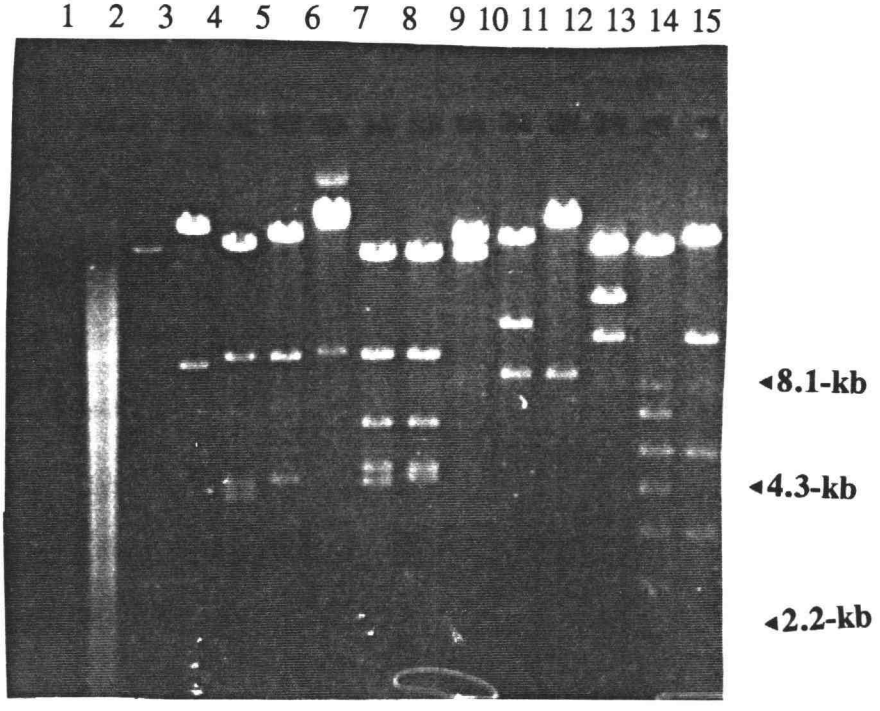
The *entB*$^+$ probe hybridized strongly to the anticipated fragments from pJEL1896 (4.3-kb *Bam*HI-*Hin*dIII), pJEL1594 and pJEL1595 (4.3-kb, 4.8-kb fragments from *Bam*HI-*Hin*dIII cut DNA) and from pJEL1596 (12.0-kb *Hin*dIII) (FIGURE 1-6). There was a slight hybridization to the fragment corresponding to pLAFR3, the vector of pJEL1594, pJEL1595, and pJEL1596. This hybridization was attributed to contamination of the probe with vector DNA. Additionally, there was hybridization to bands corresponding to contaminating chromosomal DNA of the *E. coli* host.

The *entB*$^+$ probe did not hybridize to DNA from the *ent(DC)EA*$^+$ cosmid pJEL1599. Background hybridization to the pLAFR3 vector was seen. Thus, the *entB*$^+$ fragment was distinct from the *ent(DC)EA*$^+$ region of *E. carotovora*.

Hybridization of enterobactin biosynthesis genes from *E. coli* to *ent(DC)EA*$^+$ complementing cosmids from W3C105.

Under low stringency conditions, the 6.7-kb *Eco*RI-*Hin*dIII fragment from pCP410 that contained the *entCEBA*$^+$ region of *E. coli* hybridized to the *ent(DC)EA*-complementing cosmids of W3C105 (FIGURE 1-7). As expected, the probe hybridized strongly to a genomic 6.7-kb *Eco*RI fragment of *E. coli*. The probe also

FIGURE 1-6. Southern blot analysis of *ent(DC)EA*$^+$ and *entB*$^+$ cosmids derived from genomic DNA of *Erwinia carotovora* subsp. *carotovora* W3C105.
An ethidium-bromide-stained gel is above and an autoradiogram below on the following page.The blot was probed with the 9.1-kb-*Hin*dIII fragment from *entB*$^+$ region of W3C105 cloned in pJEL1895. Lanes: 1) 1) AN194 genomic DNA, degraded; 2) W3C105 genomic DNA, *Hin*dIII-*Bam*HI; 3) pJEL1896, *Bam*HI-*Hin*dIII; 4) pJEL1594, *Hin*dIII; 5) pJEL1594, *Hin*dIII-*Bam*HI; 6) pJEL1594, *Bam*HI; 7) pJEL1595, *Hin*dIII; 8) pJEL1595, *Bam*HI-*Hin*dIII; 9) pJEL1595, *Bam*HI; 10) pJEL1596, *Hin*dIII; 11) pJEL1596, *Hin*dIII-*Bam*HI; 12) pJEL1596, *Bam*HI; 13) pJEL1599, *Hin*dIII; 14) pJEL1599, *Bam*HI-*Hin*dIII; 15) pJEL1599, *Bam*HI. The lane on the far right is λ-*Hin*dIII included as a size marker.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◂8.1-kb
◂4.3-kb
◂2.2-kb

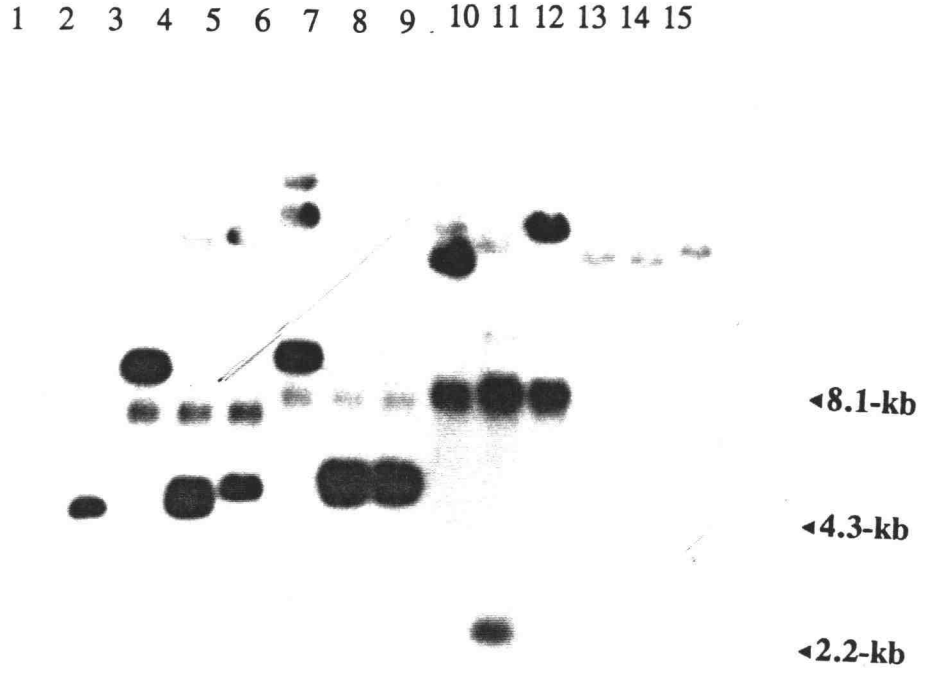

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
◂8.1-kb
◂4.3-kb
◂2.2-kb

FIGURE 1-6.

FIGURE 1-7. Southern blot analysis of *ent(DC)EA*$^+$ cosmids derived from genomic DNA of *Erwinia carotovora* subsp. *carotovora* W3C105.
An ethidium-bromide-stained gel is above and an autoradiogram below on the following page. The blot was hybridized with the 6.7-kb *Eco*RI fragment from pCP410 containing *entCEBA*$^+$ region from *E. coli*. Lanes: 1) pCP410, *Eco*RI; 2) AN194 genomic DNA, *Eco*RI; 3) W3C105 genomic DNA, *Eco*RI; 4) W3C105 genomic DNA, *Eco*RI-*Hin*dIII; 5) W3C105 genomic DNA, *Hin*dIII; 6) pJEL1602, *Eco*RI; 7) pJEL1602, *Eco*RI-*Hin*dIII; 8) pJEL1602, *Hin*dIII; 9) pJEL1597, *Eco*RI; 10) pJEL1597, *Eco*RI-*Hin*dIII; 11) pJEL1597, *Hin*dIII; 12) pJEL1598, *Eco*RI; 13) pJEL1598, *Eco*RI-*Hin*dIII; 14) pJEL1598, *Hin*dIII; 15) pJEL1599, *Eco*RI; 16) pJEL1599, *Eco*RI-*Hin*dIII; 17) pJEL1599, *Hin*dIII. The lane on the far left has the 1-kb ladder (BRL) included as a size marker.

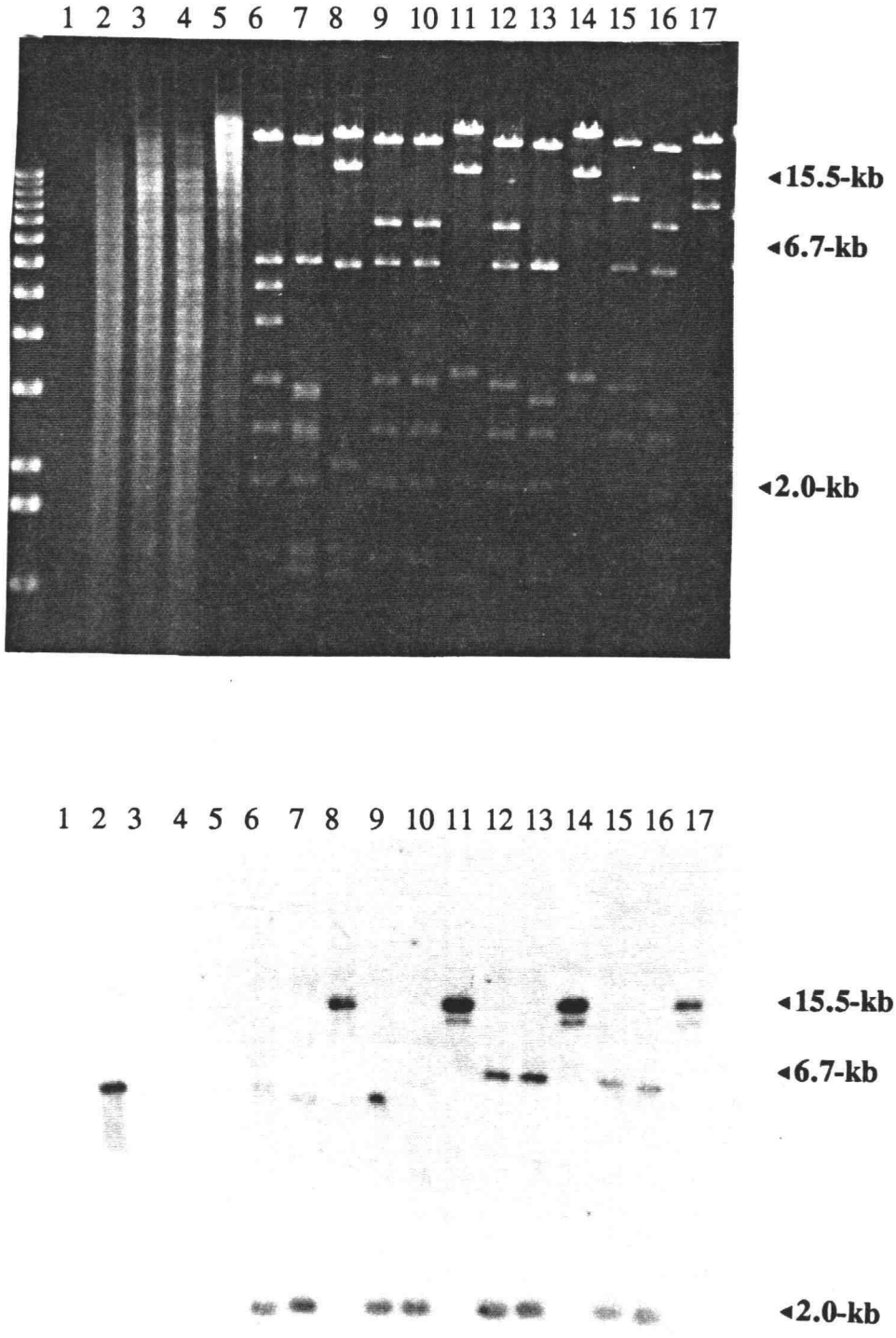

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
◂15.5-kb
◂6.7-kb
◂2.0-kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
◂15.5-kb
◂6.7-kb
◂2.0-kb

FIGURE 1-7.

hybridized to a 15-kb *Hin*dIII fragment of chromosomal DNA of W3C105. The *entCEBA*$^+$ region of *E. coli* hybridized to two *Eco*RI fragments (2.0-kb and 1.1-kb) that were internal to the 15-kb *Hin*dIII fragment in cosmids pJEL1597, pJEL1598, pJEL1599 and pJEL1602.

The *entE*$^+$ gene probe of *E. coli*, which consisted of the 2.4-kb *Pvu*II-*Eco*RI fragment of pCP1492, hybridized to the 2.0-kb *Eco*RI, 1.3-kb *Bam*HI and 5.6-kb *Kpn*I fragments in all *ent(DC)EA*$^+$ cosmids (FIGURE 1-8). The *entCEBA*$^+$ probe from pCP410 also hybridized to these fragments (FIGURE 1-7). Background hybridization was observed to the chromosomal DNA of *E. coli* that was present in all plasmid preparations.

Probes of other enterobactin-biosynthesis genes (*entA*$^+$, *entC147*$^+$, *entD*$^+$, and *entF*$^+$) and *fepA*$^+$ and *fes*$^+$ genes from *E. coli* (TABLE 1-5) did not hybridize to the *ent(DC)EA*$^+$ nor *entB*$^+$ cosmids (data not shown).

Hybridization of *ent*$^+$ regions of *Erwinia carotovora* subsp. *carotovora* W3C105 to genomic DNA from other strains of *Erwinia*.

The 2.0-kb *Eco*RI (pJEL1804) and the 5.6-kb *Kpn*I (pJEL1868) fragments hybridized to genomic DNA of the other strains of *Erwinia* that were evaluated (TABLE 1-9). There was, however, great variability in the size of the *Eco*RI and *Hin*dIII fragments to which the 2.0-kb *Eco*RI fragment and 5.6-kb *Kpn*I fragment hybridized. These probes did not hybridize to genomic DNA from *E. chrysanthemi* 3937 under low stringency conditions. Thus, while regions hybridizing to the *entEA*$^+$ region of strain W3C105 were common among strains of *E. carotovora*, they were not detected in a chrysobactin producing strain of *E. chrysanthemi*.

FIGURE 1-8. Southern blot analysis of pJEL1601 an *ent(DC)EA*$^+$ cosmid derived from *Erwinia carotovora* subsp. *carotovora* W3C105 genomic DNA.
An ethidium-bromide-stained gel is above and an autoradiogram below on the following page. The blot was hybridized with the 2.4-kb *Eco*RI-*Pvu*II fragment from pCP1492 containing *entE*$^+$ region from *E. coli*. Lanes: 1) pCP1492, *Eco*RI-*Pvu*II; 2) pCP410, *Eco*RI; 3) AN194 genomic DNA, *Eco*RI; 4) W3C105 genomic DNA, *Eco*RI; 5) W3C105 genomic DNA, *Eco*RI-*Hin*dIII; 6) pJEL1601, *Eco*RI; 7) pJEL1601, *Eco*RI-*Hin*dIII; 8) pJEL1601, *Hin*dIII; 9) pJEL1601, *Hin*dIII-*Bam*HI; 10) pJEL1601, *Bam*HI; 11) pJEL1601, *Bam*HI-*Kpn*I; 12) pJEL1601, *Kpn*I; 13) pJEL1601, *Kpn*I-*Hin*dIII; 14) pJEL1601, *Kpn*I-*Sma*I; 15) pJEL1601, *Sma*I; 16) pJEL1601, *Sma*I-*Bam*HI.

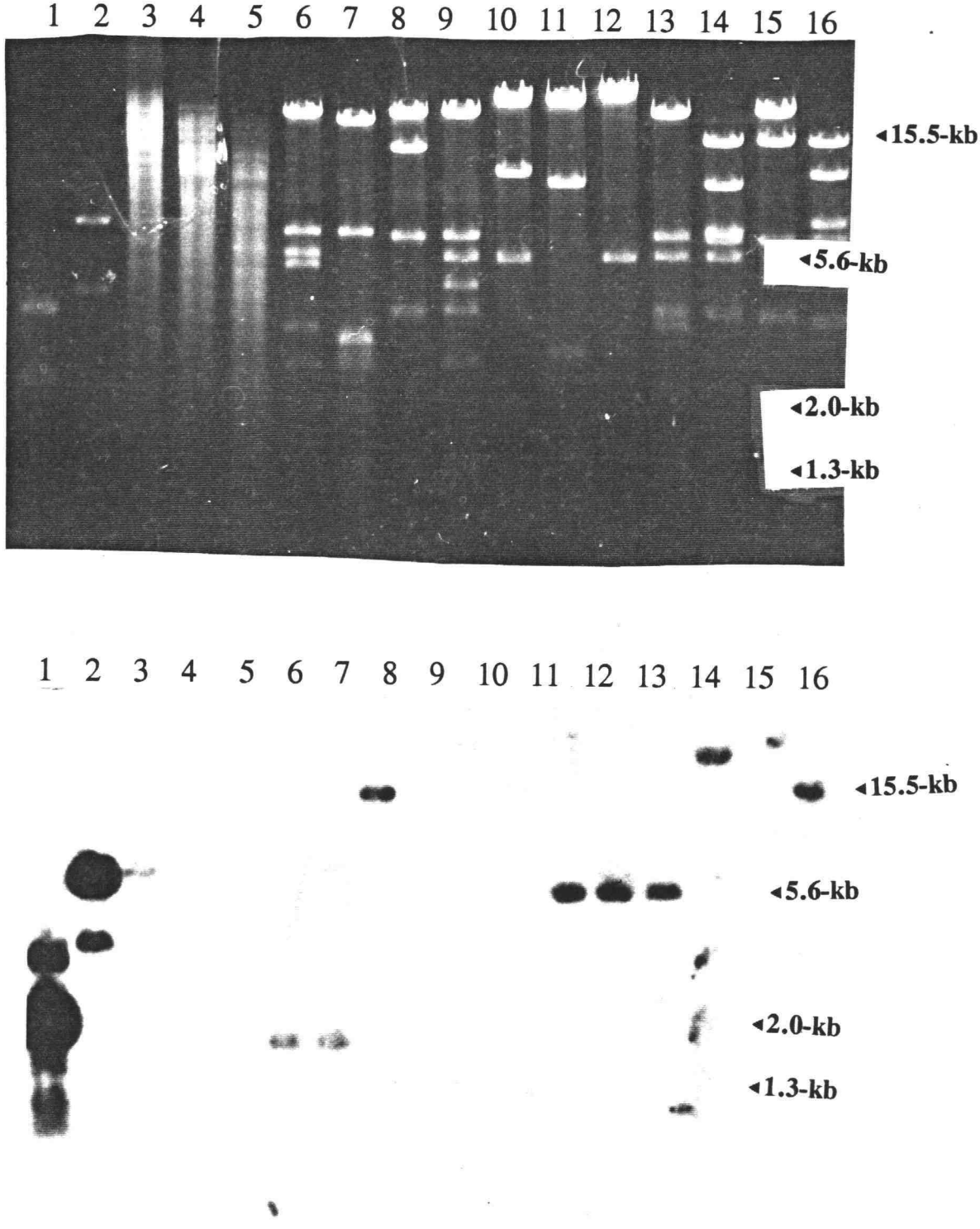

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
◂15.5-kb
◂5.6-kb
◂2.0-kb
◂1.3-kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
◂15.5-kb
◂5.6-kb
◂2.0-kb
◂1.3-kb

FIGURE 1-8.

TABLE 1-9. Hybridization of catechol biosynthesis genes from *Erwinia carotovora* subsp. *carotovora* W3C105 to genomic DNA of other *Erwinia* species

		Sizes of fragments identified by hybridization[v]			
		Probes			
		2.0 KB *Eco*RI (pJEL1804)		5.6 KB *Kpn*I (pJEL1868)	
Strains	Serotype[w]	*Eco*RI fragments	*Hin*dIII fragments	*Eco*RI fragments	*Hin*dIII fragments
E. c. subsp. *carotovora*					
W3C105	XXXIX	2.0 B[x]	14 A	1.8, 1.1 H	12.5 G
SR319	XXIX	6.0 B	NT[y]	10, 6.0 C	NT
CC501	XXIX	NT	12 A	9.8 I	NT
CC110	XXXVIII	NT	20 A	9.8 I	22 J
CC106	XI	6.0 B	22 A	10, 6.0 C	28 J
CC108	XXXVI	6.3 B	22 A	23, 6.0 C	25 J
CC101	III	6.3 B	20 A	23, 6.0 C	25 J
CC103	XL	5.8 B	20, 16 A	23, 6.0 C	15.5 J
CC102	V	4.9 L	NT	22, 4.9 E	22, 4.9 F
CC505	XXVII	3.9, 0.7 L	16 A	22, 3.9, 0.7 E	22 F
CC306	XXXIII	4.9 L	31 K	4.9 E	31 G
JL1128		NT	17 K	5.8, 4.5 H	12.5 E
274-1-2		5.4 L	21 K	5.6 E	18 E
CC303	XXXVII	5.4 L	NT	5.4 E	5.4 F
JL1132		5.4 L	19 K	15, 5.8, 4.5 H	18 E
JL1133		4.9 D	18.5 K	NT	18.5 E
JL1134		NT	19 K	7.6 H	17 E
JL1137		NT	17 K	8.2, 7.6, 4.5 H	18 E
JL1138		7.5 D	17 K	NT	18 E

TABLE 1-9. (Continued)

E. c. subsp. *atroseptica*				
SCRI-1043	NT	12 K	1.8, 5.0 H	16.5 E
JL1156	NT	NT	1.8, 7.6 H	9.7 J
E. chrysanthemi				
3937	None[z] D	None A	NT	none E
E. coli				
AN194	6.3 D	NT	6.6 H	NT

[v]Hybridization conditions were at low stringency (44-55 C; 50% formamide; 0.2 x SSC).
[w]Serotype information provided by Mary Powelson.
[x]Fragment sizes followed by the same letter were present on the same blot.
[y]NT = not tested.
[z]None = hybridization was not detected.

DISCUSSION

Catechol production is a common characteristic of strains of the plant pathogenic bacterium *E. carotovora*. In this study, 22 strains of *E. carotovora* produced catechols and siderophores, as detected by removal of iron from a dye in CAS agar. The catechol(s) produced by *E. carotovora* is functionally related to enterobactin, a catechol siderophore produced by many species of the Enterobacteriaceae (Neilands, 1981; Payne, 1988; Earhart, 1987). Catechol-producing strains of *E. carotovora* provided iron to *S. typhimurium enb-1* and *E. coli* AN93, which use enterobactin but do not use other catechol siderophores as a source of iron. This indicates that the catechol(s) produced by *E. carotovora* functions as a siderophore for other members of the Enterobacteriaceae.

I presume from these data that the catechol(s) produced by *E. carotovora* was a siderophore related to enterobactin. A genetic approach was used to explore this possibility with one strain, *E. c.* subsp. *carotovora* W3C105. From this strain, two distinct genomic regions involved in catechol siderophore production were cloned and characterized. One region was a 12.8-kb fragment with catechol biosynthesis genes arranged in the order of *ent(DC)EA*$^+$ according to subcloning and complementation data. Also present was a distinct 4.3-kb region that complemented *entB*. Because the *entB*$^+$ region did not hybridize to the cosmids containing the *ent(DC)EA*$^+$ region, and because of the lack of similarity in restriction maps, I propose that at least 17-kb of DNA separates *entB*$^+$ from *ent(DC)EA*$^+$ on the chromosome of *E. carotovora* W3C105.

In *E. coli* (Earhart, 1987) and *Shigella flexneri* (Schmitt and Payne, 1988), all genes involved in enterobactin biosynthesis, uptake and utilization are located on a

single 25-kb genomic fragment. The *entD*$^+$ gene is separated from *entCEBA*$^+$ by approximately 16.6-kb. The remaining genes (*entCEBA*$^+$) are clustered on a 7.0-kb fragment (Earhart, 1987). This arrangement of catechol biosynthesis genes is common among members of the Enterobacteriaceae that produce a variety of catechol siderophores including *A. hydrophila* (enterobactin, amonabactin), *E. coli* (enterobactin), and *E. chrysanthemi* (chrysobactin) (Rowe Byer and George Massad, Personal communication). The *entB* gene, which is generally flanked by *entE* and *entA* genes within the *ent* gene cluster, was located distal to the *ent(DC)EA* cluster of *E. carotovora*. In *E. carotovora*, the relative order of the *entC* and *entD* genes was not resolved. The relative order of *entC*, *entE*, and *entA* may be similar to other enteric bacteria except for a distal location of *entB*$^+$ in W3C105. From the evidence presented, I suggest that arrangement of genes involved in catechol biosynthesis by *E. carotovora* differs from that of other members of the Enterobacteriaceae investigated.

Generally, *ent* genes of *E. coli* did not hybridize, even under low stringency conditions, to their functional counterparts in *E. carotovora*. Only the *entE*$^+$ region of genomic DNA of *E. carotovora* W3C105 hybridized to the *entE*$^+$ gene of *E. coli* (FIGURE 1-8). Thus, the *entE*$^+$ genes of *E. carotovora* and *E. coli* may be structurally as well as functionally similar. In contrast, enterobactin-biosynthesis mutants *entD*, *entC147*, *entA*, and *entB* of *E. coli* were complemented by genomic DNA from W3C105, but they did not hybridize to their functional counterparts from *E. carotovora* and may be structurally distinct. Lack of hybridization under the low stringency conditions used in hybridization studies indicates that the sequences differ by at least 80-85% (Ausubel *et al.*, 1987). Similarly, genes for enterobactin and amonabactin production of *A. hydrophila* complement enterobactin biosynthesis mutants of *E. coli* but do not hybridize to the *E. coli* genes (Rowe Byer and George Massad, Personal communication). Thus, most enterobactin-

biosynthesis genes of *E. coli* did not hybridize to DNA of *E. carotovora*, but a siderophore similar to enterobactin may be produced by *E. carotovora*.

The *entF* mutation from *E. coli* was not complemented by genomic DNA from W3C105, nor did the *entF*$^+$ gene hybridize to W3C105 genomic DNA. The EntF protein is responsible for the activation of L-serine, a process that occurs late in the enterobactin biosynthesis pathway. The pathway of catechol siderophore production of *E. carotovora* may be divergent from the enterobactin biosynthesis pathway of *E. coli* with respect to the late steps in siderophore production. This indicates that the catechol produced by W3C105 was similar but not identical to enterobactin.

Siderophore mediated iron-uptake involves outer-membrane proteins that transport specific ferric-siderophores (Neilands, 1982). In *E. coli*, the outer-membrane receptor for ferric-enterobactin is the FepA protein, encoded by the *fepA* gene. FepA is also a receptor for the catechols produced by *E. carotovora* as was demonstrated by cross-feeding of *S. typhimurium enb-1* and *E. coli* AN93. Complementation of *fepA* mutants with cloned DNA of W3C105 and hybridization to *fepA*$^+$ genes from *E. coli* were used in attempts to identify outer-membrane receptor genes of W3C105. The *entB*$^+$ cosmids of W3C105 complemented mutants of *E. coli* for iron-limited growth, indicating that these cosmids may contain a *fepA*-like gene. The possibility that *fepA* gene is present in the *entB*$^+$ region of *E. carotovora* may be confirmed in future studies correlating the presence of a putative *fepA* gene with that of a novel outer-membrane protein that is recognized by antisera to FepA in Western blots. Western-blot analysis has been useful for identification of outer-membrane receptors for ferric-aerobactin (Ishimaru and Loper, 1992) and ferric-chrysobactin (Enard et al., 1991) in *Erwinia* spp.

The 81-kDa outer-membrane FepA protein of *E. coli* is a receptor for colicins B and D (Gutterman and Dann, 1973); Fep$^+$ strains are sensitive whereas Fep$^-$ strains

are insensitive to colicins B and D. *Erwinia carotovora* strain W3C105 was not sensitive to colicin B nor did the *ent*B$^+$ and *ent(DC)EA*$^+$ regions of W3C105 confer colicin B sensitivity to FepA$^-$ mutants of *E. coli*. An outer-membrane protein functioning as a receptor for a catechol siderophore undoubtedly was present but did not confer colicin B sensitivity to *E. carotovora*. Similarly, the aerobactin receptor protein Iut does not confer cloacin DF13 sensitivity to *E. carotovora* as it does to *E. coli* (Ishimaru and Loper, 1992). Nevertheless, introduction of a cloned *iut* gene from *E. carotovora* confers cloacin DF13 sensitivity to *E. coli*. Apparently, the confirmation of the Iut protein in the outer membrane of *E. carotovora* differs from that in *E. coli*; although the Iut protein functions as a ferric-aerobactin receptor in both bacterial species, it functions as a receptor of cloacin DF13 only in *E. coli*. At present, the possibility that a FepA-like protein from *E. carotovora* confers colicin B sensitivity to *E. coli* cannot be excluded. Nevertheless, a *fepA*-like gene that confers colicin B sensitivity was not expressed from the *entB*$^+$ or *ent(DC)EA*$^+$ regions of *E. carotovora* that were cloned in this study. A gene for the receptor of a catechol-siderophore produced by *E. carotovora* may not confer colicin B sensitivity in either *E. carotovora* or *E. coli*. In *E. coli*, the colicin B and ferric-enterobactin receptor functions are separable by mutation (McIntosh et al., 1979) and thermal sensitivity. Deletions in *fepA* commonly result in a protein that is not recognized by colicins B and D but functions in ferric-enterobactin transport (Pugsley and Schnaitman, 1979, McIntosh et al., 1979). The outer membrane receptor for the catechol siderophore produced by strain W3C105 may be analogous to the products of these *fepA* mutants, functioning as receptors for ferric-enterobactin but not for colicins B and D.

Cloned DNA from W3C105 that complemented the *fes* mutant was not identified and *fes*$^+$ regions of *E. coli* did not hybridize to *E. carotovora* DNA. Originally, it was hypothesized that Fes-mediated release of iron from the ferric siderophore complex was dependant upon the esterase activity that occurred

concurrently with iron release (O'Brien *et al.*, 1971). The esterase activity is not necessary for the release of iron via Fes-dependant process, however (Heidringer, *et al.*, 1983; Hollifield and Neilands, 1978; Veenuti, *et al.*, 1979). A ferric-siderophore reductase has been proposed to be involved in the release of iron (Hollifield and Nielands, 1978) although no direct evidence for this enzyme has been found (Earhart, 1987). *Agrobacterium tumefaciens* and *Bacillus subtillus* produce compounds that reduce the ferric-enterobactin complex and release iron (Gaines *et al.*, 1981; Lodge *et al.*, 1982; 1980). The lack of hybridization of the *fes*$^+$ gene from *E. coli* to genomic DNA of W3C105 may indicate that although Fes and the Fes-like product from W3C105 are functionally related, the genes coding for these products are less than 85% homologous. Alternatively, genes coding for a Fes-like product in W3C105 may not be linked with *entB*$^+$ or *ent(DC)EA*$^+$ and therefore did not hybridize to the cloned regions of W3C105. Another explanation for the lack of hybridization of *fes*$^+$ genes *E. coli* to DNA of W3C105 may be that there is a different mechanism involved in the release of iron in *E. coli* than in *E. carotovora.*

The catechol(s) produced by *E. carotovora* was distinct from chrysobactin. Catechol-producing strains of *E. carotovora* provided iron to *S. typhimurium enb-1* and *E. coli* AN93, which use enterobactin but do not use other catechol siderophores as a source of iron. In contrast, chrysobactin was not used as a source of iron by *enb-1* nor AN93. These cross-feeding data distinguished the catechol(s) produced by *E. carotovora* strains from chrysobactin produced by *E. chrysanthemi.* The catechol biosynthesis genes from *E. c.* subsp. *carotovora* W3C105 also appeared to be different than those for chrysobactin biosynthesis by *E. chrysanthemi* 3937. Catechol biosynthesis genes from W3C105, *ent(DC)EA*$^+$, did not hybridize to genomic DNA from strain 3937. In addition, the *entD* mutation of *E. coli* was complemented by a 16.6-kb fragment designated *ent(DC)EA*$^+$ in *E. carotovora.* A 14-kb fragment from *E. chrysanthemi* complements *entC*, *entE*, *entB*, and *entA*, but

does complement *entD* (Enard *et al.*, 1991; Franza and Expert, 1991). The arrangement of catechol siderophore-biosynthesis genes in *E. chrysanthemi* is similar to that of *E. coli*, *S. flexneri*, or *A. hydrophila* (Rowe Byer and George Massad, Personal communication) and distinct from that of *E. carotovora*. Also, restriction patterns of the catechol-biosynthesis region of *E. carotovora* W3C105 differed from that of *E. chrysanthemi* 3937 (Enard *et al.*, 1991; Franza and Expert, 1991).

An *entEA*$^+$ genomic region of W3C105 (pJEL1868) that hybridized to the *entE* gene of *E. coli*, also hybridized to genomic DNA of all of the strains of *E. carotovora* tested. Restriction fragment length polymorphisms (RFLP) occurred among these strains but did not correspond to the serological groups of the species tested (TABLE 1-9). A related pathogen, *E. chrysanthemi*, is highly polymorphic according to RFLP analysis of genes involved in production of pectic enzymes. Ten RFLP groups were identified for 52 strains tested (Boccara *et al.*, 1991). In this study, *E. carotovora* species were highly polymorphic with respect to catechol-biosynthesis genes.

In *Erwinia carotovora* subsp. *carotovora* W3C105, catechol production is inversely related to the concentration of iron available in the media (FIGURE 1-1). This is similar to the response of *E. coli* AN194 to iron. The relationship between available iron and catechol production supports the hypothesis that the catechol produced by W3C105 is an iron-regulated siderophore. In *S. typhimurium* (Ernst *et al.*, 1978) and *E. coli* (Hantke, 1981), enterobactin uptake is regulated at the transcriptional level by *fur* (*f*erric *u*ptake *r*egulator), which in turn is regulated by iron concentration (Bagg and Neilands, 1985). Iron regulation of the enterobactin biosynthesis genes in *E. coli* were demonstrated with *ent-lacZ* fusions (Fleming *et al.*, 1983; Nahilik *et al.*, 1989). Transcription of catechol biosynthesis genes may also be regulated by iron in W3C105. Future studies utilizing transcriptional fusions to catechol biosynthesis genes of *E. carotovora* will allow an investigation of

the possibility that iron regulation of catechol biosynthesis is at the level of transcription.

This study determined that *E. carotovora* produced a catechol(s) that was used as a functional siderophore(s) by *E. coli* and *S. typhimurium*. Chemical characterization of the catechol produced by *E. carotovora* is needed to establish its relationship to enterobactin and chrysobactin. The genetic characterization of catechol production of W3C105 is an important first step in determining the role of the catechol in the virulence and ecology of *E. carotovora*. In order to conclude that the catechol produced by *E. carotovora* is a siderophore, however, the role of the catechol in iron-acquisition by *Erwinia carotovora* must be demonstrated.

CHAPTER 2. GENERATION OF CATECHOL-BIOSYNTHESIS MUTANTS OF *ERWINIA CAROTOVORA*.

INTRODUCTION

Erwinia carotovora produces a catechol(s) with siderophore-like properties (Leong and Neilands, 1982; Bull, Chapter 1). Catechol(s) produced by *E. carotovora* cross-feeds mutants of *E. coli* that are deficient in the biosynthesis of the catechol siderophore enterobactin but can utilize ferric-enterobactin as a source of iron. Genes involved in catechol production of *E. carotovora* strain W3C105 complement a number of enterobactin-biosynthesis mutants, allowing for iron-independent growth of *E. coli*. Nevertheless, the role of catechol(s) in the iron-acquisition of *E. carotovora* has not been demonstrated.

Definitive evidence for the role of catechol in the iron-acquisition of *E. carotovora* strain W3C105 will require the isolation of derivative strains that do not produce catechol. Mutant strains, which utilize but do not produce a siderophore, are useful tools for demonstrating a role for exogenously-provided siderophore in iron nutrition. For example, the importance of an aerobactin iron-acquisition system of *E. carotovora* was demonstrated by contrasting the iron-limited growth of aerobactin-deficient mutants and a parental strain (Ishimaru and Loper, 1992). Similar studies, contrasting the iron-independent growth of catechol-producing and non-producing strains, will elucidate the role of catechol production in iron nutrition of *E. carotovora*. The availability of cloned catechol biosynthesis genes from W3C105 (Bull, Chapter 1) provides an opportunity to derive specific mutations in catechol production through marker-exchange mutagenesis. Marker-exchange

mutagenesis, which is used to generate well characterized mutations in the genomes of plant pathogens, has been a valuable approach for elucidating the importance of specific phenotypes to the virulence, pathogenicity, and physiology of *E. carotovora* (Ellard, *et al.*, 1989; Zink, *et al.*, 1984; Roeder and Collmer, 1985; Hinton, *et al.*, 1985; Salmond, *et al.*, 1986; Jayaswal, *et al.*, 1984). Isogeneic Cat^+ and Cat^- strains, derived by marker-exchange mutagenesis, can be compared for iron-limited growth to demonstrate the role of catechol production as an iron-acquisition system for *E. carotovora.*

Important to the demonstration that key metabolites, such as siderophores, are involved in virulence and biological control, is the demonstration of *in situ* production of siderophores. With the exception of schizokinen (Akers, 1983), siderophores have not been directly isolated from plant and soil samples. In environments in which a key phenotype can not be directly isolated, reporter gene systems are used to observe gene regulation and production of the compounds of interest (Lindgren *et al.*, 1989; Loper *et al.*, 1991; Jayaswal, *et al.*, 1984). Reporter gene systems have readily quantifiable phenotypes that can be fused to the regulatory elements of a native gene with a phenotype that is difficult to quantify and/or detect. Quantification of the product of the reporter gene is a measure of the transcriptional activity of the native promoter. The location and orientation of reporter genes fusions that result in reporter gene activity indicate the location and orientation of the native gene. Thus, reporter gene systems are used to elucidate operon structure and gene expression. The usefulness of reporter gene systems varies according to the reporter used, specificity and detection limits of assays for the reporter, and interfering activities present in samples being tested (Lindgren *et al.*, 1989). The *inaZ* reporter gene system (Lindgren *et al.*, 1989) is used to generate transcriptional fusions to the promoterless *inaZ* gene, which confers ice nucleation activity. It is attractive for use in studying compounds involved in plant-microbe interactions and biological control because detection of ice nucleation

activity is convenient, sensitive, (Lindgren *et al.*, 1989) and does not depend on enzymatic activity, constituents of environmental samples do not interfere with the assay, and background levels of ice nuclei in environmental samples are low (Lindgren, *et al.*, 1989; Lindow, 1990; Loper and Lindow, 1991; Lindow and Loper, 1990).

In this study, I describe the use of Tn*3*::Spice to mutagenize the catechol biosynthesis genes of *E. c.* subsp. *carotovora* W3C105. A role of catechol siderophore production in iron-independent growth of W3C105 was demonstrated.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains used in these studies are listed in TABLE 2-1. Plasmids are listed in TABLE 2-2. *Escherichia coli*, *Erwinia chrysanthemi* and *Salmonella typhimurium* strains were cultured routinely on Luria Bertani medium (LB) (Gerhardt *et al.*, 1981) at 37 C and *Erwinia carotovora* on LB at 27 C. LB agar was supplemented with 5-bromo-4-chloro-3 indolyl-β-d-galactopyranoside (X-Gal) (40 μg/ml) (International Biotechnologies, Inc.) and isopropyl-β-d-thiogalactopyranoside (IPTG) (100 μg/ml), in some cloning experiments. CVP (Cuppels and Kelman, 1974) and pectate agar (Beraha, 1968) were used as selective media for *Erwinia* spp.. Double diffusion agar consisted of 0.8% (w/v) agar, 0.85% NaCl, 200 ppm sodium azide (De Boer *et al.*, 1979b). RGMC medium consisted of 1.0% (w/v) tryptone, 0.1% (w/v) yeast extract (Difco Laboratories, Detroit, MI), 0.8% (w/v) NaCl, 0.1% (w/v) glucose, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1.5% (w/v) agar (Simon *et al.*, 1983). Catechol and aerobactin production was determined from cultures grown in tris minimal salts medium (TMS) (Simon and Tessman, 1963), supplemented with tryptophan (0.003% w/v), and thiamine (0.0002% w/v) and deferrated cas amino acids (0.3% w/v). The iron availability of TMS medium was varied by adding $FeCl_3$ (100 μM or 0.1 μM) or 2,2'-dipyridyl (75 μM, 90 μM, 105 μM, 120 μM, 135 μM, 150 μM, or 225 μM). Antibiotics were used at the following concentrations: ampicillin (Ap, 100 μg/ml), tetracycline (Tc, 20 μg/ml), spectinomycin (Sp, 50 μg/ml), streptomycin (Sm, 100 μg/ml), kanamycin (Km, 50 μg/ml), rifampicin (Rif, 100 μg/ml), nalidixic acid (Nal, 100 μg/ml). All chemicals were purchased through Sigma Chemical Co., (St. Louis, MO) unless otherwise specified.

TABLE 2-1. Bacterial strains used for Chapter 2

Strain	Description	Relevant characteristics	Source or reference
Escherichia coli:			
DH5α	F⁻, *endA1*, *hsd17* (r_k^-, m_k^+), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, ø80*dlacZ*Δ, m15, λ⁻	Ent⁺	Bethesda Research Laboratories
HB101	*rpsL20*, Sm^r	Ent⁺	Boyer and Roulland-Dussoix, 1969
S17-1	*pro*, *res*, *mod*⁺, Tp^r, Sm^r	Ent⁺, Tra⁺, Sm^r	Simon *et al.*, 1983
C2110	*polA*, rec⁺, *mod*⁻, Nal^r	Ent⁺	Stachel *et al.*, 1983
AN194	F⁻, *tonA23*, *proC14*, *leu16*, *trpE38*, *thi-1*, Sm^r	Ent⁺	Langman, *et al.*, 1972
AN193	*entA403* derivative of AN194, Sm^r	EntA403⁻, Sm^r	J. B. Neilands
AN192	*entB402* derivative of AN194, Sm^r	EntB402⁻, Sm^r	J. B. Neilands
AN191	*entC401* derivative of AN194, Sm^r	EntC401⁻, Sm^r	J. B. Neilands
AN93	*entE405* derivative of AN194, Sm^r	EntE405⁻, Sm^r	Schwyn and Neilands, 1987
AN90	*entD* derivative of AN194, Sm^r	EntD⁻, Sm^r	Cox *et al.*, 1970
MT147	*entC147*::Km derivative of AB1515	EntC147⁻, Km^r	Ozenberger *et al.*, 1989
JL1628	Spontaneous Rif^r mutant of AN193	EntA403⁻, Rif^r	Ishimaru, unpublished
JL1753	Spontaneous Rif^r mutant of AN93	EntE405⁻, Rif^r	This study
JL1754	Spontaneous Rif^r mutant of AN191	EntC401⁻, Rif^r	This study
JL1773	Spontaneous Rif^r mutant of AN90	EntD⁻, Rif^r	This study
JL1774	Spontaneous Rif^r mutant of AN117	EntF⁻, Rif^r	This study
JL1794	Spontaneous Rif^r mutant of AN192	EntB402⁻, Rif^r	This study
JL1886	Spontaneous Rif^r mutant of MT147	EntC147⁻, Rif^r	This study

TABLE 2-1. (Continued)

Salmonella typhimurium:			
enb-1	Derivative of LT-2, *asc-1*, Sm^r	Ent^-, Sm^r	Pollack *et al.*, 1970
enb-7	Derivative of LT-2, *asc-1*, Sm^r	Ent^-, $DHBA^-$, Sm^r	Pollack *et al.*, 1970
JL1882	Spontaneous Rif^r mutant of *enb-1*	Ent^-, Sm^r, Rif^r	This study
JL1893	Spontaneous Rif^r mutant of *enb-7*	Ent^-, $DHBA^-$, Sm^r, Rif^r	This study
Erwinia carotovora subsp. *carotovora*:			
W3C105	Field isolate	Cat^+, Iuc^+, Iut^+	Xu and Gross, 1980
JL1148	Spontaneous Rif^r mutant of W3C105	Cat^+, Iuc^+, Iut^+, Rif^r	Ishimaru, unpublished
JL11178	Derivative of W3C105, Δ(*iuc*)	Cat^+, Iuc^-, Iut^+	Ishimaru and Loper, 1992
JL11182	Derivative of W3C105, Δ(*iuc-iut*)	Cat^+, Iuc^-, Iut^-	Ishimaru and Loper, 1992
JL11146	JL1148::Tn*3*-Spice marker-exchange mutant with pJEL1752	Cat^-, Iuc^+, Iut^+, Rif^r, Sp^r	This study
JL11197	JL1148(pJEL1703)	Cat^+, Iuc^+, Iut^+, $IceC^+$, Km^r	
JL11199	W3C105(pJEL1752)	Cat^+, Iuc^+, Iut^+, Sp^r	This study
JL11200	JL11178(pJEL1752)	Cat^+, Iuc^-, Iut^+, Sp^r	This study
JL11201	JL11182(pJEL1752)	Cat^+, Iuc^-, Iut^- Sp^r	This study
JL11202	W3C105::Tn*3*-Spice marker-exchange mutant with pJEL1752	Cat^-, Iuc^+, Iut^+,	This study
JL11206	JL11178::Tn*3*-Spice marker-exchange mutant with pJEL1752	Cat^-, Iuc^-, Iut^+	This study

TABLE 2-1. (Continued)

JL11207	JL11182::Tn*3*-Spice marker-exchange mutant with pJEL1752	Cat$^-$, Iuc$^-$, Iut$^-$	This study
JL11208	JL11178(pJEL1534)	Cat$^+$, Iuc$^+$, Iut$^+$	This study
JL11209	JL11182(pJEL1534)	Cat$^+$, Iuc$^+$, Iut$^+$	This study
JL11210	JL11202(pJEL1602)	Cat$^+$, Iuc$^+$, Iut$^+$	This study
JL11211	JL11206(pJEL1534)	Cat$^-$, Iuc$^+$, Iut$^+$	This study
JL11212	JL11206(pJEL1602)	Cat$^+$, Iuc$^-$, Iut$^+$	This study
JL11213	JL11207(pJEL1534)	Cat$^-$, Iuc$^+$, Iut$^+$	This study
JL11214	JL11208(pJEL1602)	Cat$^+$, Iuc$^-$, Iut$^-$	This study
SRI193	Field isolate	Cat$^+$	A. Kelman
SR319	Field isolate	Cat$^+$	Salmond and Hinton,
Erwinia chrysanthemi:			
3937	Field isolate	Cbs$^+$	Enard *et al.*, 1989

Ent$^+$ and Ent$^-$, enterobactin producer or nonproducer, respectively; Nal^r, nalidixic acid resistant; Iuc$^+$ and Iuc$^-$, aerobactin producer or nonproducer, respectively; Iut$^+$ and Iut$^-$, possesses or lacks, respectively, the outer membrane receptor for ferric aerobactin; Cat$^+$ and Cat$^-$, catechol producer or nonproducer, respectively; Cbs$^+$, chrysobactin producer.

TABLE 2-2. Plasmids used for Chapter 2

Plasmid	Description	Source or reference
pSShe	*tnpA*$^+$, Cm^r	Stachel *et al.* (1985)
pRK2013	IncP, TraRK2$^+$, Km^r	Ditta *et al.* (1980)
pTn*3*-Spice	*inaZ*$^+$, Ap^r, Sp^r, Sm^r	Lindgren *et al.* (1989)
pJEL1703	*iceC*$^+$, Km^r	Lindow and Loper, (1990)
pJEL1602	*ent(DC)EA*$^+$, Tc^r	Bull, Chapter I
pJEL1742	pJEL1602::Tn*3*-Spice, *entA*$^-$, *ent(DC)E*$^+$, Tc^r, Sp^r	This study
pJEL1751	pJEL1602::Tn*3*-Spice, *entA*$^-$, *ent(DC)E*$^+$, Tc^r, Sp^r	This study
pJEL1752	pJEL1602::Tn*3*-Spice, *entE*$^-$, *ent(DC)A*$^+$, Tc^r, Sp^r	This study
pJEL1755	pJEL1602::Tn*3*-Spice, *entA*$^-$, *ent(DC)E*$^+$, Tc^r, Sp^r	This study
pJEL1804	2.0-kb *Eco*RI fragment subcloned from pJEL1602, hybridized to *entE* of *E. coli*, *ent(DC)EA*$^-$, in pUC8, Ap^r	Bull, Chapter I

Detection of siderophore production.

Siderophore production by bacterial strains was detected by observation of orange halos surrounding bacterial colonies grown on CAS agar. CAS agar, a universal siderophore detection medium, was prepared as described (Schwyn and Neilands, 1987). Catechol and hydroxamate were detected in supernatants of bacterial cultures grown for 24-48 h in TMS. Catechol was detected and quantified as described by Arnow (1937) or Rioux *et al.* (1983). Hydroxamate was detected and quantified by published methods (Csáky, 1948; Atkin *et al.*, 1970).

Enterobactin bioassay.

Enterobactin production was detected by a cross-feeding bioassay that relies upon the inability of indicator strains of *E. coli*, *S. typhimurium* and *E. c.* subsp. *carotovora*, which had mutations in the catechol siderophore biosynthesis pathway, to grow unless enterobactin or DHBA, an intermediate in the biosynthesis of enterobactin, was provided from an exogenous source (Pollack *et al.*, 1970). Molten TMS agar containing 2,2'-dipyridyl (150 μM) was seeded with an indicator strain (10^6 cfu/ml) that had been grown overnight with shaking in TMS broth. Strains to be tested for enterobactin production also were grown overnight with shaking in TMS broth. Ten microlitres of overnight test culture was spotted onto the surface of solidified, seeded TMS medium and incubated at 27 C. After 24-48 h, plates were observed for growth of an indicator strain surrounding DHBA- and enterobactin-producing colonies. Strains AN194, which produces enterobactin, and AN192, which produces neither enterobactin nor DHBA, were test strains included as controls in all experiments.

Serology.

Serological relatedness of strains was tested by methods adapted from De Boer

et al. (1979b). The antiserum, derived as monoclonal antibodies of serogroup 39, was provided by Mary Powelson. Double diffusion tests were done in agar plates, into which eight wells (3 mm diameter) were cut 4 mm from a well in the center. Bacteria were grown 24 h on LB, washed, and suspended in 1 ml sterile deionized water to a concentration of 10^9-10^{10} cells/ml. One drop of phenol was added to the cell suspension and mixed thoroughly. Two adjacent peripheral wells were filled with 100 μl of a bacterial suspension and the center well was filled with 100 μl of antiserum. *Erwinia carotovora* subsp. *carotovora* W3C105 (serogroup 39) and *E. c.* subsp. *atroseptica* W3C37 (unknown serogroup) were included as positive and negative controls, respectively. Plates were incubated in plastic containers at room temperature. Results were recorded after two days.

Recombinant DNA techniques.

Mobilization of recombinant cosmids into *E. coli* or Rif^r strains of *E. carotovora* was accomplished by tri-parental matings using the helper plasmid pRK2013 (Figurski and Helinski, 1979). In matings with *E. c.* subsp. *carotovora* W3C105, the mobilizing strain *E. coli* S17-1 was used as a donor (Simon *et al.*, 1983). Strain S17-1 has transfer functions (*tra*$^+$) cloned into the chromosome, and serves as a donor of mobilizable plasmids. Aqueous suspensions of donor strain S17-1(pJEL1703) and recipient strain W3C105 were mixed 1:1 (v:v) and 100 μl of the mixture was spread on RGMC agar. Plates were incubated overnight at 27 C. An aliquot of the mating mixture was spread onto CVP amended with kanamycin. Transconjugants were confirmed by plasmid isolation and serology. Transformation of *E. coli* and *S. typhimurium* was performed according to the procedure described for *E. coli* DH5α by Bethesda Research Laboratories (BRL, Bethesda, MD).

Plasmids were isolated from *E. coli*, *S. typhimurium*, and *E. carotovora* by an alkaline-lysis extraction procedure and were further purified by cesium chloride

density-gradient centrifugation for Southern hybridization analyses and cloning experiments (Sambrook *et al.*, 1989). Genomic DNA was isolated according to published methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1987). Restriction endonucleases were used according to recommendations of the supplier (BRL). Restriction fragments were separated by gel electrophoresis at 50 V for 3 h or at 20 V overnight in 0.7% agarose gels (SeaKem LE, FMC BioProducts, Rockland, ME).

Nucleic acid isolation and hybridization.

DNA was transferred from agarose gels to nylon membranes (Nytran, Schleicher & Schuell [S&S], Keene, NH) by standard methods (Sambrook *et al.*, 1989). Probes used in hybridization studies were the 3.6-kb *Eco*RI fragment containing the *inaZ*$^+$ gene from pTn*3*-Spice or the 8.8-kb *Kpn*I fragment containing 1.8-kb of *inaZ*$^+$ from pJEL1752. Fragments used as probes were purified from agarose gels (SeaKem GTG, FMC BioProducts) by adsorption and elution from NA-45 DEAE membranes (S&S). Probes were prepared by nick translation of the fragments with ^{32}P-dCTP (New England Nuclear, Boston, MA) and were used at concentrations of 0.025 μg/ml. Hybridization conditions were of moderate stringency (55-62 C, 50% formamide and 0.2 X SSC) (Sambrook *et al.*, 1989).

Transposon mutagenesis of W3C105 cloned sequences.

Tn*3*-Spice was used to insertionally inactivate catechol biosynthesis genes (Lindgren *et al.*, 1989). Cosmid pJEL1602 contained a genomic region from W3C105 that complemented *entD*, *entC147*, *entE*, and *entA* mutations of *E. coli* and *enb*-1 and *enb*-7 mutants of *Salmonella typhimurium* (Bull, Chapter1; Bull and Loper, 1991; Bull *et al.*, 1989). This region was designated *ent(DC)EA*$^+$ (Bull, Chapter 1). Cosmid pJEL1602 was mutagenized with Tn*3*::Spice. Cosmid pJEL1602 was mobilized by mating into *E. coli* HB101(pTn*3*-Spice, pSShe). Tri-

parental matings were done with *E. coli* HB101(pSShe, pTn*3*-Spice, pJEL1602) serving as the donor strain, *E. coli* DH5α(pRK2013) as the helper, and *E. coli* C2110 as the recipient. To avoid screening sibling colonies, ninety-six individual matings were performed using a rapid multiple-mating technique. The multiple-mating technique involved inoculating an LB agar plate with lawn of a 1:1 mix of the helper and recipient bacteria. The donor bacteria were grown for 18 h on LB agar and were inoculated onto the lawn of the helper-recipient mixture using a 48-pronged replica-plating devise. Two multiple matings were performed yielding 48 individual matings per plate and 96 matings total. After 24 h at 27 C, the 48 pronged replica-plating devise was used to transfer cells from individual matings to LB agar amended with spectinomycin and tetracycline. Plates were incubated for 24 h and colonies were transferred twice more to the same media. Colonies resistant to tetracycline and spectinomycin were transferred with the replica-plating devise onto LB agar amended with spectinomycin and tetracycline in wells of a 96-well tissue-culture dish (Costar, Cambridge, MA) where they were stored at -80 C. The multiple mating technique was repeated using AN93 (*entE*), AN192 (*entA*) or AN193 (*entC104*) as recipients, C2110 (pJEL1602::Tn*3*-Spice) as donors and DH5α (pRK2013) as a helper. Transconjugants were selected on LB amended with streptomycin, spectinomycin, and tetracycline or on LB amended with rifampicin, spectinomycin, and tetracycline. Cosmids from four transconjugants that were not complemented for siderophore production, as detected on CAS agar, were isolated for further study. The four identified cosmids were tested for complementation of other enterobactin mutants. The Tn*3*-Spice insertions were mapped by restriction endonuclease and Southern hybridization analyses.

Derivation of Cat$^-$, Cat$^-$Iuc$^-$ and Cat$^-$Iuc$^-$Iut$^-$ strains of *E. carotovora*.

A marker-exchange mutagenesis technique modified from Roeder and Collmer (1985) was used to construct directed mutations in catechol-biosynthesis genes of *E.*

carotovora W3C105, JL1148(Rif^r W3C105), JL11178 (Iuc$^-$) and JL11182 (Iuc$^-$Iut^r). Cosmid pJEL1752 (pJEL1602::Tn*3*-Spice; *entE*$^-$), which was pLAFR3 containing inactivated catechol-biosynthesis genes from W3C105, was used in marker-exchange experiments. The Tn*3*-Spice insert conferred resistance to spectinomycin while resistance to tetracycline was conferred by pLAFR3. Cosmid pLAFR3 is used successfully as a vector for genomic fragments to be rescued by homologous recombination into W3C105 genome (Ishimaru and Loper, 1992). Cosmid pLAFR3 is not stably maintained in *E. carotovora* W3C105, and is lost from 90% of cells after 3 days in the absence of selection for tetracycline (Ishimaru and Loper, 1992). Transconjugants W3C105(pJEL1752), JL1148(pJEL1752), JL11178(pJEL1752) and JL11182(pJEL1752) were grown at 27 C with shaking in LB broth (200 ml in 1000 ml flask) in the absence of antibiotics. After 12 h, 0.1 ml of the culture was transferred to fresh LB broth (200 ml in 1000 ml flask). After 6 successive transfers, dilutions of the culture were spread onto LB agar amended with tetracycline or spectinomycin. Spectinomycin-resistant, tetracycline-sensitive colonies were isolated as putative marker-exchange mutants. The identity of the mutants as derivatives of *E. carotovora* W3C105 was confirmed on CVP agar and by serology. The *inaZ*$^+$ gene was used as a probe in Southern hybridizations to ensure that mutants had single insertions of Tn*3*-Spice.

Characterization of mutants.

Putative marker-exchange mutants were compared to the wildtype W3C105 for the production of pits on CVP agar, growth on a minimal medium (TMS), growth rate in LB broth, growth under iron-limiting conditions, and serological relatedness (De Boer *et al.*, 1979b). Generation times of mutants and parental strains were determined by the change in OD_{600nm} of cultures grown with shaking in LB broth at 27 C.

Ice-nucleation activity of bacteria containing cosmids with Tn*3*-Spice insertions and marker-exchange mutants.

Ice nucleation activity was measured at 7 C or 5 C by a droplet-freezing technique similar to that previously described (Lindow, 1990). Strains were grown at 27 C for 48 h in TMS broth (5 ml) amended with iron or 2,2'-dipyridyl. The effect of growth media on ice nucleation activity of *E. carotovora* was tested by evaluating cultures grown for 48 h at 27 C on LB, TMS, Pectate, KB (King *et al.*, 1954), NA (Difco), and PDA (Difco).

RESULTS

Analysis of Tn*3*-Spice insertions within cloned catechol biosynthesis genes of *E. carotovora*.

Each of 96 individual matings of HB101(pSShe, pTn*3*-Spice, pJEL1602) and C2110 yielded colonies that were resistant to nalidixic acid, tetracycline, and spectinomycin. These cells were presumably C2110(pJEL1602::Tn*3*-Spice) transconjugants, each with an independent insertion of Tn*3*-Spice. Of the 96 cosmids with Tn*3*-Spice insertions, three (pJEL1742, pJEL1751, pJEL1755) did not complement the *entA* mutation of *E. coli* nor *enb-1* or *enb-7* mutations of *S. typhimurium* (TABLE 2-3). One cosmid (pJEL1752) did not complement the *entE* mutation of *E. coli* nor the *enb-7* mutation of *S. typhimurium*. All four cosmids complemented *entC* and *entD*.

All four Tn*3*-Spice insertions were in a 15.5-kb *Hin*dIII fragment of pJEL1602. The Tn*3*-Spice insertions inactivating *entA* were located within the 5.6-kb *Kpn*I fragment in pJEL1742, pJEL1751, pJEL1755 (FIGURE 2-1). Orientations of the Tn*3*-Spice insertions were determined from Southern blots (FIGURE 2-2) probed with the 3.6-kb *Eco*RI fragment containing *inaZ*.

Mapping of Tn*3*-Spice insertions.

The Tn*3*-Spice insertion of pJEL1742 was located within a 5.6-kb *Bam*HI fragment and a 1.3-kb *Eco*RI fragment of pJEl1602 (data not shown). The *inaZ*$^+$ gene hybridized to an 11.0-kb *Kpn*I fragment and 4.7-kb *Kpn*I fragment of pJEL1742 (FIGURE 2-2). The 11.0-kb fragment contained 8.4 kb of Tn*3*-Spice (FIGURE 2-4) and 2.6 kb of target sequence. The 4.7-kb fragment contained 1.8 kb of Tn*3*-Spice and 2.9 kb from pJEL1602. A 15.0-kb *Hin*dIII fragment of

TABLE 2-3. Complementation of enterobactin-biosynthesis mutants with genomic regions cloned from W3C105 and mutagenized with Tn*3*-Spice

	Catechol-siderophore production[x]						
	Mutants of *E. coli*					Mutants of *S. typhimurium*	
Cosmid[y]	AN93 (*entE405*)	AN193 (*entA403*)	AN191 (*entC104*)	MT147 (*entC147*)	AN90 (*entD*)	*enb-1*	*enb-7*
pJEL1602[z]	+	+	+	+	+	+	+
pJEL1742	+	-	-	+	+	-	-
pJEL1751	+	-	-	+	+	-	-
pJEL1752	-	+	+	+	+	+	-
pJEL1755	+	-	-	+	+	-	-

[x]Catechol-siderophore production was detected by observation of a halo surrounding a colony grown on CAS agar, cross-feeding of strain AN93 and *enb-1*, and by catechol assays (Arnow, 1937; Rioux *et al.*, 1984). + = catechol siderophore produced; - = no catechol siderophore produced.
[y]Presence of plasmids in enterobactin mutants was confirmed by extraction.
[z]Plasmids were mobilized into *E. coli* (except MT147, *entC147*) by conjugal transfer and into *S. typhimurium* and *E. coli* MT147 by transformation.

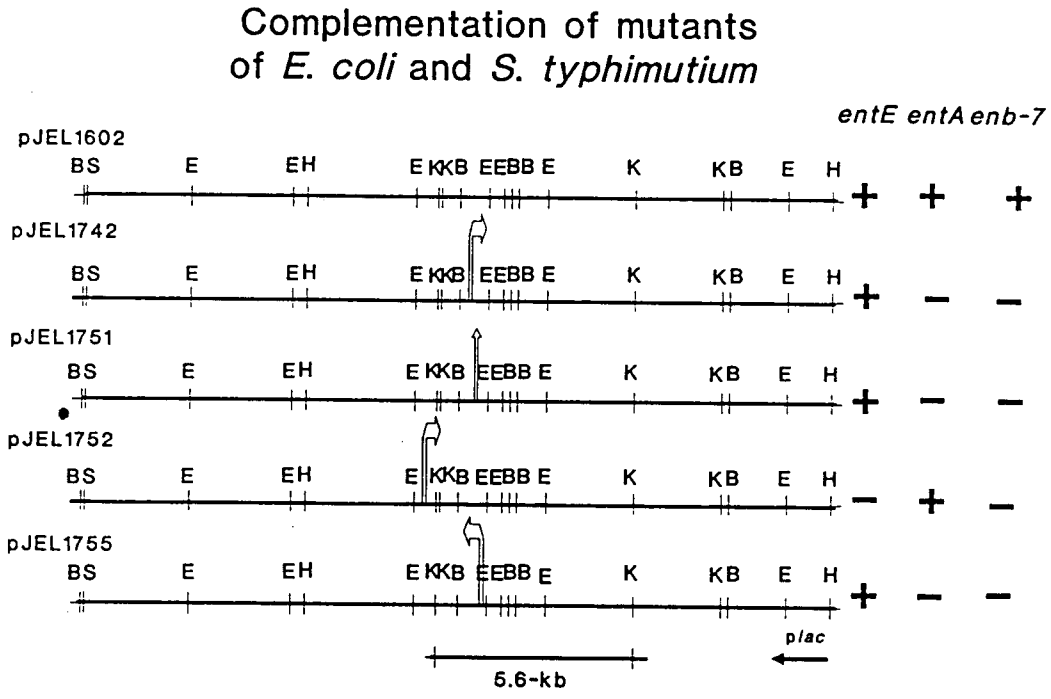
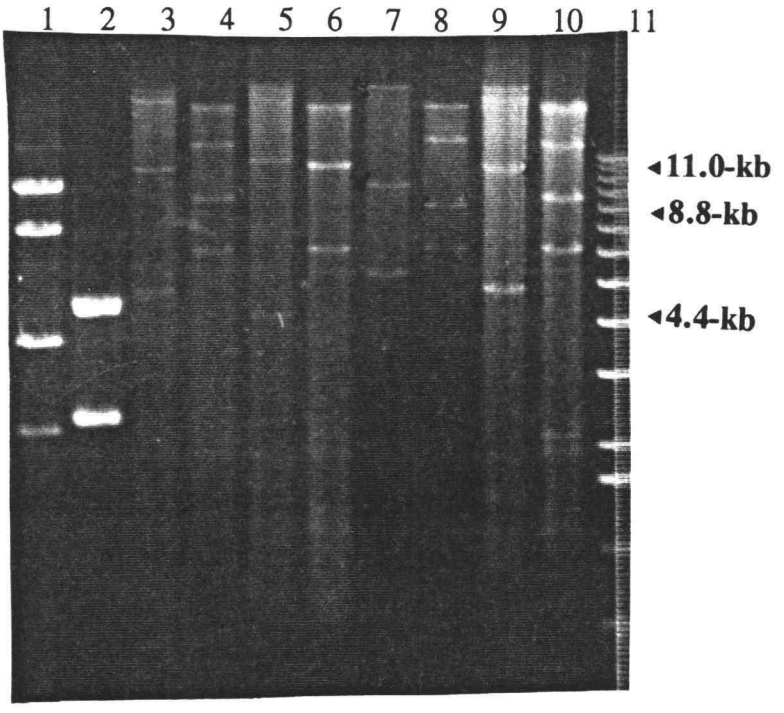


FIGURE 2-1. Physical maps of cloned catechol biosynthesis genes from W3C105 with Tn*3*-Spice insertions.
Complementation (+) and lack of complementation (-) corresponded to siderophore production assayed on CAS agar, catechol production assayed by methods of Arnow (1937) and Rioux *et al.* (1983) and by cross-feeding bioassays. Horizontal lines refer to genomic DNA derived from W3C105. Cosmid vectors are not shown. The orientation of the p*lac* is shown. Arrows indicate the location of Tn*3*-Spice insertions and the relative orientation of the *inaZ*$^+$ gene. The orientation of the Tn*3*-Spice insertion of pJEL1751 was not determined. Restriction-endonuclease cut sites are abbreviated; *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Kpn*I (K).

FIGURE 2-2. Southern blot analysis of catechol biosynthesis genes from W3C105 containing Tn*3*-Spice insertions.
An ethidium-bromide stained gel is above and an autoradiogram is below on the following page. The blot was hybridized with the 3.6-kb *Eco*RI fragment from pTn*3*-Spice containing the *inaZ*$^+$ gene. Lanes: 1) pTn*3*-Spice and pSShe, *Eco*RI; 2) pJEL1804, *Eco*RI; 3) pJEL1742, *Kpn*I; 4) pJEL1742, *Hin*dIII; 5) pJEL1751, *Kpn*I; 6) pJEL1751, *Hin*dIII; 7) pJEL1752, *Kpn*I; 8) pJEL1752, *Hin*dIII; 9) pJEL1755, *Kpn*I; 10) pJEL1755, *Hin*dIII; 11) 1-kb ladder.


1 2 3 4 5 6 7 8 9 10 11
11.0-kb
8.8-kb
4.4-kb

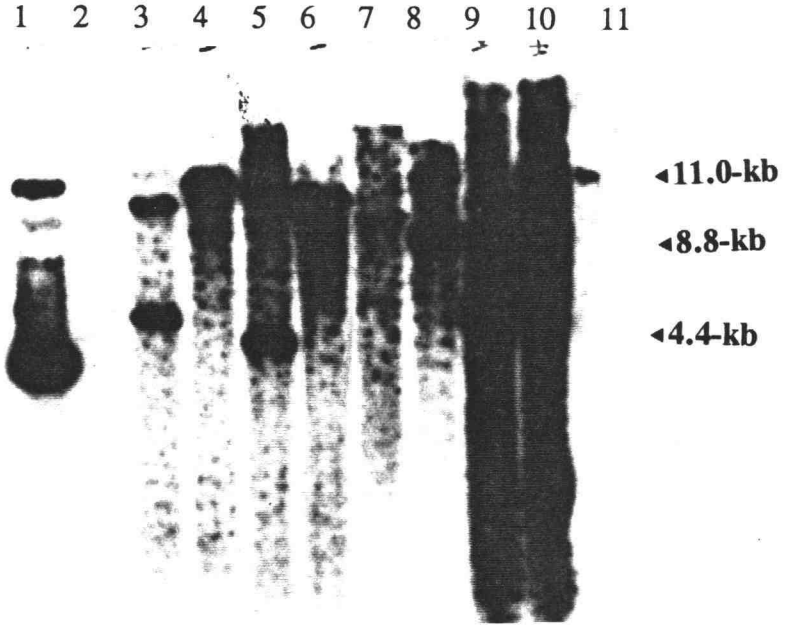

1 2 3 4 5 6 7 8 9 10 11
11.0-kb
8.8-kb
4.4-kb

FIGURE 2-2.

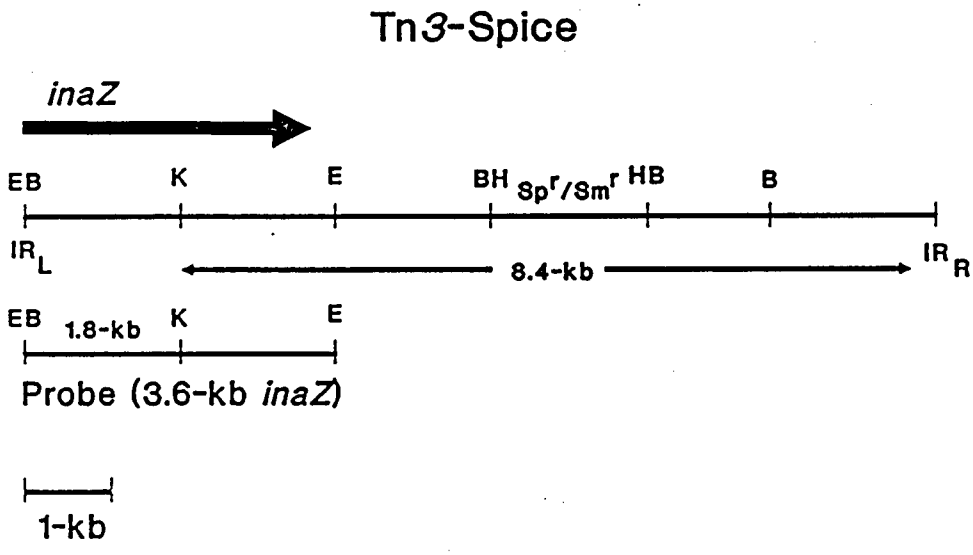


FIGURE 2-3. Physical map of Tn*3*-Spice.
Horizontal lines refer to DNA derived from pTn*3*-Spice. Restriction-endonuclease cut sites are abbreviated: *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Kpn*I (K).

pJEL1742 hybridized to the *inaZ*$^+$ gene of Tn*3*-Spice (FIGURE 2-2); 5.4 kb of the 15.0-kb fragment was contributed by Tn*3*-Spice (FIGURE 2-3). The remaining 9.4 kb of this fragment was target sequence. In order for the insertion to map 9.4 kb from a *Hin*dIII cut site and be within 5.6-kb *Kpn*I, 5.7-kb *Bam*HI and 1.3 *Eco*RI fragments, the insertion must be 9.4 kb from the right border of the 15.5-kb *Hin*dIII fragment and oriented from the right to the left as shown (FIGURE 2-1). The 9.0-kb *Hin*dIII fragment of pJEL1742 which did not hybridize to *inaZ* contained 2.9 kb from Tn*3*-Spice and 6.0 kb of the target DNA. The Tn*3*-Spice insertion of pJEL1742 was 6.0 kb from the left border of the 15.5-kb *Hin*dIII fragment of pJEL1602 (FIGURE 2-1).

The Tn*3*-Spice insertion of pJEL1751 was located 1.2 kb from a *Bam*HI site within the 5.6-kb *Bam*HI fragment of pJEL1602 (data not shown). Two *Kpn*I fragments of 4.4 kb and 11.9 kb hybridized to the *inaZ*$^+$ gene from Tn*3*-Spice (FIGURE 2-3). The 11.9-kb *Kpn*I fragment contained 8.4 kb of Tn*3*-Spice and 3.5 kb of target sequence. The 4.4-kb fragment contained a 1.8-kb fragment corresponding to the beginning of the promoterless *inaZ*$^+$ gene of Tn*3*-Spice and 2.6 kb from pJEL1602. This indicated that the insertion was 3.5 kb from a *Kpn*I site within the 5.6-kb *Kpn*I fragment of pJEL1602. An 11.5-kb *Hin*dIII fragment of pJEL1751 hybridized to *inaZ*$^+$. Two ca. 11.5-kb *Hin*dIII fragments were present in pJEL1751. One fragment must contain 5.4 kb of DNA including the *inaZ*$^+$ gene from Tn*3*-Spice indicating that the insertion was ca. 6.1 kb from a *Hin*dIII site within the 15.5-kb *Hin*dIII fragment of pJEL1751 (FIGURE 2-1). From these data, the insertion was localized but the orientation of the insertion could not be determined.

In the cosmid pJEL1755, the Tn*3*-Spice insertion was again within the *entEA*$^+$ 5.6-kb *Kpn*I fragment and a 5.6-kb *Bam*HI fragment of pJEL1602 (FIGURE 2-1). The *inaZ*$^+$ gene hybridized to 11-kb and 4.8-kb *Kpn*I fragments of pJEL1755,

indicating that the insertion was ca. 2.6 kb from a *Kpn*I site in the 5.6-kb *Kpn*I fragment of pJEL1602. This localized the insert 2.6 kb from the right *Kpn*I site of the 5.6-kb *Kpn*I fragment of pJEL1602 with the orientation of the *inaZ*$^+$ gene going from right to left. A 14.4-kb *Hin*dIII fragment of pJEL1755 hybridized to *inaZ*; thus, this fragment must contain a 5.4-kb fragment from Tn*3*-Spice (FIGURE 2-2) and 6.4 kb from the 15.5-kb *Hin*dIII fragment of pJEL1602 (FIGURE 2-1). The only way for the insertion to be within the 5.6-kb *Kpn*I fragment, the 5.6-kb *Bam*HI fragment, and 6.4 kb from a *Hin*dIII site is if the insertion is 6.4 kb from the left *Hin*dIII site within the 15.5-kb *Hin*dIII fragment in the orientation shown (FIGURE 2-1).

The Tn*3*-Spice insertion of pJEL1752 was located outside of the 5.6-kb *Kpn*I fragment, and within the 2.0-kb *Eco*RI and 9.0-kb *Bam*HI fragments of pJEL1602 (FIGURE 2-1). The insertion was 1.1 kb from a *Bam*HI site within the 9.0-kb *Bam*HI fragment of pJEL1602 (data not shown). An 8.8-kb *Kpn*I fragment and the vector-containing *Kpn*I fragment hybridized to *inaZ*$^+$ (FIGURE 2-2). These hybridization data were compatible with the *Eco*RI and *Bam*HI analysis only when the 8.8-kb *Kpn*I fragment contained the 8.4-kb region from Tn*3*-Spice. Thus, the insertion was localized to 0.3 kb to the left of a *Kpn*I site in pJEL1602 (FIGURE 2-1). The orientation of the *inaZ*$^+$ gene was from left to right with the gene fused at 0.2 kb from the left border of the 2.0-kb *Eco*RI fragment and within 0.3 kb of the left border of the 5.6-kb *Kpn*I fragment. The insertion was located ca. 3.5-kb from a *Hin*dIII site within the 15.5-kb *Hin*dIII fragment of pJEL1602.

Derivation of Cat$^-$, Cat$^-$Iuc$^-$ and Cat$^-$Iuc$^-$Iut$^-$ mutants of *E. carotovora*.

In four marker-exchange experiments involving W3C105(pJEL1752), JL1148(pJEL1752), JL11178(pJEL1752) and JL11182(pJEL1752), Tc^{s}Sp^r colonies were obtained from cultures grown without antibiotic selection. These colonies lost

pJEL1752 but retained Tn*3*-Spice, presumably due to recombination between homologous sequences in plasmid and genomic DNA. The Tn*3*-Spice insertion of pJEL1752 was introduced into the chromosome of W3C105 and derivatives (JL1148, JL11178, JL11182) by marker exchange mutagenesis. Cosmid pJEL1752 no longer complemented *S. typhimurium* strain *enb-7*, which has a mutation occurring prior to the production of DHBA in the enterobactin-biosynthesis pathway (Pollack *et al.*, 1970). Of the putative marker-exchange mutants tested, none produced catechol as detected by the methods of Rioux *et al.* (1983) or Arnow (1937). Tn*3*-Spice insertion mutants derived from strains deficient in aerobactin production (JL11178, Iuc$^-$Iut$^+$; or JL11182, Iuc$^-$Iut$^-$) were Cat$^-$Iuc$^-$Iut$^+$ (JL11206) or Cat$^-$Iuc$^-$Iut$^-$ (JL11207); these mutants did not produce halos on CAS agar because neither aerobactin nor catechol was produced. Catechol production was restored to Tn*3*-Spice mutants by introduction of pJEL1602, which contains cloned catechol-biosynthesis genes from W3C105 (TABLE 2-4). Aerobactin production was restored to Cat$^-$Iuc$^-$Iut$^+$ and Cat$^-$Iuc$^-$Iut$^-$ mutants by introduction of pJEL1534, which contains the cloned aerobactin-biosynthesis and uptake genes from W3C105. The mutants and parental strain W3C105 gave reactions of identity to antiserum 39, indicating that antigen reacting to antiserum 39 was not altered by the Tn*3*-Spice insertions. The mutants and W3C105 formed pits on CVP agar and grew on pectate agar indicating that the Tn*3*-Spice insertion did not inactivate pectic enzyme production. Mutants grew on the minimal medium TMS and generation times of the mutants and parental strains did not differ in LB broth.

Analysis of insertions in Cat$^-$, Cat$^-$Iuc$^-$ and Cat$^-$Iuc$^-$Iut$^-$ mutants of *E. carotovora*.

An 8.8-kb *Kpn*I fragment from pJEL1752, which contained part of the *inaZ*$^+$ gene of Tn*3*-Spice (FIGURE 2-2) and ca. 0.5 kb of DNA from W3C105, did not hybridize genomic DNA of JL1148 (W3C105 Rif^r) presumably because DNA was degraded (FIGURE 2-4). The probe hybridized to a 27 kb of the genomic DNA

TABLE 2-4. Relevant characteristics of catechol-biosynthesis and aerobactin-biosynthesis mutants of *Erwinia carotovora* subsp. *carotovora* W3C105

E. carotovora[y]	Derivation of strain	Siderophore production[x]		
		Catechol	Aerobactin	CAS
W3C105[z]	wildtype	+	+	+
JL11202	Cat⁻ derivative of W3C105	-	+	+
JL11199	JL11202 (pJEL1602)	+	+	+
JL11178	Iuc⁻Iut⁺ derivative of W3C105	+	-	+
JL11208	JL11178 (pJEL1534)	+	+	+
JL11206	Cat⁻Iuc⁻Iut⁺ derivative of W3C105	-	-	-
JL11211	JL11206 (pJEL1534)	-	+	+
JL11212	JL11206 (pJEL1602)	+	-	+
JL11182	Iuc⁻Iut⁻ derivative of W3C105	+	-	+
JL11209	JL11182 (pJEL1534)	+	+	+
JL11207	Cat⁻Iuc⁻Iut⁻ derivative of W3C105	-	-	-
JL11213	JL11207 (pJEL1534)	-	+	+
JL11214	JL11207 (pJEL1602)	+	-	+

[x]Siderophore production was detected by observation of a halo surrounding a colony grown on CAS agar, cross-feeding of strain AN93 (*entE*), by catechol assays (Arnow, 1937; Rioux *et al.*, 1983) and by a hydroxamate assay (Csáky, 1948). + = siderophore produced; - = no siderophore produced.

[y]Presence of plasmids in strains of *E. carotovora* was confirmed by extraction. Plasmids were mobilized into *E. carotovora* by conjugal transfer.

[z]Serological relatedness of strains was determined by methods adapted from De Boer and Kelman (1978).

FIGURE 2-4. Southern blot analysis of catechol biosynthesis genes of catechol mutants of JL1148 (Rif^r, W3C105).
An ethidium-bromide stained gel is above and an autoradiogram is below on the following page. The blot was hybridized with an 8.8-kb *Kpn*I fragment of pJEL1752, which contained part of the *inaZ*$^+$ gene and 0.5-kb of DNA from W3C105. Lanes: 1) 1-kb ladder; 2) λ, *Hin*dIII; 3) JL1148, *Kpn*I; 4) pJEL1602, *Kpn*I; 5) pJEL1752, *Kpn*I; 6) JL1148(pJEL1752); The following were all cut with *Kpn*I; 7) JL1148::pJEL1752 marker-exchange mutant (MEM)-1; 8) JL1148::pJEL1752 MEM-2; 9) JL1148::pJEL1752 MEM-3; 10) JL1148::pJEL1752 MEM-4; 11) JL1148::pJEL1752 MEM-5; 12) JL11146; 13) JL1148::pJEL1752 MEM-7; 14) JL1148::pJEL1752 MEM-8; 15) JL1148::pJEL1752 MEM-9; 16) JL1148::pJEL1752 MEM-10.

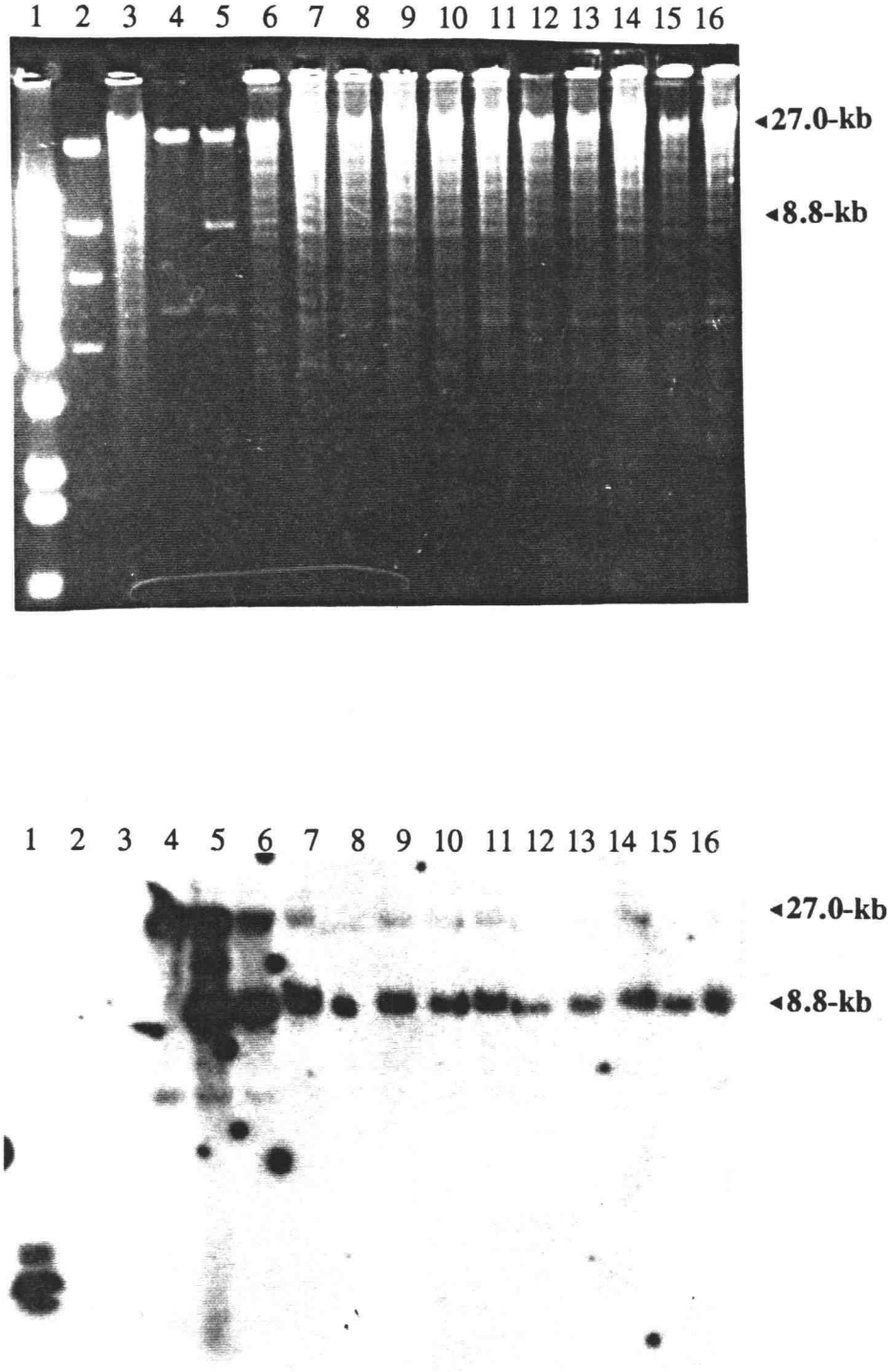
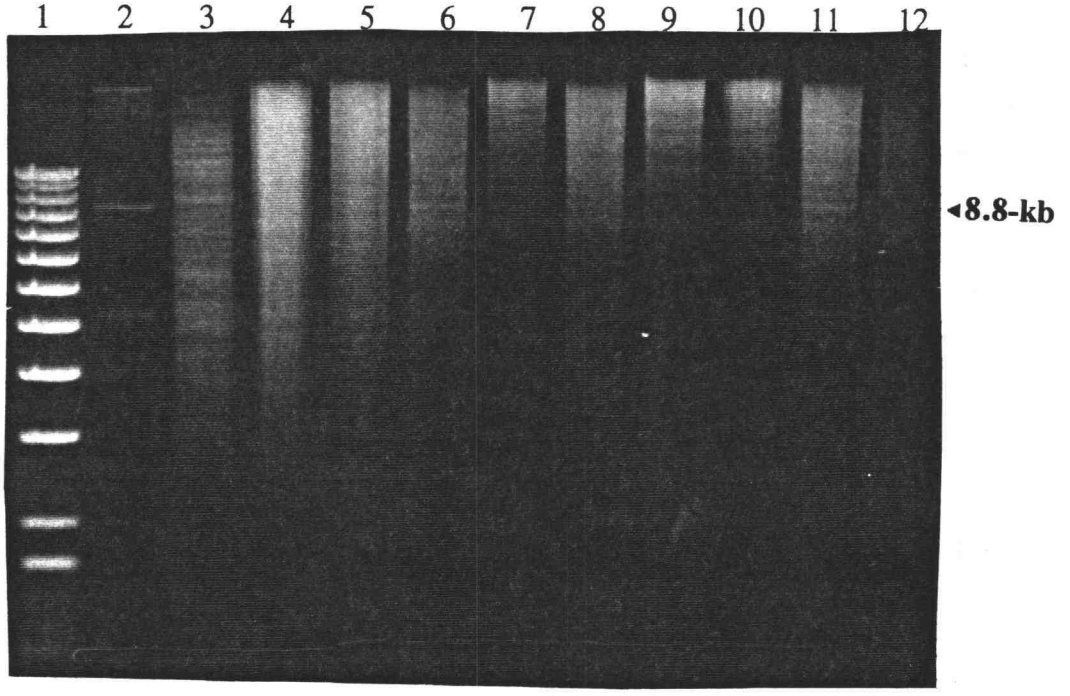

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
◂27.0-kb
◂8.8-kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
◂27.0-kb
◂8.8-kb

FIGURE 2-4.

was contained in a 33-kb *Kpn*I fragment of pJEL1602 that also included the vector pLAFR3 (lane 4). In addition, 5.6-kb and 0.5-kb *Kpn*I fragments of pJEL1602 hybridized weakly to the probe, indicating that the probe was probably contaminated with DNA from pJEL1752. The 8.8-kb *Kpn*I fragment of pJEL1752 strongly hybridized to the probe but the 33-kb fragment also hybridized strongly whereas the 5.6-kb *Kpn*I fragment hybridized wealy. The probe also hybridized to the 8.8-kb *Kpn*I fragment of JL1148(pJEL1752) and in marker-exchange mutants. In marker-exchange mutants, a 22-kb *Kpn*I fragment also hybridized weakly to the probe. This fragment corresponded to the remainder of the 27-kb *Kpn*I fragment from JL1148. Thus, restriction fragments of genomic DNA of marker-exchange mutants were of the size predicted for strains with genomic insertion of Tn*3*-Spice located in a site corresponding to the insertion of pJEL1752. From these data, I conclude that homologous recombination occurred between the genomic regions of JL1148 and pJEL1752.

The presence of single Tn*3*-Spice insertions in the predicted location in the genome of rifampicin-sensitive Cat mutants of *E. carotovora* was also evaluated in Southern blots with the *inaZ*$^+$ probe. As expected the *inaZ*$^+$ hybridized to a 8.8-kb *Kpn*I fragment in W3C105(pJEL1752), JL11202, JL11206, JL11207; no fragment hybridizing to the *inaZ*$^+$ probe was observed in genomic DNA of W3C105, JL1148, JL11178, and JL11182 (FIGURE 2-5). A 22-kb *Kpn*I fragment that hybridized to the *inaZ*$^+$ gene was also expected but was not observed in genomic DNA of most Tn*3*-Spice insertion mutants. A weakly-hybridizing *Kpn*I fragment of ca. 22-kb was observed only in JL11206. The absence of the fragment in other mutants was attributed to the low sensitivity of this Southern blot. Nevertheless, the presence of a single 8.8-kb *Kpn*I fragment hybridizing to the *inaZ*$^+$ gene indicated that a single Tn*3*-Spice insertion was present in the predicted location in the genomes of the mutants JL11146, JL11202, JL11206 and JL11207.

FIGURE 2-5. Southern blot analysis of catechol biosynthesis genes of catechol mutants of W3C105.
The blot was hybridized with the 3.6-kb *Eco*RI fragment from pTn*3*-Spice containing the *inaZ*$^+$ gene. An ethidium-bromide stained gel is above and an autoradiogram below on the following page. Lanes: 1) 1-kb ladder; 2) pJEL1752, *Kpn*I; 3) AN194, *Kpn*I; 4) W3C105, *Kpn*I; 5) JL1148, *Kpn*I; 6) JL11199, *Kpn*I; 7) JL11202, *Kpn*I; 8) JL11178, *Kpn*I; 9) JL11206, *Kpn*I; 10) JL11182, *Kpn*I; 11) JL11207, *Kpn*I; 12) JL11201, *Kpn*I.



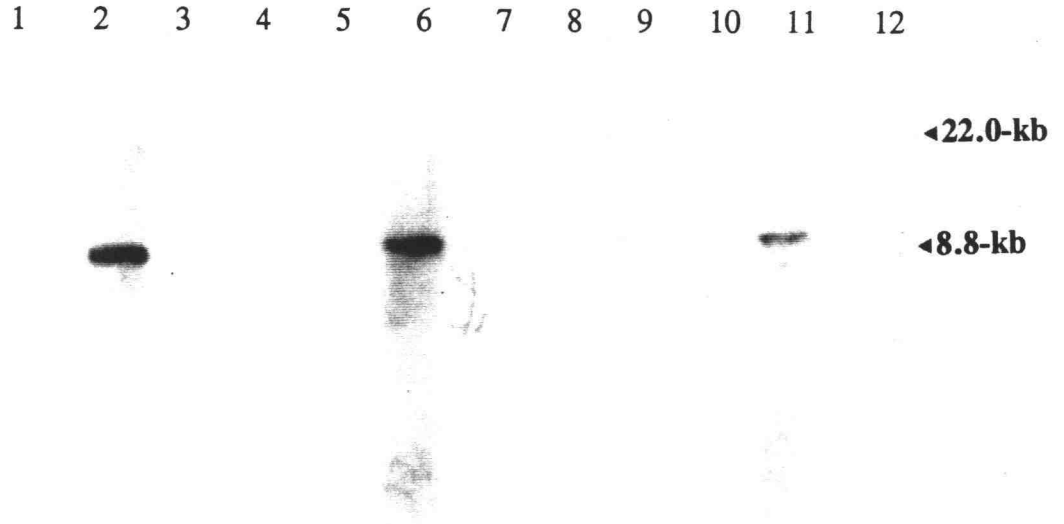


FIGURE 2-5.

Growth of *E. carotovora* on iron-limiting media.

Growth of mutant and wildtype strains varied on TMS with low available iron (TABLE 2-5). W3C105 and JL1148, which produce catechol and aerobactin, grew on TMS agar amended with 225 μM 2,2'-dipyridyl. Strains of JL11202 (Cat^- Iuc^+Iut^+), producing only aerobactin, also grew on TMS agar amended with 225 μM 2,2'-dipyridyl. Aerobactin-deficient mutants JL11182 ($Cat^+Iuc^-Iut^-$), JL11178 ($Cat^+Iuc^-Iut^+$), JL11206 ($Cat^-Iuc^-Iuc^+$), and JL11207 ($Cat^-Iuc^-Iut^-$) did not grow at 2,2'-dipyridyl levels of 135 μM or greater. These results demonstrate that aerobactin was involved in iron acquisition by W3C105.

Catechol-producing strains JL11178 ($Cat^+Iuc^-Iut^+$) and JL11182 ($Cat^+Iuc^-Iut^-$) grew at 135 μM 2,2'-dipyridyl whereas strains deficient in the production of both aerobactin and catechol, JL11206 ($Cat^-Iuc^-Iuc^+$) and JL11207 ($Cat^-Iuc^-Iut^-$), grew only at concentrations of 2,2'-dipyridyl of 120 μM or less (TABLE 2-5). These results demonstrate that the catechol produced by W3C105 was involved in iron acquisition.

Cross-feeding of siderophore non-producing strains of *E. carotovora*.

Strains JL11206 ($Cat^-Iuc^-Iut^+$) and JL11207 ($Cat^-Iuc^-Iut^-$) did not grow on TMS amended with 150 μM 2,2'-dipyridyl. Strain JL11206 did not produce aerobactin nor catechol but utilized both as siderophores (TABLE 2-6). JL11207 also did not produce aerobactin nor catechol and did not utilize aerobactin as a siderophore. Catechol-producing derivatives of W3C105 cross-fed JL11207 whereas derivatives producing only aerobactin did not cross-feed JL11207; thus, genes involved in uptake of aerobactin were dysfunctional in JL11207. Therefore, JL11207 was used as a specific indicator for the catechol siderophore(s) produced by W3C105. In addition, JL11206 and JL11207 were cross-fed by *E. carotovora* SRI193 or SR319, which produced catechol(s) and by *E. coli* AN194, which produced enterobactin. Thus, W3C105 derivatives utilized enterobactin and catechols produced by other

TABLE 2-5. Growth of siderophore mutants on TMS amended with 2,2'-dipyridyl

Producer strain	Phenotype	Growth on TMS 2,2'-dipyridyl level (μM)						
		75	90	105	120	135	150	225
W3C105	Cat^+, Iuc^+, Iut^+	+	+	+	+	+	+	+
JL11202	Cat^-, Iuc^+, Iut^+	nt	nt	nt	nt	nt	+	+
JL11178	Cat^+, Iuc^-, Iut^+	+	+	+	+	+	-	-
JL11208	Cat^+, Iuc^+, Iut^+	+	nt	nt	nt	nt	+	+
JL11206	Cat^-, Iuc^-, Iut^+	+	+	+	$+^w$	-	-	-
JL11211	Cat^-, Iuc^+, Iut^+	+	nt	nt	nt	nt	+	+
JL11212	Cat^+, Iuc^-, Iut^+	+	+	+	+	$+^w$	-	-
JL11182	Cat^+, Iuc^-, Iut^-	+	+	+	+	$+^w$	-	-
JL11209	Cat^+, Iuc^+, Iut^+	+	+	+	+	+	+	+
JL11207	Cat^-, Iuc^-, Iut^-	+	+	+	$+^w$	-	-	-
JL11213	Cat^-, Iuc^+, Iut^+	+	+	+	+	+	+	+
JL11212	Cat^+, Iuc^-, Iut^-	+	+	+	+	$+^w$	-	-
SRI193	Cat^+	+	+	+	+	+	+	nt
SR319	Cat^+	+	+	+	+	+	+	nt

Cells from LB broth cultures were harvested and adjusted to 0.1 OD_{600nm}. Five microliters of bacterial suspension was spotted onto TMS agar. Growth was recorded after 3 and 5 days. + = growth; $+^w$, = weak growth; - = no growth. The experiment had 3 replications per treatment and was repeated.

TABLE 2-6. Cross-feeding of catechol mutants of *Escherichia coli* and Cat⁻Iuc⁻, and Cat⁻Iuc⁻Iut⁻ mutants of *Erwinia carotovora*

		Cross-feeding zone (cm)[y] Indicator strain			
		Mutant of *E. carotovora*		Mutant of *E. coli*	
Producing strain	Siderophore produced	JL11206 (Cat⁻Iuc⁻)	JL11207 (Cat⁻Iuc⁻Iut⁻)	AN93 (*entE405*)	AN193 (entA403)
E. c. subsp. *carotovora*:					
W3C105	Aerobactin Catechol	(1.6)	(0.25)	+[z]	+
JL11178	Catechol	(0.50)	(0.20)	+	+
JL11182	Catechol	(0.30)	(0.20)	+	+
Jl11202	Aerobactin	(1.45)	-	-	-
JL11206	None	-	-	-	-
JL11207	None	-	-	-	-
SRI193	Catechol	(1.00)	(0.95)	+	+
SR319	Catechol	(1.55)	(1.33)	+	+
E. chrysanthemi:					
PMV3937	Chrysobactin	-	-	-	-
E. coli:					
AN194	Enterobactin	(1.00)	(0.90)	+	+
AN93	DHBA	+	+	-	+

[y]Molten TMS containing 2,2'-dipyridyl (150 μM) was seeded with 10^6 cfu/ml of the indicator strain. A producing strain was spotted onto the surface of solidified, seeded TMS medium and incubated at 26 C. After 24-48 h, plates were observed for growth of the indicator strain surrounding colonies of producing strains. Indicator and producing strains were grown in TMS broth overnight prior to use. Zone of cross-feeding was measured from the edge of the producing colony to the boundary of growth of the indicator strain. Cross-feeding of strains of *E. carotovora* and *E. coli* were conducted as separate experiments. Each experiment had 3 replications per treatment and the experiments were repeated.

[z] + = cross-feeding of indicator strain was observed; - = no cross-feeding was observed. Sizes of zones indicated as "+" were not measured.

strains of *E. carotovora*. The size of the zones of growth of JL11206 surrounding AN194, SRI193 and SR319 were similar to the size of the zones surroundingaerobactin producing strains, JL11202, W3C105 and JL1148. The size of zones surrounding JL11178 and JL11182, which produced only catechol were smaller. JL11206 and JL11207 were not cross-fed by *E. chrysanthemi* 3937, indicating that chrysobactin was not used as a siderophore by W3C105.

Ice nucleation activity of *E. coli* containing plasmids with Tn*3*-Spice insertions.

Plasmids pJEL1752 and pJEL1755 conferred iron-regulated ice nucleation activity to *E. coli* strain AN193, an *entA* derivative of AN194. In contrast, ice nucleation activity conferred by plasmids pJEL1742 and pJEL1751 to AN193 did not vary greatly with the level of available iron (TABLE 2-7). Similarly, ice nucleation activity expressed by the Ent^+ strain AN194 harboring pJEL1751 was not regulated by iron, whereas that expressed by AN194 harboring pJEL1752 was iron-regulated.

Ice nucleation activity of *E. carotovora* containing plasmids with Tn*3*-Spice insertions.

Catechol-biosynthesis mutants, which contained a genomic insertion of Tn*3*-Spice located at a site analogous to the insertion in pJEL1752, did not express detectable ice nucleation activity on LB, pectate, KB, NA or PDA agar. The limit of detection for ice nucleation activity was -7.60 $\log_{10}$ (ice nuclei/cell). The Cat^- strain JL11202, when grown on TMS amended with 75 μM 2'2-dipyridyl, expressed low levels of ice nucleation activity (-7.60 $\log_{10}$ [ice nuclei/cell]). Strains of *Erwinia* that harbored the plasmid pJEL1703, which has an *iceC* gene with a native promoter from *P. syringae*, also did not express detectable levels of ice nucleation activity on LB, pectate, KB, NA, PDA or TMS media.

TABLE 2-7. Ice nucleation activity of strains of *Escherichia coli* that harbor cosmids containing an insertion of Tn*3*-Spice

E. coli Strain (cosmid)[x]	Phenotype	Ice nucleation activity [$\log_{10}$ (ice nuclei/cell)][w]		
		75 μM 2,2'-dipyridyl	0.001 μM $FeCl_3$	0.1 μM $FeCl_3$
AN193 (pJEL1742)[y]	Ent$^-$	-4.24	-5.40	-6.93
AN193 (pJEL1751)	Ent$^-$	-5.07	-6.94	NA[z]
AN193 (pJEL1752)	Ent$^+$	-0.40	-1.25	-4.85
AN193 (pJEL1755)	Ent$^-$	-0.40	-1.29	-4.85
AN194 (pJEL1742)	Ent$^+$	NA	NA	NA
AN194 (pJEL1751)	Ent$^+$	-3.37	-4.65	-4.90
AN194 (pJEL1752)	Ent$^+$	-2.31	-3.69	-7.49

[w]Ice nucleation activity was measured at -7 C by a droplet freezing technique similar to that previously described (Lindow, 1990). Strains were grown at 27 C for 48 h in TMS broth (5 ml) amended with iron or 2,2'-dipyridyl. Values presented are the means of three replicates of each culture.

xPlasmids were mobilized into *E. coli* by conjugal transfer.

[y]Presence of plasmids in strains of *E. coli* was confirmed by extraction.

[z]The limit of detection for ice nucleation activity was -7.60 $\log_{10}$ (ice nuclei/cell) in this study. NA = ice nucleation activity below limit of detection.

DISCUSSION

Catechol production is a common trait among strains of *E. carotovora* (Leong and Neilands, 1982; Bull, Chapter 1). Although catechols produced by *E. carotovora* have "siderophore-like properties" (Bull, Chapter 1), demonstration of their involvement in iron acquisition by *E. carotovora* has been lacking. In this study, mutants of *E. c.* subsp. *carotovora* W3C105 that were defective in catechol production were used to demonstrate the role of the catechol in iron acquisition. Several lines of evidence demonstrate the role of catechol in iron-acquisition of W3C105: 1) Mutants of W3C105 that produced only catechol but did not produce aerobactin (JL11178, Cat^+Iuc^-, or JL11182, $Cat^+Iuc^-Iut^-$) produced halos on CAS agar whereas mutants that produced neither catechol nor aerobactin (JL11206, Cat^-Iuc^-, or JL11207, $Cat^-Iuc^-Iut^-$) did not produce halos. 2) Mutants of W3C105 that produced only catechol grew on media with lower available iron than did mutants that produced neither catechol nor aerobactin. 3) Mutants of W3C105 deficient in catechol production and aerobactin production and uptake were cross-fed by W3C105, mutants of W3C105 that produced no aerobactin, and other strains of *E. carotovora* or *E. coli*. The catechol siderophore enterobactin was used as siderophore by $Cat^-Iuc^-Iut^-$ derivatives of strain W3C105, supporting the hypothesis that W3C105 has an outer-membrane receptor protein that recognizes enterobactin (Bull, Chapter 1). From these data, I conclude that the catechol produced by W3C105 is a siderophore involved in iron acquisition by W3C105. This was the first demonstration of the involvement of a catechol produced by *E. carotovora* in iron acquisition.

The catechol(s) produced by *E. carotovora* differed from chrysobactin, the

catechol siderophore produced by *E. chrysanthemi*, as demonstrated by several findings: 1) Chrysobactin, produced by *E. chrysanthemi* 3937, was not used as a siderophore by mutants of W3C105; therefore, W3C105 did not have an iron-acquisition system for chrysobactin. 2) Catechol biosynthesis genes from W3C105 were functionally similar to enterobactin biosynthesis genes from *E. coli* and *S. typhimurium*. Cloned sequences from *E. carotovora* complemented the *entD* mutation, which has not been complemented with the cloned chrysobactin biosynthesis genes of *E. chrysanthemi*. 3) The catechol from *E. carotovora* is used as a siderophore by *entE* mutants of *E. coli* and *enb-1* mutants of *S. typhimurium*, whereas chrysobactin was not used as a siderophore by these strains. From these results I conclude that the catechol siderophore produced by *E. c.* subsp. *carotovora* W3C105 is distinct from chrysobactin. Chemical characterization of the catechol is needed to further analyze the relationship of the catechol produced by *E. carotovora* to enterobactin.

The *entA* gene homolog for catechol siderophore production cloned in pJEL1602 was localized by Tn*3*-Spice insertion mutagenesis. Three Tn*3*-Spice insertions that inactivated *entA*$^+$ activity mapped to within a 1 kb region of the 5.6-kb *Kpn*I fragment that confers the EntEA$^+$ phenotype. Although *entE* mutations of *E. coli* were complemented by the 5.6-kb *Kpn*I fragment (Bull, Chapter 1), the Tn*3*-Spice insertion that inactivated *entE*$^+$ activity was located 0.3-kb outside of the 5.6-kb *Kpn*I fragment. This insertion was located in a 2.0-kb *Eco*RI fragment that hybridized to *entE*$^+$ gene from *E. coli* (Bull, Chapter 1). The structural *entE* gene may be present on the 5.6-kb *Kpn*I fragment whereas regulatory elements may be localized outside this fragment, in the region where Tn*3*-Spice inserted into pJEL1752. Thus, while the Tn*3*-Spice insertions of pJEL1742, pJEL1751 and pJEL1755 further localized the *entA*-homolog of *E. carotovora*, the insertion inactivating the *entE* activity was probably in a region regulating gene expression rather than in the structural *entE* gene of strain W3C105.

Ice nucleation activity was iron-regulated for both pJEL1752 (*entE*$^-$) and pJEL1755 (*entA*$^-$), even though *inaZ*$^+$ was in opposite orientations in these cosmids. Thus, *entE* and *entA* may be transcribed in opposite directions in *E. carotovora* strain W3C105. In contrast, in *E. coli* genes in the DHBA operon (*entCEBA)* are transcribed in the same direction (Fleming *et al.*, 1983; Nahilik *et al.*, 1989). Promoters of other enterobactin-biosynthesis and uptake genes, however, are oriented opposite to the orientation of the DHBA operon of *E. coli* (Fleming *et al* ., 1983). Alternatively, the *inaZ*$^+$ gene inserted in pJEL1755 may have been using the p*lac* promotor from pLAFR3 for transcription giving no information concerning the relative orientation of the *entE*$^+$ and *entA*$^+$ promoters of W3C105. Read-through from the vector promoter was unlikely, however, since there was at least 15 kb of DNA from that promoter to the location of the Tn*3*-Spice insertion. One of the identified iron-regulated promoters may be located between the insertions of pJEL1755 and pJEL1742; although Tn*3*-Spice was in the same orientation in the two cosmids, pJEL1742 did not confer iron-regulated ice nucleation activity to *E. coli* whereas pJEL1755 did. These Tn*3*-Spice insertions in the catechol-biosynthesis genes of *E. carotovora* W3C105 provide cursory information regarding the presence and possible location of iron-regulated promoters. Saturation mutagenesis and sequencing are required to characterize the complex regulation of this region.

In *E. carotovora*, ice nucleation activity expressed from the introduced *inaZ* fusions or from an *iceC* gene of *P. syringae* was low or not detected. The low ice nucleation activity was not expected because many Gram-negative bacteria express ice nucleation activity efficiently (Lindgren *et al.*, 1989). Nevertheless, ice nucleation activity was expressed inefficiently by *Agrobacterium* spp. (Lindgren *et al.*, 1989) and *P. solanacearum* (Timothy Denny, personal communication) and may also be expressed weakly by *E. carotovora*. It is possible that the promoter from *P. syringae* and the promoter from the catechol biosynthesis region of *E. carotovora* were not expressed by strain W3C105 under the conditions tested. It is also

possible that the ice nucleation gene was expressed but the InaZ protein was not active in the outer membrane of *E. carotovora*. The hydrophobic environment of the outer membrane is critical to the proper assembly and structural organization of an oligomeric ice protein complex; a membrane that does not support the formation of this complex will not enable its expression as an ice nucleus (Govindarajan and Lindow, 1988).

Genetic tools developed for *E. carotovora* are useful in studying molecular aspects of plant-pathogen interactions (Ellard, *et al.*, 1989; Zink, *et al.*, 1984; Roeder and Collmer, 1985; Hinton, *et al.*, 1985; Salmond, *et al.*, 1986; Jayaswal, *et al.*, 1984). Mutagenesis of key phenotypes in *E. carotovora* enhanced the understanding of the role of these phenotypes in virulence and pathogenicity (Ellard, *et al.*, 1989; Zink, *et al.*, 1984; Roeder and Collmer, 1985; Hinton, *et al.*, 1985; Salmond, *et al.*, 1986; Jayaswal, *et al.*, 1984). A similar approach has been used to demonstrate the role of siderophores produced by fluorescent pseudomonads in biological control (Loper, 1988). Mutagenesis of genes involved in siderophore production by *E. carotovora* results in strains useful for determining the role of siderophores produced by this pathogen in virulence and sensitivity to biological control. Mutants of W3C105 have been generated that no longer produce or utilize aerobactin (Ishimaru and Loper, 1992), no longer produce catechol, and no longer produce catechol and aerobactin. These strains will be useful in determining the role of siderophores in the ecology and virulence of *E. carotovora*.

CHAPTER 3. ROLE OF SIDEROPHORE PRODUCTION BY *ERWINIA CAROTOVORA* IN VIRULENCE AND INSENSITIVITY TO BIOLOGICAL CONTROL.

INTRODUCTION

Iron is an essential element needed in trace amounts by most microorganisms. Although iron is the fourth most abundant element in the earths crust, at neutral pH it is present in the soil as insoluble colloidal hydroxides that are not biologically available (Lindsay, 1979). In iron-limiting environments, microorganisms employ strategies involving high-affinity iron uptake systems to obtain iron. Siderophore-mediated iron uptake is often involved in iron acquisition in low iron environments. Siderophores are low molecular weight, iron-chelating agents that are produced under iron-limiting conditions, excreted into the environment, where they chelate iron and transport iron as a ferric-siderophore complex to the producing organisms (Neilands 1981; Neilands 1982). The ferric-siderophore complex is transported internally cells via specific outer membrane receptors followed by the release of iron for the use of the cell (Neilands, 1982).

Iron acquisition by vertebrate pathogens is an important factor in virulence and the establishment of infection (Weinberg, 1986). Enteric bacteria including *Escherichia coli* produce siderophores with two types of chelating moieties: hydroxamates and catechols. The hydroxamate siderophore aerobactin is key to virulence of *E. coli* in vertebrate hosts (Payne, 1988; Weinberg, 1986).

Many phytopathogenic bacteria produce siderophores or compounds having

siderophore-like properties (Cody and Gross, 1987; Ong *et al.*, 1979; Loper *et al.*, 1984; Expert and Toussaint, 1988; Ishimaru and Loper, 1992; Bull *et al.*, 1989; Bull and Loper, 1991). The function of siderophores in the ecology and virulence of several plant pathogens has been studied. The pathogen *Agrobacterium tumefaciens* does not require the production of agrobactin for infection or disease production (Leong and Neilands, 1981; Ong *et al.*, 1979). Similarly pyoverdin$_{pss}$ produced by *Pseudomonas syringae* pv. *syringae*, is not required for virulence (Cody and Gross, 1987a; 1987b; Loper and Lindow, 1987). *Erwinia chrysanthemi*, a phytopathogenic member of the Enterobacteriaceae, produces a catechol siderophore, chrysobactin, which is important in systemic virulence of this pathogen on *Saintpaulia ionantha* (Expert and Toussaint, 1985; Enard *et al.*, 1988; Persmark *et al.*, 1989). In addition, catechol production is a common trait among strains of *Erwinia carotovora* (Leong and Neilands, 1982; Bull *et al.*, 1989; Bull, Chapter 1; Ishimaru and Van Buren, 1991). In general, siderophore production by members of the Enterobacteriaceae is important in virulence. Nevertheless, the role of siderophores produced by *E. carotovora* in virulence or ecology of the pathogen is unknown.

In addition to the potential involvement of the siderophores produced by *Erwinia carotovora* subsp. *carotovora* W3C105 in virulence, it has been hypothesized that siderophore production by W3C105 may be involved in reduced sensitivity of this pathogen to biological control (Loper and Ishimaru, 1991). It is hypothesized that siderophores are produced by biological control agents under iron-limiting conditions. Siderophores are excreted into the soil where they chelate iron and provide it specifically to producing strains, thus making the limited supply of iron in the soil even more limiting to the growth of a pathogen (Buyer and Leong, 1986; Neilands and Leong, 1986; Loper and Buyer, 1991; Loper and Ishimaru, 1991). The role of siderophores produced by a pathogen in iron-

competition with biological control agents is not known and has received little attention.

Mutants are now available that produce only a catechol siderophore (Cat^+Iuc^+), produce only aerobactin (Cat^+Iuc^+), produce neither aerobactin nor catechol (Iuc^-Cat^-), or can not produce nor utilize aerobactin ($Cat^+Iuc^-Iut^-$) (Bull, Chapter 2; Ishimaru and Loper, 1992). Through studies comparing these mutants with the parental strain W3C105 ($Cat^+Iuc^+Iut^+$), the role of siderophore production by W3C105 in virulence and insensitivity to biological control may be elucidated. It is important to design bioassays which will detect subtle differences in virulence and relative sensitivity of these pathogens to biological control.

Several bioassays for virulence have been used to determine differences among strains of *Erwinia*. Virulence of the pathogen (amount of soft rot produced) has been compared (Johnson *et al.*, 1989; Smith and Bartz, 1990). Soft rot bioassays have also been useful in determining what phenotypes are essential for pathogenicity of *Erwinia* spp. (Chatterjee and Starr, 1977; Lei et al., 1985a; 1985b; Zink and Chatterjee, 1985; Roeder and Collmer, 1985; Murata *et al.*, 1990). Soft rot bioassays may be useful in determining the role of siderophore production in virulence of *E. carotovora*.

In a related pathogen, *E. chrysanthemi*, siderophore production is involved in systemic virulence (Enard *et al.*, 1989; Persmark *et al.*, 1989). Aerial stem rot is a disease caused by *E. carotovora* which has a systemic component. An aerial stem rot bioassay may be useful for detecting differences between the parental strain and siderophore mutants of *E. carotovora*.

Biological control of *E. carotovora* has been demonstrated by monitoring several different responses including but not limited to pathogen population size

(Kloepper and Schroth, 1981a; Xu and Gross, 1986b), maceration of potato tuber tissue (Axelrood *et al.*, 1988), and plant emergence (Xu and Gross, 1986a). Two strains of *E. carotovora* (W3C105 and W3C37) differ in sensitivity to biological control in plant emergence assays (Xu and Gross, 1986a). Soft rot bioassays and population studies also may be useful in determining differences in sensitivity to biological control, but this has not been tested. Strains W3C37 and W3C105 will be useful in determining if differences in sensitivity to biological control can be detected in these bioassays.

In order to determine if siderophores are important to virulence or biological control, we must develop assays that will detect differences among strains. This study presents several bioassays that were used to test sensitivity to biological control and evaluate virulence of a pathogen. In addition, results support the hypothesis that siderophore production contributes to the virulence of *E. carotovora* W3C105.

METHODS AND MATERIALS

Bacterial strains and media.

Bacterial strains used in these studies are listed in TABLE 3-1. All bacteria were grown at 27 C. *Erwinia carotovora* was cultured routinely on Luria Bertani medium (LB) (Gerhardt *et al.*, 1981) and *Pseudomonas* spp. were cultured on King's Medium B (KMB) (King *et al.*, 1954). Antibiotics were used at the following concentrations: ampicillin (Ap, 100 μg/ml), tetracycline (Tc, 20 μg/ml), spectinomycin (Sc, 50 μg/ml), streptomycin (Sp, 100 μg/ml), kanamycin (Km, 50 μg/ml), rifampicin (Rif, 100 μg/ml). All chemicals were purchased through Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Potato tubers.

Russet Burbank potato tubers were used in all experiments. Tubers were obtained from the Columbia River Basin, Oregon (Table 3-2). Potatoes were stored at 4 C and were used within 1 yr of harvest. Larger tubers (>10 cm) were used for in population studies and soft-rot experiments with two or more inoculation sites per tuber. Small tubers (<10 cm), with no visible wounding or disease symptoms, were selected for soft-rot experiments with one inoculation site per tuber.

Tubers were removed from 4 C storage 24 h prior to inoculation and were kept at room temperature. Potatoes were washed gently with tap water to remove excess dirt. Tubers were surface disinfested by soaking tubers in 0.05% sodium hypochlorite solution (10% Clorox, Proctor and Gamble, Cincinnati, OH) for 20 min. The tubers were rinsed well and air dried. In some soft-rot experiments,

TABLE 3-1. Bacterial strains used for Chapter 3

Bacteria	Description	Relevant Characteristics[w]	Source or Reference
Erwinia carotovora subsp. *carotovora*:			
W3C105	Field isolate	Cat^+, Iuc^+, Iut^+	Xu and Gross, 1986a
SR319	Field isolate	Cat^+	A. Kelman
JL1148	Spontaneous rifampicin-resistant mutant of W3C105	Cat^+, Iuc^+, Iut^+	Ishimaru, unpublished
JL1154	Spontaneous rifampicin-resistant mutant of SR319	Cat^+	Ishimaru, unpublished
JL11146	JL1148::Tn*3*-Spice, recombinant-exchange mutant with pJEL1752[x], Sp^r, Cm^r	Cat^-, Iuc^+, Iut^+	Bull, Chapter 2
JL11202	W3C105::Tn*3*-Spice, recombinant-exchange mutant with pJEL1752, Sp^r, Cm^r	Cat^-, Iuc^+, Iut^+	Bull, Chapter 2
JL11178	Derivative of W3C105, Δ(*iuc*)	Cat^+, Iuc^-, Iut^+	Ishimaru et al., 1992
JL11182	Derivative of W3C105, Δ(*iuc*, *iut*)	Cat^+, Iuc^-, Iut^-	Ishimaru et al., 1992
JL11206	JL11178::Tn*3*-Spice, recombinant-exchange mutant with pJEL1752, Sp^r, Cm^r	Cat^-, Iuc^-, Iut^+	Bull, Chapter 2
JL11207	JL11182:Tn*3*-Spice, recombinant-exchange mutant with pJEL1752, Sp^r, Cm^r	Cat^-, Iuc^-, Iut^-	Bull, Chapter 2

TABLE 3-1. (Continued)

Erwinia carotovora subsp. *atroseptica*			
W3C37	Field isolate	Cat^+	Xu and Gross, 1986a
JL1152	Spontaneous rifampicin-resistant mutant of W3C37	Cat^+	Ishimaru, unpublished
Pseudomonas fluorescens			
W4F151	Biovar III, antibiosis group III[y],	Sid (high)[z]	Xu and Gross, 1986a
W4F382	Biovar III, antibiosis group III,	Sid (high)	Xu and Gross, 1986a
W4F156	Biovar III, antibiosis group III,	Sid (high)	Xu and Gross, 1986a
W4F166	Biovar III, antibiosis group I	Sid (high)	Xu and Gross, 1986a
W4F164	antibiosis group I	Sid (high)	Xu and Gross, 1986a
W4F35	Biovar III, antibiosis group III,	Sid (high)	Xu and Gross, 1986a
W4F111	Biovar III, antibiosis group I,	Sid (high)	Xu and Gross, 1986a
W4F131	antibiosis group IV,	Sid (high)	Xu and Gross, 1986a
Pf-5	antibiosis group III	Sid (high)	Howell and Stipanovic, 1980

TABLE 3-1. (Continued)

Pseudomonas putida			
W4P396	antibiosis group I,	Sid (high)	Xu and Gross, 1986a
W4P5	antibiosis group III,	Sid (high)	Xu and Gross, 1986a
W4P144	antibiosis group III,	Sid (high)	Xu and Gross, 1986a
W4P63	antibiosis group III,	Sid (medium)	Xu and Gross, 1986a

[w]Catechol production (Cat^+), aerobactin production (Iuc^+), aerobactin uptake (Iut^+).
[x]Cosmid pJEL1752 consists of pLAFR3 containing catechol biosynthesis genes of W3C105 with a Tn*3*-Spice insertion.
[y]Inhibition of strain W3C37 of *E. carotovora* subsp. *atroseptica* on: only KMB agar (group I strains), only potato-dextrose agar (group II strains), both KMB agar and PDA (group III strains), and neither KMB agar nor PDA (group IV strains) (Xu and Gross, 1986a).
[z]Strains of *Pseudomonas* that grow on media containing high concentrations of ethylenediamine-di-(o-hydroxyphenyl acetic acid) ($\geq$1,000 μg/ml), medium concentrations (>250-<1,000 μg/ml), or low concentrations ($\leq$250 μg/ml) (Xu and Gross, 1986a).

TABLE 3-2. Source of potato tubers used in experiments

Lot Designation	Date received	Description	Source
A	Sept., 1987	Russet Burbank, large tubers from potato processing house in Pendleton, Oregon.	John Walchi via Dr. Mary Powelson
B	Sept., 1988	Russet Burbank, large tubers from potato processing house in Pendleton, Oregon.	John Walchi via Dr. Mary Powelson
C	Sept., 1990	Russet Burbank, small tubers that were too small for grading.	Lamb-Weston via Luther Fitch
D	Sept., 1991	Russet Burbank, small tubers that were too small for grading.	Lamb-Weston via Luther Fitch
E	Spring, 1992	Norgold Russet, 6-week-old plants from Seed Potato Virus Certification Program.	Bob Henderson, Oregon State University

tubers were soaked in 0.8% NaCl for 3 h to equilibrate osmotic potential. These tubers were rinsed briefly prior to inoculation and were not air dried.

Population assays.

Potatoes were inoculated by suspending the tubers for 10-15 min in bacterial suspensions (0.1 OD_{600nm}) in 0.5% methylcellulose or deionized water. Bacteria were grown 18-24 h prior to use. In population studies in which a tuber was inoculated with more than one strain, the density of each strain was equal to that of a bacterium in inoculum containing only one strain.

Potatoes were placed on wire racks in a mist chamber (RH=100%) or on wet paper towels inside a closed vegetable crisper in a growth chamber. Potatoes were incubated at 22 C. Six inoculated and one noninoculated tuber were sampled at 2 h, 1, 2, 3, 5 and 7 days after inoculation. Tubers were sampled by slicing two 2-cm strips from each tuber surface. From each tuber slice, two cork-borer samples (3.1 cm diameter) were removed. Each cork-borer sample was sonicated for 20 s in 5 ml of sterile water. One-hundred-microliter aliquots from serial dilutions were spread on LB amended with rifampicin and cycloheximide. Colonies were counted after plates were incubated for 2 to 3 days at 27 C. The experiments were completely randomized designs with subsampling. Six replications each with 4 subsamples were made per treatment. The experiments were not repeated.

Erwinia carotovora subsp. *atroseptica* JL1152 (W3C37, Rif^r), *E. carotovora* subsp. *carotovora* JL1148 (W3C105, Rif^r), and *P. fluorescens* Pf-5 (JL3871) grew on KMB amended with tetracycline. Both strains of *E. carotovora* were resistant to rifampicin and sensitive to nalidixic acid in LB, NaGly and KMB. *Pseudomonas fluorescens* Pf-5 was resistant to nalidixic acid but sensitive to rifampicin in LB, NaGly and KMB. Therefore, in experiments involving mixed populations of *E.*

carotovora and *P. fluorescens*, the population size of Pf-5 was estimated by dilution plating on KMB amended with nalidixic acid, and that of *E. carotovora* was estimated on LB amended with rifampicin.

Soft rot assays.

For soft rot studies, potatoes were inoculated by one of two methods. Method A was modified from De Boer and Kelman (1978). A bacterial suspension (100 μl) containing a known concentration of bacteria was drawn into a 200 μl pipet tip (Costar, Cambridge, MA). The pipet tip was then stuck 2 cm into the tuber where it remained during incubation of the tubers. Inoculation sites were distal to lenticles and eyes of the tubers. Potato tubers were placed in a growth chamber on wet paper towels inside a vegetable crisper. For some experiments tubers were placed in individual weigh boats containing a paper towel folded and wet with water (20 ml) to prevent contamination between tubers. The crispers were sealed with parafilm. Tubers were incubated at 22 C for 4 days, 15 C for 7 days, 10 C for 10 days.

Method B was adapted from Axelrood et al. (1988). A cork-borer plunger was used to push a 2-cm-deep hole into the tuber. Excess fluid was removed from the hole by sucking fluid up with a pipet tip and 10 μl of a bacterial suspension was placed in the hole. Tubers were wrapped in a moist paper towel with the inoculation point remaining upright. The paper towel and tuber were then sealed with a layer of plastic wrap. Tubers were incubated in crispers sealed with parafilm and incubated at 22 C for 4 days. Inoculum for both inoculation methods was prepared from overnight cultures of bacteria adjusted to 0.1 OD_{600nm} in $MgSO_4$ (0.1 M).

Disease was evaluated by quantifying the mass of rotted tissue. The rot from an

individual inoculation point was scooped from the potato and weighed. In experiments in which pipet tips were used for inoculation, the pipet tip was removed from the site of inoculation and any moisture remaining in the pipet tip was added to the sample. Experiments were designed and analyzed as completely randomized experiments.

Stem rot assays.

Six-week-old Norgold Russet potato plants were obtained from the Virus Free Certification Program (Oregon State University). Plants were transplanted into 20 cm^2 pots containing moist peat moss (Redi-Earth Peat-Lite Mix, W. R. Grace and Co., Cambridge, MA). A compound leaf, located approximately 5-10 cm from the top of the plant, was removed from the stem at the base of the petiole. The wound was deepened by poking the area once with an instrument having five 4-cm needles. Ten-microliters of a bacterial suspension (0.05 OD_{600nm} or 0.10 OD_{600nm}) were placed on a sterile filter paper disk (Whatman Int. Ltd., Maidstone, England). The disk was placed on the wound and was held in place with tape. Plants were placed in a greenhouse at 18 C on a 12-h light-dark cycle. For 2 days after inoculation, the plants were sealed in an opaque plastic bag. Disease rating began 3 days after inoculation. A 0-4 disease rating scale was used to evaluate disease severity (Table 3-3).

In experiment I, plants were rated at 3 days, 4 days, and 5 days after inoculation. In the repeat of one experiment, plants were rated at 3 days after inoculation only. Blocking in both experiments was based on plant size.

TABLE 3-3. Disease rating scheme for aerial stem rot experiments

Disease Rating	Symptoms
0	Lacking symptoms, identical to water control
1	Blackening at point of inoculation, but not spreading
2	Lesion spreading to larger than 2 cm diameter of the inoculation filter disk
3	Lesion as in 2, and at least one upper leaf curling and/or at least one upper leaf wilted.
4	Dead plant

RESULTS

Development of an assay to evaluate population dynamics on potato tubers.

Population dynamics of *E carotovora.* There was no significant difference at 2 h after inoculation between population sizes of JL1152 on potato tubers inoculated with JL1152 suspended in methylcellulose (1.58 log [cfu/cm^2]) or water (1.08 log [cfu/cm^2]). Bacteria from either methylcellulose or water suspensions were detected on 63% of the 24 samples assayed for each treatment (detection limit = 1.39 log [cfu/cm^2]). Potatoes were inoculated with aqueous-bacterial suspensions in other experiments.

Population sizes of *E. carotovora* subsp. *atroseptica* JL1152 and *E. carotovora* subsp. *carotovora* JL1148 increased from the initial population size on potato tubers incubated in mist chambers (FIGURE 3-1). On surfaces of tubers incubated in the mist chamber, the population size of JL1152 increased by 3 days and remained stable after 3 days. The population size of JL1148 increased between 3 and 5 days after inoculation. On potatoes incubated in the growth chamber, the population sizes of JL1152 or JL1148 did not increase throughout the 7 days of the experiment. Because the population size of both strains increased when inoculated tubers were incubated in mist chambers, further studies monitoring population size were conducted in mist chambers.

Effect of *P. fluorescens* on population dynamics of *E. carotovora.* The population dynamics of *E. carotovora* subsp. *atroseptica* JL1152 inoculated in combination with *P. fluorescens* Pf-5 were similar to those on tubers inoculated with JL1152 alone (FIGURE 3-2). The population size of JL1152 was not smaller on tubers

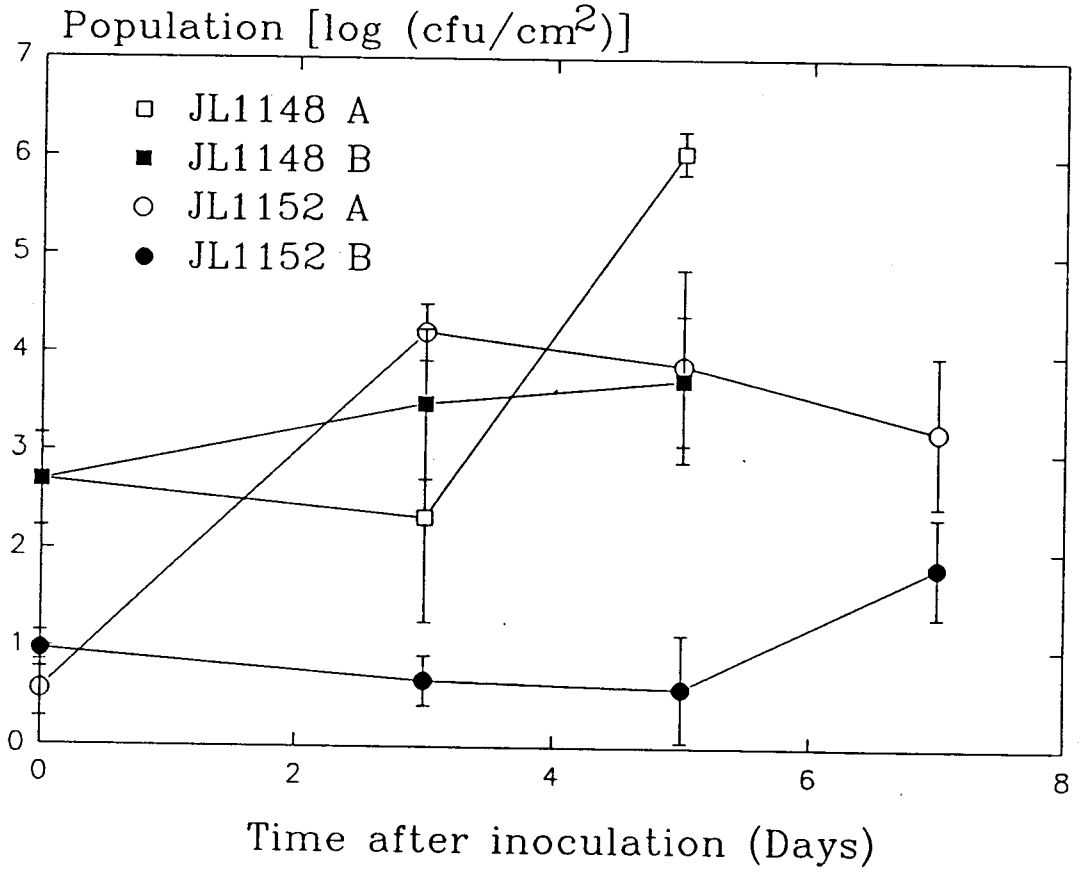


FIGURE 3-1. Population dynamics of *Erwinia carotovora* subsp. *carotovora* JL1148 (W3C105, Rif^r) and *E. carotovora* subsp. *atroseptica* JL1152 (W3C37, Rif^r) on potato tubers incubated in mist chambers or growth chambers at 22 C. Potatoes used in these experiments were from lot A. Tubers were incubated A) in mist chambers at 100% RH or B) in growth chambers in crispers. Population studies involving JL1148 and JL1152 were done separately. Populations are means of six replication having four subsamples each. Error bars represent standard error around the mean for each population.

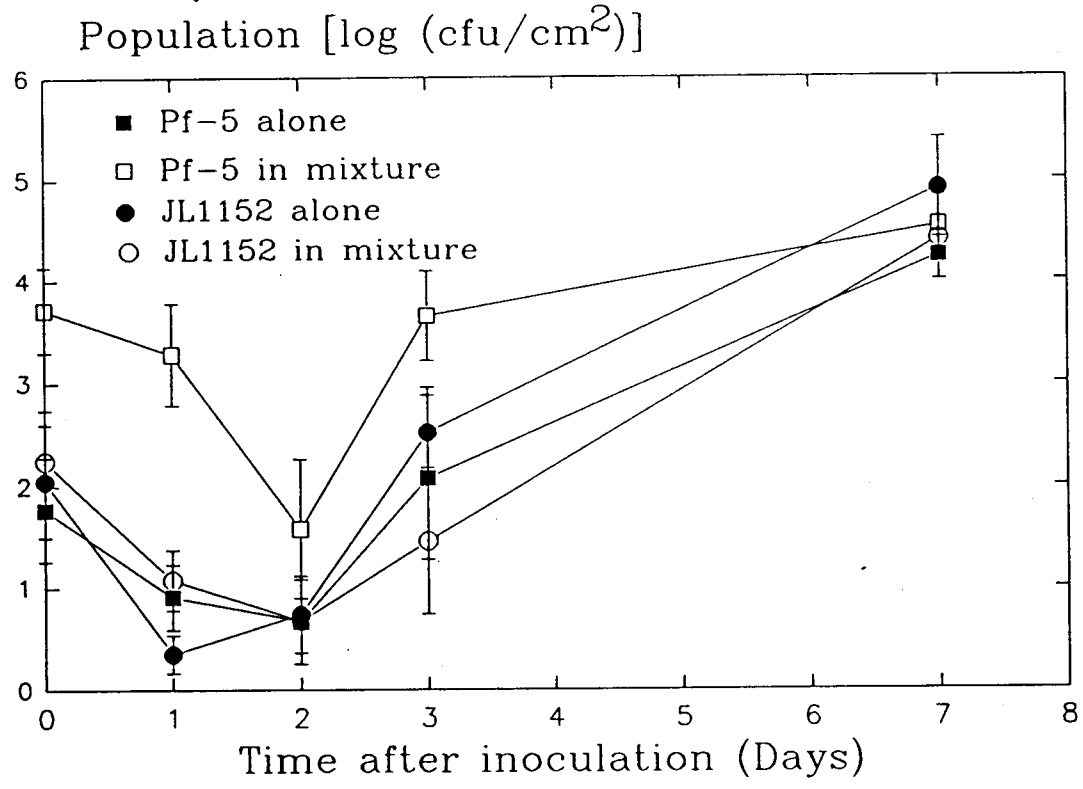


FIGURE 3-2. Population dynamics of *Erwinia carotovora* subsp. *atroseptica* JL1152 (W3C37, Rif^r) and *Pseudomonas fluorescens* Pf-5 inoculated alone and in combination on potato tubers.
Tubers were incubated at 22 C in mist chambers at 100% RH. Potatoes used in this experiment were from lot A. Population are means of six replications each with four subsamples. Error bars represent standard error around the mean for each population.

coinoculated with Pf-5 than on tubers inoculated with JL1152 alone. Populations of Pf-5 and JL1152 dropped from initial levels for 2 days after inoculation. Between 2 and 7 days after inoculation populations of both strains increased regardless of whether they were inoculated singly or in combination.

No correlation was detected between populations of Pf-5 and JL1152 on individual periderm disks when data from all days was analyzed by regression analysis. Slight correlations between populations of Pf-5 and JL1152 were detected 2 h after inoculation (day 0, R-squared=0.8288), 2 days after inoculation (R-squared=0.8396), and 3 days after inoculation (R-squared=0.6015). No correlation was detected at 1 day (R-squared=0.2449) nor at 7 days after inoculation (R-squared=0.3324). Thus, samples that had a large population of the biological control agent did not necessarily have a large population of the pathogen. The presence of populations of Pf-5 did not reduce the population size of JL1152 on potato tuber surfaces. Thus, under the conditions of this study Pf-5 did not inhibit growth of JL1152 on the tuber surface. These results suggest that monitoring populations of *E. carotovora* on potato surfaces was not an adequate assay for the detection of biological control activity or that biological control was not occurring on the tuber surface.

Development of an assay to assess tuber soft rot.

Effect of inoculum density on tuber soft rot. Amount of potato tuber tissue macerated increased with increasing inoculum concentrations of *E. carotovora* (FIGURE 3-3). At a given inoculum concentration W3C37 caused less tissue maceration than SR319 and W3C105 in this experiment. In other experiments W3C37 caused more rot than W3C105 at a given inoculum concentration. The lowest coefficient of variation (CV) was detected at the 5.3 and 6.3 log (cfu/ml)

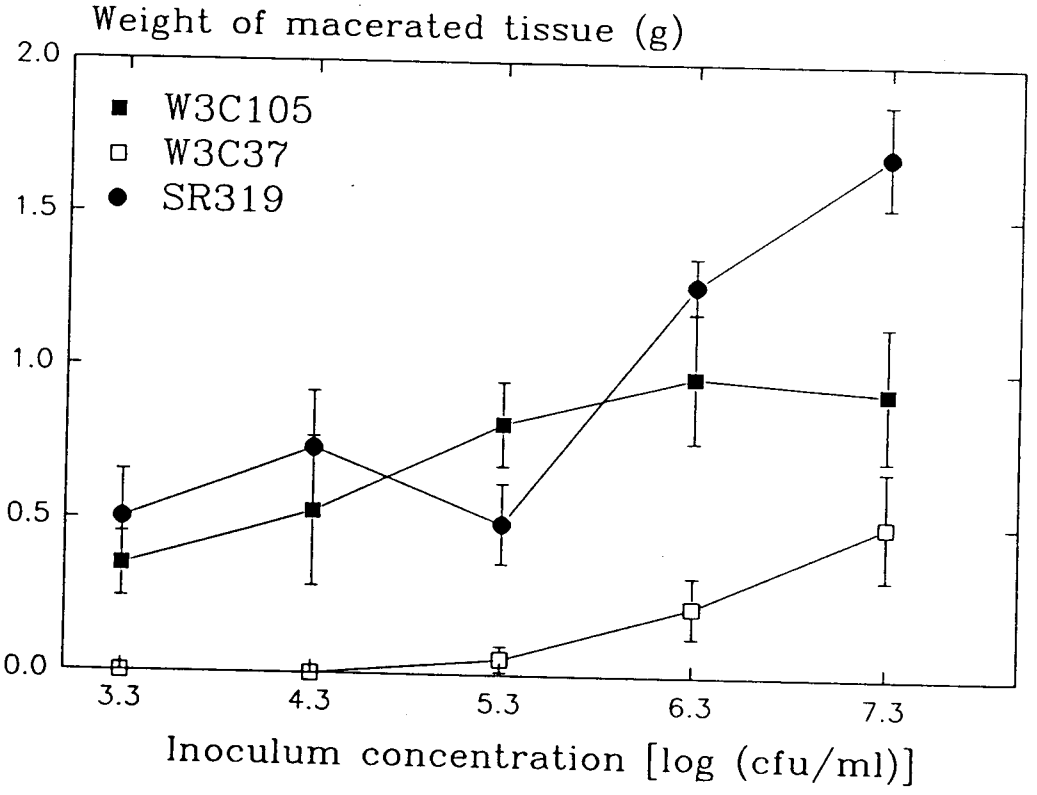


FIGURE 3-3. Potato tuber maceration on Russet Burbank potatoes inoculated with increasing concentrations of *E. carotovora* subsp. *carotovora* (SR319 and W3C105) or *E. carotovora* subsp. *atroseptica* (W3C37) and incubated at 22 C for 4 days in growth chambers in crispers.
Potato tubers used in this experiment were from lot B. Potatoes were inoculated by a method modified from De Boer and Kelman (1978) as described in Table 3-4. Weight values are means from four replications. Error bars represent standard error around the treatment means.

(CV=16-37%). Inoculum concentrations of 5.3, 6.3, and 7.3 log (cfu/ml) were chosen for further soft rot bioassays.

Effect of incubation conditions on tuber soft rot. Potatoes inoculated with W3C37 had greater quantities of macerated tissue than did potatoes inoculated with W3C105 (TABLE 3-4). When potatoes were incubated at 22 C for 4 days, there were no differences between the weight of macerated tissue produced by inoculation with W3C37 or W3C105. The CV was the lowest (52.9%) when potatoes were incubated at 22 C for 4 days; therefore, further soft rot experiments were conducted under these conditions.

Potato tuber maceration initiated by spontaneous rifampicin-resistant mutants. The amount of macerated tissue in tubers inoculated with W3C37, W3C105, and SR319, or spontaneous rifampicin-resistant derivatives of these strains did not differ significantly (TABLE 3-5). The similarity between the amount of tissue maceration caused by rifampicin-resistant mutants and parental strains, in addition to similarity of generation times of the parental strains and rifampicin-resistant mutants (Bull, Chapter 2) led to the assumption that the mutants were similar to the parental strains and could be used to represent the parental strains in soft rot bioassays. The rifampicin-resistant mutant JL1148 and JL11146, the catechol-biosynthesis (Cat^-) derivative of JL1148 (Cat^+), were used in subsequent experiments.

Tuber soft rot caused by siderophore mutants of *E. carotovora* subsp. *carotovora*.

In two sets of experiments, potatoes inoculated with mutants deficient in catechol production, JL11146 (Cat^-Rif^R) or JL11202 (Cat^-Rif^R), developed soft rot (TABLE 3-6; 3-7; FIGURE 3-4; 3-5). Thus, catechol production by *E. carotovora* subsp. *carotovora* was not necessary for soft rot development by *E. carotovora*.

TABLE 3-4. Maceration of Russet Burbank potato tubers inoculated with strains of *Erwinia carotovora*

Inoculum[v]	Inoculum concentration[w]	Weight of Macerated Tissue (g)[st] Temperature (C)[u] 22	15	10
W3C37[w]	6.50	1.41AB[xy]	2.87A	2.27A
	7.50	2.91A	3.25A	3.20A
W3C105[z]	6.50	0.21B	0.00B	0.05B
	7.50	1.15AB	1.29AB	0.37B

[s]Potatoes used were from lot A, each with two inoculation sites per tuber.
[t]Treatments within each temperature were completely randomized and each experiment at each temperature was conducted separately.
[u]Potatoes at 10 C, 15 C and 22 C were incubated 10, 7, and 4 days, respectively. Potatoes were incubated in growth chambers sealed in crispers.
[v]Method for inoculation of potatoes was modified from De Boer and Kelman (1978). Bacterial strains grown on LB overnight at 27 C, were adjusted to an 0.1 OD_{600nm} in 0.1 M $MgSO_4$ and diluted in 0.1 M $MgSO_4$ to the designated concentration. Potatoes were inoculated by inserting a 200 μl pipet tip containing 100 μl of inoculum into the tuber, where it remained throughout the experiment.
[w]*Erwinia carotovora* subsp. *atroseptica* W3C37.
[x]Weight data are means from four replications.
[y]Means followed by the same letter are not significantly different according to Tukey's separation of means at the $\underline{P}=0.05$ level.
[z]*Erwinia carotovora* subsp. *carotovora* W3C105.

TABLE 3-5. Maceration of Russet Burbank potato tubers inoculated with field strains and rifampicin-resistant mutants of *Erwinia carotovora* and incubated for 4 days at 22 C in growth chambers

Weight of macerated tissue[tuv]					
E. carotovora subsp. *atroseptica*[w]		*E. c.* subsp. *carotovora*[x]			
W3C37 Rif[s]	JL1152 W3C37 Rif[r]	W3C105 Rif[s]	JL1148 W3C105 Rif[r]	SR319 Rif[s]	JL1154 SR319 Rif[r]
4.75A[yz]	4.55A	3.40A	4.00A	3.05A	4.47A

[t]Potatoes used in this experiment were from lot A; each tuber had two inoculations.
[u]The experiment was complete randomized design.
[v]Method A was modified from De Boer and Kelman (1978), described (TABLE 3-4).
[w]Strain JL1152 is a rifampicin-resistant (Rif[r]) derivative of *E. carotovora* subsp. *atroseptica* strain W3C37.
[x]Strain JL1148 is a Rif[r] derivative of *E. carotovora* subsp. *carotovora* strain W3C105; Strain JL1154 is a Rif[r] derivative of *E. carotovora* subsp. *carotovora* strain SR319.
[y]Weight values are means from 6 replications.
[z]Means followed by the same letter are not significantly different according to Tukey's separation of means at the $\underline{P}=0.05$ level.

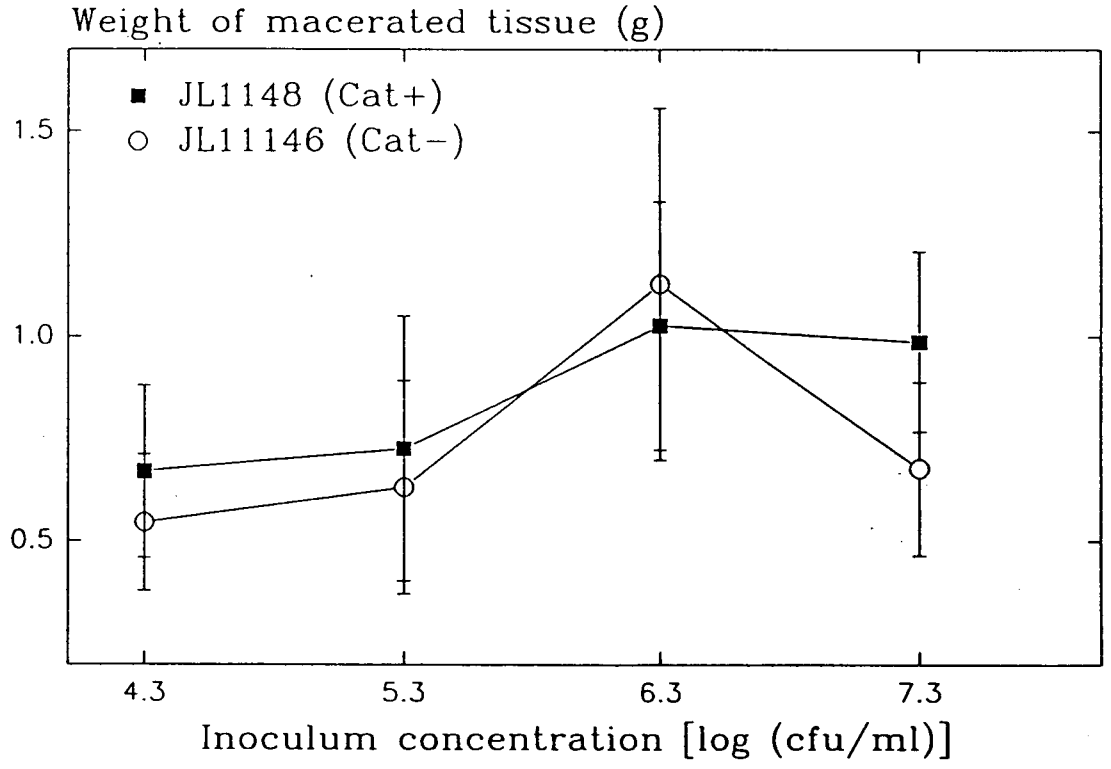


FIGURE 3-4. Maceration of Russet Burbank potato tubers inoculated with increasing concentrations of *Erwinia carotovora* subsp. *carotovora* JL1148 (Cat^+, Rif^r) and JL11146 (Cat^-, Rif^r).
Potatoes were from lot D. Potatoes were inoculated by a method modified from De Boer and Kelman (1978) as described (TABLE 3-4). Macerated tissue was removed and weighed 4 days after tubers were inoculated and placed in growth chambers at 22 C. Weights of macerated tissue are the means of 10 replications. Error bars represent standard error around the treatment means.

TABLE 3-6. Maceration of Russet Burbank potato tubers inoculated with *Erwinia carotovora* subsp. *carotovora* W3C105 (Cat^+), JL1148 (Cat^+, Rif^r) or JL11146 (Cat^-, Rif^r) and incubated for 4 days at 22 C in growth chambers

	Weight of macerated tissue (g)[s]				
	Experiment[t]				
Inoculum[u]	1	2	3	4	Means[v]
W3C105[w]	2.40A[x]	6.14A	1.71A	1.68A	2.08A
JL1148[y]	2.20A	4.45A	0.93B	0.78B	1.27B
JL11146[z]	2.40A	2.08B	0.76B	0.90B	1.20B

[s]Potatoes used in experiments 1 and 2 were from lot C; potatoes used in experiments 3 and 4 were from lot D.
[t]Experiments were complete randomized designs. Experiment 1 had 6 replications per treatment. Experiments 2, 3 and 4 had 10 replications per treatment.
[u]Method A was modified from De Boer and Kelman (1978), described (TABLE 3-4).
[v]Means are pooled from experiments 1, 2, and 4, which had equal variances. There was no significant interaction between experiments 1, 2, and 4, and treatment effects ($P=0.83$).
[w]*E. carotovora* subsp. *carotovora* W3C105 (Cat^+).
[x]Means followed by the same letter are not significantly different according to Tukey's comparison of means at the $P=0.05$ level.
[y]JL1148 is a rifampicin-resistant mutant of *E. carotovora* subsp. *carotovora* W3C105.
[z]JL11146 is a Cat^- mutant of JL1148.

TABLE 3-7. Maceration of Russet Burbank potato tubers inoculated with *Erwinia carotovora* subsp. *carotovora* W3C105, or siderophore production mutants

Inoculum[t]	Siderophore Production[u]	Weight of macerated tissue (g)[qr] Experiment[s] 1[v]	2[w]
W3C105[x]	Cat^+, Iuc^+, Iut^+	1.31A[yz]	0.96A
JL11202	Cat^-, Iuc^+, Iut^+	1.51A	0.89A
JL11178	Cat^+, Iuc^-, Iut^+	1.06A	1.10A
JL11182	Cat^+, Iuc^-, Iut^-	1.66A	1.13A
JL11206	Cat^-, Iuc^-, Iut^+	1.36A	0.90A
JL11207	Cat^-, Iuc^-, Iut^-	1.32A	0.91A
CV		59.7%	47.5%

[q]Potatoes used in these experiments were from lot E. Potatoes were soaked in 0.8% NaCl for 3 h prior to inoculation.
[r]Experiments were completely randomized.
[s]Data from each inoculation method was analyzed separately.
[t]Inoculum was prepared from strains grown overnight on TMS agar (0.001 μM $FeCl_3$) at 27 C. Inoculum was adjusted to 0.1 OD_{600nm} in 0.1 M $MgSO_4$. Inoculum concentration was adjusted such that concentrations were 6.3 log (cfu/ml).
[u]Catechol production, aerobactin production and aerobactin uptake are denoted as Cat^+, Iuc^+, and Iut^+, respectively.
[v]Experiment 1 was conducted by Method A modified from De Boer and Kelman (1978). Potatoes were incubated in separate weigh boats for 4 days at 22 C in growth chambers.
[w]Experiment 2 was conducted by Method B modified from Axelrood *et al.* (1988).
[x]All mutants were derived through marker-exchange mutagenesis of *Erwinia carotovora* subsp. *carotovora* W3C105.
[y]Weights are means of ten replications per treatment.
[z]Means within a column that are followed by the same letter are not significantly different according to Tukey's separation of means at the $P=0.05$ level.

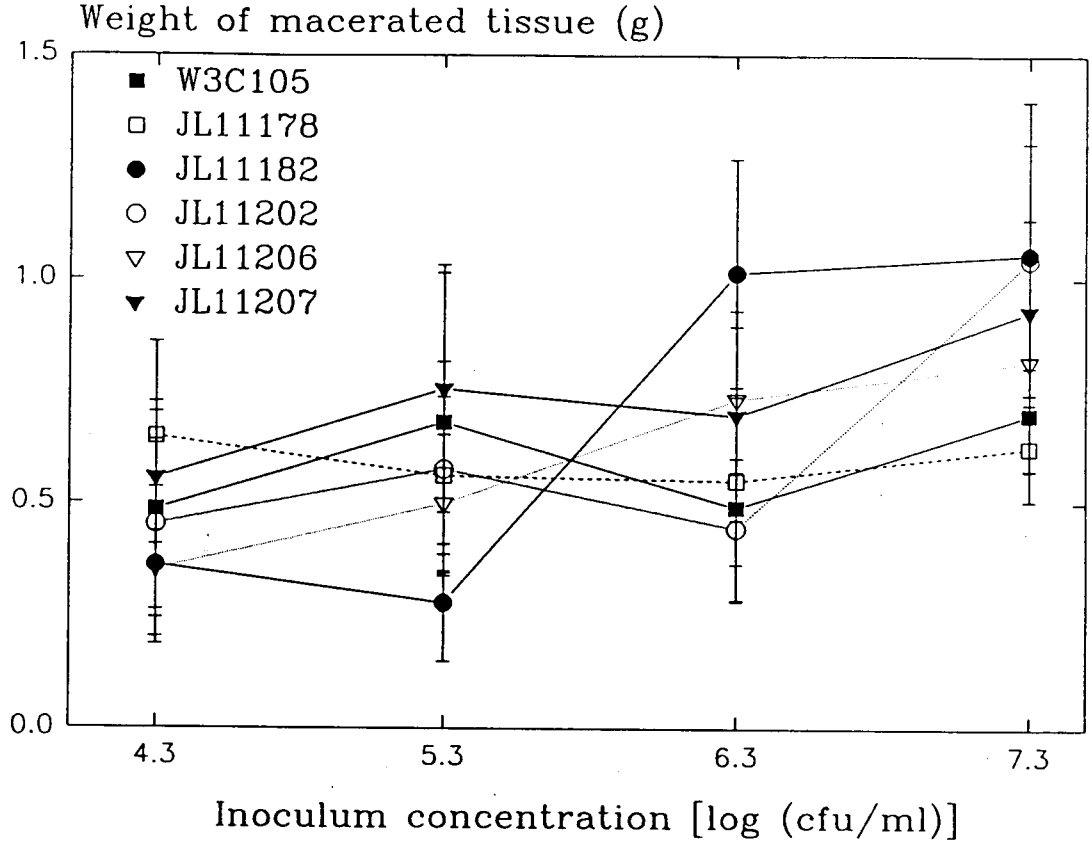


FIGURE 3-5. Maceration of Russet Burbank potato tubers inoculated with increasing concentrations of *Erwinia carotovora* subsp. *carotovora* W3C105 ($Cat^+Iuc^+Iut^+$) or derivatives, JL11202 ($Cat^-Iuc^+Iut^+$), JL11178 ($Cat^+Iuc^-Iuc^+$), JL11182 ($Cat^+Iuc^-Iut^-$), JL11206 ($Cat^-Iuc^-Iut^+$), or JL11207 ($Cat^-Iuc^-Iut^-$). Potatoes were from lot E. Potatoes were inoculated by a method modified from De Boer and Kelman (1978) as described (TABLE 3-4). Macerated tissue was removed and weighed 4 days after tubers were inoculated and placed in growth chambers at 22 C. Weights of rot are means from ten replications per treatment. Error bars represent standard error around the treatment means.

Inoculation of tubers with increasing concentrations of the rifampicin-resistant strains JL1148 (Cat^+) or JL11146 (Cat^-) did not result in greater amounts of macerated tissue (FIGURE 3-4). No differences were detected between potatoes inoculated with the highest concentration of cells, 7.3 log (cfu/ml) or the lowest inoculum level, 4.3 log (cfu/ml). There was no significant difference between the amount of rot in tubers inoculated with JL1148 or JL11146 at any inoculum concentration tested.

In three out of four experiments, the amount of rot in potatoes inoculated with JL11146 (Cat^-) was not significantly different than the amount of rot from potatoes inoculated with JL1148 (Cat^+) (TABLE 3-6). In experiment 2, tubers inoculated with JL11146 had significantly less rot than those inoculated with JL1148. Experiments 1, 2, and 4 had homogeneous variances and no significant interactions between treatment and experimental means ($p=0.83$). When the data from three of four experiments (Experiments 1, 2, and 4) were analyzed together (representing 26 replications), no significant difference in the amount of rot was detected among potatoes inoculated with JL1148 (1.27 g) and potatoes inoculated with JL11146 (1.20 g). Thus, under the conditions of these experiments, production of catechol did not contribute significantly to the virulence of *E. carotovora* W3C105.

In two out of four experiments, potatoes inoculated with JL1148 (Rif^r) had less rot than potatoes inoculated with the parental strain W3C105 (Table 3-6). In an earlier experiment, no difference was detected between tubers inoculated with these Rif^r and Rif^s strains (TABLE 3-5). When the data from three of four experiments (experiments 1, 2, and 4) (TABLE 3-6) were analyzed together, the amount of rot in potatoes inoculated with JL1148 (1.27 g) was significantly less than that of potatoes inoculated with W3C105 (2.08 g). Thus, in contrast to preliminary studies, JL1148 was less virulent than W3C105 soft rot studies.

Due to the complications associated with rifampicin-resistant strains, a second

set of experiments evaluated the virulence of rifampicin-sensitive strains with mutations in catechol siderophore production (Cat^+) and aerobactin production (Iuc^+) or uptake (Iut^-). Potatoes inoculated with strains JL11206 ($Cat^-Iuc^-Iut^+$) or JL11207 ($Cat^-Iuc^-Iut^-$), which produced no siderophores, developed soft rot (TABLE 3-7; FIGURE 3-5). Thus, siderophore production is not essential for pathogenicity of *E. carotovora*. In addition, the weight of macerated tissue did not differ among potatoes inoculated with the parental strain W3C105 ($Cat^+Iuc^+Iut^+$), a catechol production mutant JL11202 (Cat^-Iuc^+), aerobactin mutants JL11178 (Cat^+Iuc^-) and JL11182 ($Cat^+Iuc^-Iut^-$) or strains that produced no siderophore, JL11206 (Cat^-Iuc^-) and JL11207 ($Cat^-Iuc^-Iut^-$) (TABLE 3-7). Thus, siderophore production was not essential for soft-rot development by W3C105. Further, strains producing siderophores were not more virulent in soft rot production than strains that did not produce siderophores.

Biological control of soft rot.

Biological control of tuber soft rot caused by *E. carotovora* subsp. *atroseptica* W3C37 was not achieved by inoculation of potato tubers with strains of *P. fluorescens* or *P. putida* strains (TABLES 3-8; 3-9; 3-10). Biological control was evaluated using two methods of inoculation but disease control was not achieved using either method (TABLE 3-8). Tubers coinoculated with W3C37 and *P. fluorescens* W4F382 had significantly greater amounts of macerated tissue than did tubers coinoculated with W3C37 and *P. putida* W4P63 or W4P540 (Method A). There were no significant differences in the amount of tissue macerated among all treatments when Method B was used to inoculate tubers. The coefficients of variation of Method A and Method B were 44.5% and 31.3%, respectively. Method A was used for remaining experiments because some differences were detected among treatments and it was less cumbersome than Method B.

TABLE 3-8. Maceration of Russet Burbank potato tubers inoculated with strains of *Erwinia carotovora* subsp. *atroseptica* W3C37 in the presence and absence of fluorescent pseudomonads and incubated for 4 days at 22 C in growth chambers

	Weight of macerated potato tissue (g)[qr]	
	Inoculation Method[s]	
Inoculum[tu]	A[v]	B[w]
W3C37[x] alone	2.79AB[yz]	4.7A
W3C37 + *Pseudomonas putida*:		
+ W4P5	2.31AB	4.5A
+ W4P144	2.24AB	4.5A
+ W4P63	1.56B	4.0A
+ W4P540	1.62B	4.1A
W3C37 + *Pseudomonas fluorescens*:		
+ W4F151	1.96AB	4.1A
+ W4F382	3.02A	3.6A
+ Pf-5	1.84AB	3.7A

[q]Potatoes used in these experiments were from lot B.
[r]Experiments were completely randomized designs.
[s]Treatments from each method of inoculation were analyzed separately.
[t]Inoculum was prepared from strains grown overnight on LB agar at 27 C adjusted to 0.1 OD_{600nm} in 0.1 M $MgSO_4$. Inoculum concentration of W3C37 was 6.3 log (cfu/ml) and of *P. fluorescens* Pf-5 was 7.3 log (cfu/ml).
[v]Method A was modified from De Boer and Kelman (1978) as described (TABLE 3-4).
[w]Method B was modified from Axelrood *et al.* (1990).
[x]*Erwinia carotovora* subsp. *atroseptica* W3C37.
[y]Weights are means of six replications per treatment.
[z]Means within a column that are followed by the same letter are not significantly different according to Tukey's separation of means at the $\underline{P}=0.05$ level.

TABLE 3-9. Maceration of Russet Burbank potato tubers inoculated with strains of *Erwinia carotovora* in the presence or absence of *Pseudomonas fluorescens* Pf-5 and incubated at 22 C in growth chambers

Strain	Presence of Pf-5[v]	Weight of Macerated Tuber Tissue (g)[st] Inoculum concentration of *E. carotovora*[u] (Log cfu/ml) 5.7	6.7
W3C37[w]	- Pf-5	0.76AB[xy]	1.31A
	+ Pf-5	0.89A	0.93AB
W3C105[z]	- Pf-5	0.52AB	0.60B
	+ Pf-5	0.23B	0.49B

[s]Potatoes used in this experiment were from lot B, each tuber having two inoculations.
[t]Experiments complete randomized designs.
[u]Method A was modified from De Boer and Kelman (1978), described (TABLE 3-4).
[v]The concentration of *P. fluorescens* Pf-5 was 7.3 log (cfu/ml).
[w]*Erwinia carotovora* subsp *atroseptica* W3C37.
[x]Weights are the means of five replications per treatment.
[y]Means followed by the same letter are not significantly different ($P=0.05$) according to Tukey's separation of means.
[z]*Erwinia carotovora* subsp. *carotovora* W3C105.

TABLE 3-10. Maceration of Russet Burbank potato tubers inoculated with *Erwinia carotovora* subsp. *atroseptica* W3C37 in the presence or absence of *Pseudomonas fluorescens* Pf-5 and incubated at 10 C, 15 C or 22 C in growth chambers

Concentration of W3C37[vw]	Presence of Pf-5[x]	Weight of Macerated Tissue (g)[st] Temperature (C)[u]		
		22	15	10
6.50	- Pf-5	0.82A[yz]	2.17A	0.89A
6.50	+ Pf-5	0.66A	1.51A	0.89A
7.50	- Pf-5	1.29A	3.53A	1.57A
7.50	+ Pf-5	0.53A	2.75A	1.25A

[s]Potatoes used were from lot A; each tuber had two inoculation sites.
[t]Experiments were completely randomized designs.
[u]Potatoes at 10 C, 15 C and 22 C were incubated 10, 7, and 4 days respectively.
[v]Method A was modified from De Boer and Kelman (1978), described (TABLE 3-4).
[w]*Erwinia carotovora* subsp. *atroseptica* W3C37.
[x]The concentration of *P. fluorescens* Pf-5 was 7.3 log (cfu/ml).
[y]Weights are means of six replications per treatment.
[z]Means followed by the same letter are not significantly different according to Tukey's separation of means at the p=0.05 level.

Pseudomonas fluorescens Pf-5, like the other biocontrol agents did not reduce the amount of rot produced by either W3C37 or W3C105 (TABLE 3-9). For either inoculum density used, the amount of tissue macerated was not significantly different for potatoes inoculated with *E. carotovora* or coinoculated with Pf-5. In addition, biological control was not achieved under three different incubation conditions tested (TABLE 3-10). The amount of rot in tubers inoculated only with W3C37 did not differ from the amount of rot in tubers coinoculated with W3C37 and Pf-5 regardless of the conditions under which the tubers were incubated. The coefficient of variation for samples incubated at 22 C for 4 days, 15 C for 7 days, or 10 C for 10 days were 59.2%, 73.6% and 62.7%, respectively. Thus, under the conditions of these studies biological control of soft rot was not achieved.

Sensitivity of *E. carotovora* subsp. *carotovora* siderophore mutants to biological control by fluorescent pseudomonads.

As in previous studies, the amount of rot in potatoes inoculated with W3C105 did not differ significantly from that in tubers inoculated with the siderophore mutants JL11182 ($Cat^+Iuc^-Iut^-$), JL11202 ($Cat^-Iuc^+Iut^+$) or JL11207 ($Cat^-Iuc^-Iut^-$). Less potato tissue was macerated in tubers in which W3C105 was coinoculated wit *P. fluorescens* W4F166 than in tubers inoculated only with W3C105 (FIGURE 3-6). In contrast, *P. putida* strain W4P396 had no significant effect on severity of soft rot caused by W3C105. *Pseudomonas fluorescens* strain W4F166 , however, did not suppress the severity of soft rot caused by the siderophore deficient mutants. Thus, siderophore mutants of W3C105 were not more sensitive to biological control than was W3C105. The results from this experiment need to be confirmed by repeating the experiment.

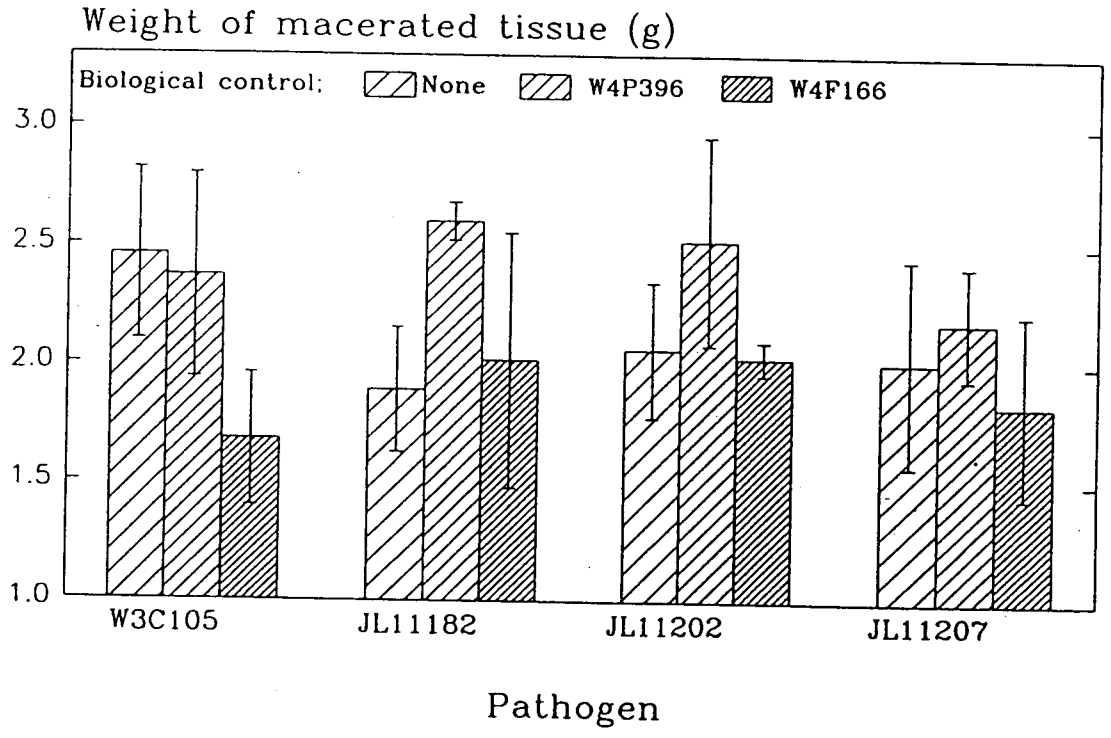


FIGURE 3-6. Maceration of Russet Burbank potato tubers inoculated with *Erwinia carotovora* subsp. *carotovora* W3C105 derivatives in the presence or absence of fluorescent pseudomonads.
Potatoes used in this experiment were from lot D. The experiment had 8 replications per treatment. Inoculum concentrations of strains of *E. carotovora* were 6.3 log (cfu/ml) and that of *P. fluorescens* Pf-5 was 7.3 log (cfu/ml). Inoculation method A was modified from De Boer and Kelman (1978) as described (TABLE 3-4). Phenotypes were W3C105 ($Cat^+Iuc^+Iut^+$), JL11182 ($Cat^+Iuc^-Iut^-$), JL11202 ($Cat^-Iuc^+Iut^+$), JL11207 ($Cat^-Iuc^-Iut^-$). Weights of macerated tissue are means of eight replications per treatment. Error bars represent standard errors around treatment means.

Aerial stem rot caused by siderophore-minus mutants of *E. carotovora* subsp. *carotovora.*

Inoculation of 6-week-old Norgold Russet potato plants with 10 μl of bacterial suspensions at densities of either 0.1 OD_{600nm} or 0.05 OD_{600nm} gave different results. For plants inoculated with 0.05 OD_{600nm} suspensions, less aerial stem rot was observed in plants inoculated with mutants producing no siderophores, JL11206 or JL11207, than in plants inoculated with W3C105 (FIGURE 3-7). Differences between siderophore-producing and non-producing strains were observed throughout the duration of the experiment. Plants inoculated with strains producing one siderophore, JL11202 ($Cat^-Iuc^+Iut^+$), JL11178 ($Cat^+Iuc^-Iut^+$), and JL11182 ($Cat^+Iuc^-Iut^-$) did not have different disease ratings compared to plants inoculated with W3C105. In a second experiment in which plants were inoculated at this inoculum concentration, the disease progressed faster and most of the plants were dead by day three, presumably due to infection by *Rhizoctonia solani* (data not shown).

For plants inoculated with inoculum at 0.1 OD_{600nm}, a different result was obtained. Of the two mutants that no longer produced any siderophore, only JL11207 ($Cat^-Iuc^-Iut^-$) caused less aerial stem rot than wildtype strain W3C105; JL11206 ($Cat^-Iuc^-Iut^+$) caused wildtype levels of disease (FIGURE 3-7). Inoculation of stems with either of the aerobactin-uptake mutants, JL11207 ($Cat^-Iuc^-Iut^-$) or JL11182 ($Cat^+Iuc^-Iut^-$), caused less aerial stem rot than did inoculation with W3C105.

From these data, I conclude that siderophore production is important in the development of aerial stem rot. Nevertheless, the relative importance of the catechol siderophore and aerobactin iron-acquisition systems to the virulence of *E. carotovora* in aerial stem rot was not conclusive. The results from this experiment must be confirmed by repeating the experiment.

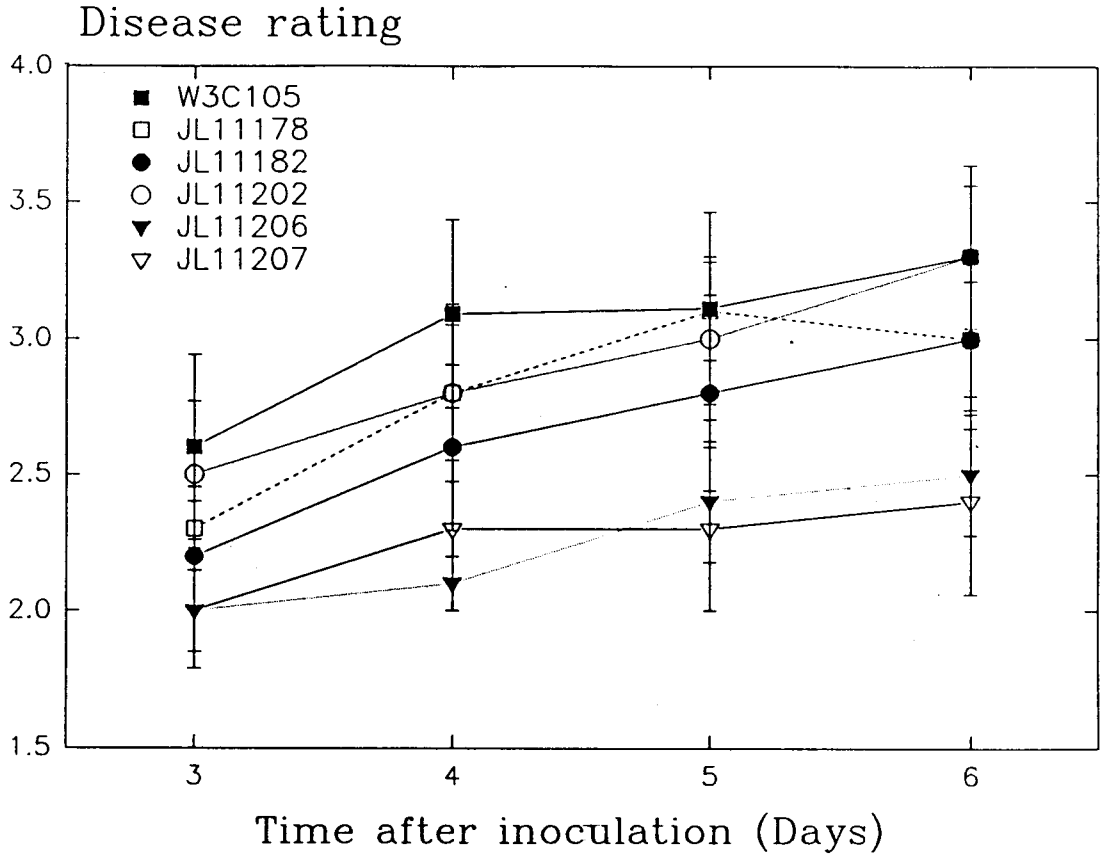


FIGURE 3-7. Dynamics of aerial stem rot development on potatoes inoculated with *Erwinia carotovora* subsp. *carotovora* W3C105 and siderophore mutants at 0.05 OD600nm.

Disease was rated on a scale of 1-4 with plants receiving 1 as a rating having no movement of the disease from the initial site of inoculation and plants receiving a 4 as a rating being dead. Phenotypes were W3C105 (Cat+Iuc+Iut+), JL11178 (Cat+Iuc-Iut+), JL11182 (Cat+Iuc-Iut-), JL11202 (Cat-Iuc+Iut+), JL11206 (Cat-Iuc-Iut+), JL11207 (Cat-Iuc-Iut-). Disease ratings are means of ten replications per treatment. Error bars represent standard errors around treatment means.

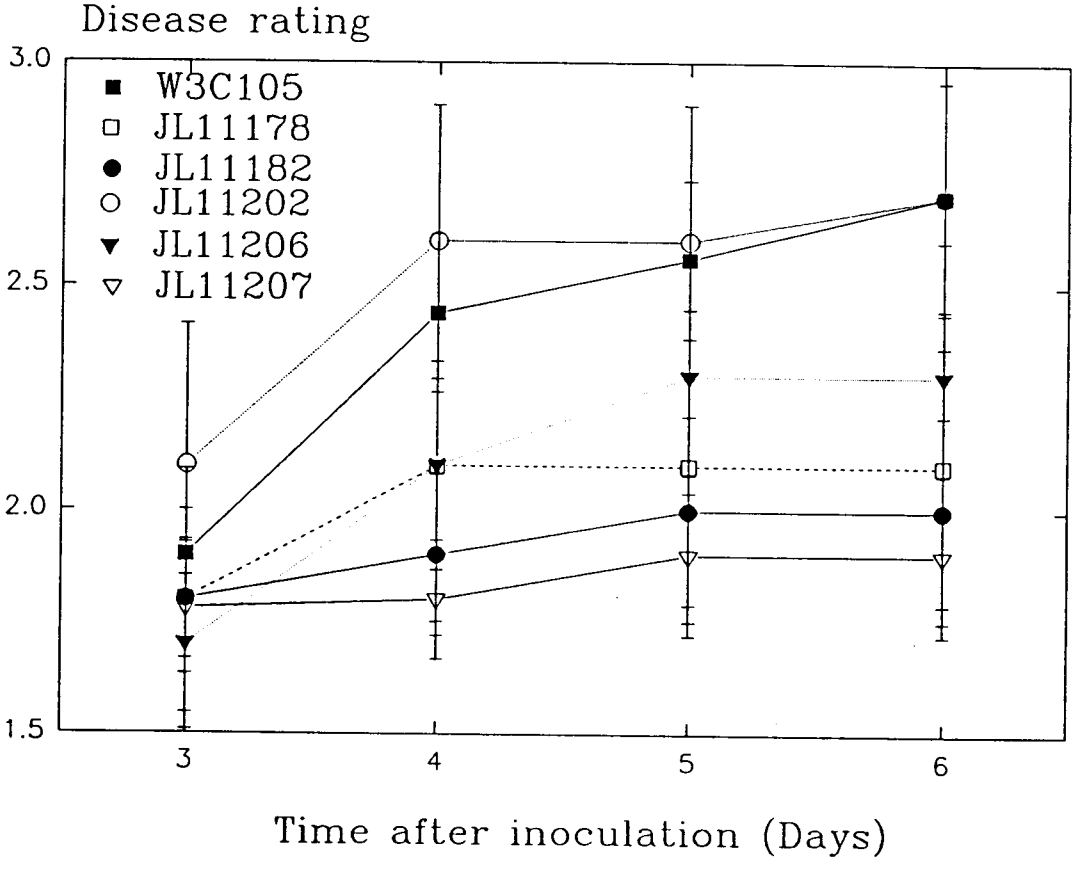


FIGURE 3-8. Dynamics of aerial stem rot development on potatoes inoculated with *Erwinia carotovora* subsp. *carotovora* W3C105 and siderophore mutants at 0.1 OD600nm.

Disease was rated on a scale of 1-4 with plants receiving 1 as a rating having no movement of the disease from the initial site of inoculation and plants receiving a 4 as a rating being dead. Phenotypes were W3C105 (Cat+Iuc+Iut+), JL11178 (Cat+Iuc-Iut+), JL111202 (Cat-Iuc+Iut+), JL11182 (Cat+Iuc-Iut-), JL11206 (Cat-Iuc-Iut+), JL11207 (Cat-Iuc-Iut-). Disease ratings are means of ten replications per treatment. Error bars represent standard errors around treatment means.

DISCUSSION

Catechol siderophore production is a trait common among strains of *Erwinia carotovora*. In a related pathogen, *E. chrysanthemi*, siderophore production is important in systemic virulence (Enard *et al.*, 1988). In these studies, siderophore production may contribute to tuber soft rot but was important in aerial stem rot caused by *E. carotovora*. Siderophore mutant JL11207 ($Cat^-Iuc^-Iut^-$) was less virulent in aerial stem rot assays than the parental strain W3C105 ($Cat^+Iuc^+Iut^+$). At a low inoculum level (0.05 OD_{600nm}), JL11206 ($Cat^-Iuc^-Iut^+$) also was less virulent than W3C105. From this result, I suggest that iron acquisition by either siderophore is adequate to maintain virulence and that strains lacking siderophores are less virulent. Thus, the role of catechol production in virulence of W3C105 is not the specific role of the catechol but a function of iron acquisition. Although catechol production is a common trait among strains of *E. carotovora*, the importance of this compound may be attributable to its properties as a siderophore but not specifically to the catechol nature of the siderophore.

The role of the catechol siderophore in virulence is not conclusive. Using a second inoculum concentration (0.10 OD_{600nm}), aerobactin uptake mutants JL11182 ($Cat^+Iuc^-Iut^-$) and JL11207 ($Cat^-Iuc^-Iut^-$), regardless of catechol production, were less virulent than W3C105. The reduced virulence of JL11182 and JL11207 can be attributed to the lack of aerobactin uptake and utilization by these strains. The results of this experiment do not rule out the involvement of the catechol siderophore in virulence in the aerial stem rot system because all six strains used in this study could utilize catechol siderophores produced by other strains of *E. carotovora* and other members of the Enterobacteriaceae (Bull, Chapter 2). It is possible that changes in virulence due to catechols were not detected because these

strains could utilize catechols from exogenous sources. In addition, these experiments must be repeated before any definite conclusions can be made.

Tuber soft-rot bioassays have been used to compare aggressiveness (quantity of tissue rotted/unit time) (Johnson *et al.*, 1989; Smith and Bartz, 1990) and pathogenicity (ability to cause soft rot) (Chatterjee and Starr, 1977; Lei *et al.*, 1985a; 1985b; Zink and Chatterjee, 1985; Roeder and Collmer, 1985; Murata *et al.*, 1990) among strains of *E. carotovora*, *E. chrysanthemi* and mutants no longer producing key compounds. Unlike pectic enzyme production, siderophore production was not necessary for tuber soft rot development. This study has shown that siderophores produced by *E. carotovora* are not essential for pathogenicity with respect to soft rot of potato tubers.

Strains of *E. carotovora* producing neither aerobactin nor the catechol siderophore were as virulent as the parental strain W3C105, which produced both siderophores. Siderophores may not be important in tuber soft rot because iron may not be a limiting factor to the growth of pathogens in potato tuber tissue. Alternatively, when cellular iron is low, increased amounts of pectic enzymes may be produced thus generating equal tissue maceration from fewer cells. Iron regulation of pectic enzymes occurs in a related pathogen, *E. chrysanthemi*, such that in low-iron conditions increased pectic enzymes are produced (Sauvage *et al.*, 1991). Pectic enzyme production by siderophore-minus mutants of *E. carotovora* may be increased in low iron environments. In tubers, the growth rate of mutants may not be as high as that of the parental strain but increased pectic enzyme production by the mutants may compensate for a lower growth rate resulting in the production of an equivalent amount of rot. The role of increased pectic enzyme production by siderophore mutants in soft rot development could be assayed by comparing population dynamics of the pathogen during soft rot development and iron regulation of pectic enzyme production in mutants and W3C105.

It can not be concluded from these data that there was no role of the catechol siderophore nor of siderophore production in general in aggressiveness of *E. carotovora*. Because all strains tested in the soft rot bioassays utilize catechols from exogenous sources, it can not be ruled out that the mutants were obtaining iron from catechol siderophores produced by other members of the Enterobacteriaceae that may have contaminated potato tubers. Not until mutants in catechol siderophore uptake are generated, can the role of catechol siderophore production in virulence be fully evaluated.

Spontaneous rifampicin-resistant mutants are used commonly in ecological studies to mimic the strains from which they were derived (Weller and Saettler, 1978). In this study, a spontaneous rifampicin-resistant mutant (JL1148) differed from W3C105 based on virulence in soft rot bioassays. The parental strain and the rifampicin-resistant mutant did not differ with respect to generation time in LB. Unfortunately, the difference between JL1148 and W3C105 was not detected in preliminary tests comparing virulence of these strains. I recommend that spontaneous rifampicin-resistant mutants be tested for similarity to parental strains with respect to ecological properties as well as generation time in culture.

Differences in virulence among strains of *E. carotovora* have been described by other researchers. The weight of rot in tubers inoculated with 37 different strains of *E. carotovora* vary from 3 g to 40 g when tubers are incubated for 4 days at 30 C (Smith and Bartz, 1990). Although virulence of field strains of *E. carotovora* differed, the differences were not consistent among experiments. For example, in one study W3C105 was more virulent than W3C37 (FIGURE 3-3), while in other studies W3C37 was more virulent (TABLE 3-4). The variability within these two studies may be explained by differences in the tuber status at the time of the experiment. Factors including oxygen status (Maher and Kelman, 1983), Ca^{+} content (McGuire and Kelman, 1984), osmotic condition (De Boer and Kelman,

1978) and other physiological characteristics of tubers will alter the amount of tissue macerated upon inoculation with a given pathogen (Pérombelon and Kelman, 1980). It is possible that variation among potatoes from different lots also influenced the amount of rot produced, resulting in variable rot among replicate tubers. It is not known what effect potatoes from different lots had on the variation among experiments.

In these experiments, virulence of *E. carotovora* was assayed using a variety of different temperature/time regimes, methods of inoculation and/or inoculum densities of the pathogen. *Erwinia carotovora* subsp. *atroseptica* is favored by temperatures 15 C or lower while *E. carotovora* subsp. *carotovora* is favored by temperatures above 15 C (Kelman and Pérombelon, 1980). In addition, at lower temperatures potatoes were incubated longer in order to obtain the same magnitude of rot as obtained at 4 days at 22 C. This is a similar finding to other studies showing that rot develops slower at 10 C than at 16 C (De Boer and Kelman, 1978).

The two inoculation methods used in these studies presumably differed with respect to oxygen status of the tuber and bacteria, consistency of water film on potato surface, and contact of the pathogen to the tuber. Method B (Axelrood *et al.* 1988) had a low coefficient of variation in all experiments.

There was no significant correlation between increased inoculum concentrations and weight of macerated tissue in any of the studies, although there were significant differences between rot produced when 7.3 log (cfu/ml) and 3.3 log (cfu/ml) was used to inoculate tubers. Other researchers using the percentage of wounds that develop decay at 22 C as a measure of aggressiveness detect a significant correlation between the percentage of wounds that developed decay and the inoculum concentration. Decay consistently developed when wounds in potatoes were inoculated with as low as 5.0 log (cfu/wound) of bacteria (De Boer and Kelman,

1978). Because results from soft rot studies conducted in this research were extremely variable, I would recommend evaluating the percentage of wounds that developed decay as a measure of virulence.

Biological control of *E. carotovora* W3C105, siderophore mutants or W3C37 was not demonstrated in population studies on potato tuber surfaces. Inoculation of potato tubers with fluorescent pseudomonads did not reduce the population size of *E. carotovora*. The application of pseudomonads to seed pieces reduces the populations of *E. carotovora* on roots and the number of daughter tubers infested with *E. carotovora* (Kloepper, 1983). Gross (1988), however, detected no differences between populations of *E. carotovora* on tubers and roots of plants treated with fluorescent pseudomonads and on untreated plants. Populations on tubers vary greatly over the tuber surface (Rhodes and Logan, 1984). Population means may be misleading because populations vary on different portions of the tuber surface. A lognormal distribution of bacteria may exist such that portions of the tuber surface have low populations of pseudomonads while few sections may have relatively high populations (Loper *et al.*, 1984a). It is hypothesized that the level of biological control achieved increases as the population of the biological control agent increases. For at least two diseases, disease severity decreases as population size of the control agent increases (Leben *et al.*, 1988; Bull et al., 1991). In contrast, in this study, large populations of the fluorescent pseudomonads on a tuber sample did not correlate to small population size of the pathogen.

Biological control of soft rot caused by *E. carotovora* subsp. *carotovora* W3C105 or *E. carotovora* subsp. *atroseptica* W3C37 was achieved by one antagonist in only one of four biological control experiments conducted. Biological control of soft rot produced by siderophore mutants of W3C105 was not achieved. Biological control of *E. carotovora* is achieved using *E. carotovora* subsp. *betavasculorum* as an antagonist in similar soft rot bioassays (Axelrood *et al.*,

1988). An anaerobic environment favors the development of soft rot; in the studies reported here it is likely that bacteria experienced oxygen-limiting conditions. Because fluorescent pseudomonads are strict aerobes, they may not control soft rot caused by *E. carotovora* in oxygen-limited environments.

Strain W3C37 is more sensitive to biological control by fluorescent pseudomonads than is strain W3C105 (Xu and Gross, 1986a). Strain W3C37 produces only a catechol siderophore whereas W3C105 produces both a catechol siderophore and aerobactin (Bull, chapter 1; Ishimaru and Loper, 1992). It has been hypothesized that aerobactin production contributes to the lower sensitivity of strain W3C105 to biological control (Ishimaru and Loper, 1991). In soft rot assays, differences in sensitivity of W3C105 and W3C37 to biological control were not detected presumably because biocontrol was not achieved under the conditions of these experiments. In order to determine if siderophore production is a factor in the sensitivity of *E. carotovora* to antagonism by fluorescent pseudomonads, the biological control of non-emergence of potato should be evaluated, as was described by Xu and Gross (1986a). The siderophore mutants derived and characterized here will be invaluable tools in such future studies.

This research has demonstrated that siderophores are not pathogenicity factors in soft rot development or aerial stem rot caused by *E. carotovora*. The siderophores have a role in virulence in relation to aerial stem rot but the relative role of the catechol siderophore and aerobactin have not been determined from this research. In order to determine the relative roles of these siderophores, mutants in catechol siderophore uptake must be generated.

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