

Role of Iron in Rhizobacteria-Mediated Induced Systemic Resistance of Cucumber

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ABSTRACT

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Seed treatment with the rhizosphere bacterium *Serratia marcescens* strain 90-166 suppressed anthracnose of cucumber, caused by *Colletotrichum orbiculare*, through induced systemic resistance (ISR). When the iron concentration of a planting mix was decreased by addition of an iron chelator, suppression of cucumber anthracnose by strain 90-166 was significantly improved. Strain 90-166 produced 465 ± 70 mg/liter of catechol siderophore, as determined by the Rioux assay in deferrated King's medium B. The hypothesis that a catechol siderophore produced by strain 90-166 may be responsible for induction of systemic resistance by this strain was tested by evaluating disease suppression by a mini-Tn5-*phoA* mutant deficient in siderophore production. Sequence analysis

of genomic DNA flanking the mini-Tn5-*phoA* insertion identified the target gene as *entA*, which encodes an enzyme in the catechol siderophore biosynthetic pathways of several bacteria. Severity of anthracnose of cucumbers treated with the *entA* mutant was not significantly different ($P = 0.05$) from the control, whereas plants treated with wild-type 90-166 had significantly less disease ($P = 0.05$) than the control. Total (internal and external) population sizes of 90-166 and the *entA* mutant on roots did not differ significantly ($P = 0.05$) at any sample time, whereas internal population sizes of the *entA* mutant were significantly lower ($P = 0.05$) than those of the wild-type strain at two sampling times. These data suggest that catechol siderophore biosynthesis genes in *Serratia marcescens* 90-166 are associated with ISR but that this role may be indirect via a reduction in internal root populations.

Additional keywords: systemic acquired resistance.

Certain strains of rhizosphere bacteria can suppress plant diseases by inducing resistance responses in the plant through a process termed induced systemic resistance (ISR) (3,46,48). The mechanisms involved in ISR are just beginning to be elucidated (10,14,22,23,28,33,34), and appear to vary among bacterial strains and pathosystems. In at least some systems, however, ISR is influenced by iron availability. For example, we previously reported that ISR induced by *Serratia marcescens* strain 90-166 against cucumber anthracnose is reduced when iron is made more available in the planting medium (34). Similarly, Leeman et al. (22) reported that ISR induced by *Pseudomonas fluorescens* WCS374 against Fusarium wilt of radish is inversely related to iron availability of the planting substrate. Furthermore, the production of at least two bacterial metabolites with proposed roles in ISR are inversely regulated by iron. Several strains of rhizosphere bacteria produce salicylic acid (SA) (14,22,28,34) in environments in which iron is not plentiful, and SA contributes to ISR mediated by a subset of the strains that produce it. The vast majority of microorganisms produce siderophores (6,49), a diverse class of high-affinity iron chelating compounds that are produced in response to iron limitation, and exported from the cell where they chelate ferric iron. Ferric-siderophore complexes are recognized and bound by specific outer-membrane receptor proteins, and iron is transported into the cell where it becomes available for metabolic functions. Among the siderophores produced by rhizo-

sphere bacteria, only the pyoverdines (also called pseudobactins) produced by the fluorescent pseudomonads have been implicated in ISR (15,28).

The rhizosphere bacterium *Serratia marcescens* 90-166 can induce resistance to plant disease caused by *P. syringae* pv. *lachrymans*, *Colletotrichum orbiculare*, and *Erwinia tracheiphila* in a field environment (34). Because the capacity of 90-166 to induce resistance is diminished under iron-replete conditions (34), we are investigating the contribution of iron-regulated metabolites produced by the bacterium to ISR. We previously demonstrated that SA had a minor role in ISR induced by 90-166; three mutants of 90-166 lacking SA production retained the capacity to induce resistance of cucumber against anthracnose caused by the pathogen *C. orbiculare* (34). We also described a mutant of 90-166 that is deficient in the production of a siderophore and in ISR (34). The siderophore produced by 90-166 has not been identified, but other strains of *Serratia marcescens* produce the catechol siderophore enterobactin (4,52). Like other catechol siderophores produced by members of the Enterobacteriaceae, enterobactin is derived from chorismate via isochorismate and dihydroxybenzoic acid (DHBA; Sigma Chemical, St. Louis) (1,16,25–27,29,36,37,41,50). Enzymes encoded by *entC*, *entB*, and *entA* (or their homologs in other bacterial species) catalyze the formation of DHBA from chorismate (1,16,25–27,29,36,37,41,50) and a multi-enzyme complex composed of the products of *entD*, *entE*, *entF*, and *entG* catalyzes the synthesis of enterobactin from three molecules each of DHBA and serine (30,38,39). In pseudomonads, SA is produced from chorismate and isochorismate via PchB (43). It is unknown if the same pathway is utilized by *Serratia marcescens* to produce SA, but there may be a link between SA and siderophore production. The objectives of this study were to (i) characterize the siderophore-deficient mutant of

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90-166, (ii) evaluate the siderophore-deficient mutant for disease suppression via ISR and root colonization, and (iii) further investigate the relationship between iron availability and ISR induced by 90-166.

MATERIALS AND METHODS

Bacterial strains. *Serratia marcescens* strain 90-166 was used for all experiments. This strain has intrinsic resistance to tetracycline and ampicillin at 100 and 500 µg/ml, respectively, and produces SA at 2.89 ± 0.95 µg/ml in stationary phase cultures grown in King's Medium B (KMB). *Serratia marcescens* 90-166-2882 (34) is a mini-Tn5-*phoA* insertion mutant of 90-166 selected for lack of siderophore production on the universal siderophore detection medium containing chrome azurol S (42). Mutant 90-166-2882 produces SA at 1.86 ± 0.63 µg/ml (34). All other strains used are described in Table 1.

Quantification of catechol siderophore production. Catechol production was quantified according to the colorimetric assay described by Rioux et al. (35). Test strains were grown in either minimal medium Davis (MMD; Difco Laboratories, Detroit) broth amended with succinate (15 mM) and 1 g/liter of ammonium sulfate (MMDN) or deferrated KMB broth for 48 h at 25°C with shaking (200 rpm). Deferration of media was accomplished by serial extraction of KMB (adjusted to pH 9.0) with chloroform plus (1%, wt/vol) 8-hydroxyquinoline (Sigma) in a separatory funnel (6). KMB was extracted until the green coloration of the chloroform layer was no longer evident. Residual 8-hydroxyquinoline was removed by extraction with chloroform at pH 9.0 and 7.0, respectively. Mini-Tn5-*phoA* mutants were grown in MMDN amended with 50 µg/ml of kanamycin and 100 µg/ml of tetracycline. Cultures were centrifuged, and supernatants were filtered to remove bacterial cells. DHBA was used to set a standard curve for quantification of catechol siderophore production using a spectrophotometer ($\lambda = 510$ nm) (detection limit 4 mg/liter). Controls included either broth and water in place of culture supernatant, or water in place of 1,10-phenanthroline solution (35). 1,10-Phenanthroline is necessary for the colorimetric reaction in the Rioux assay, and replacing this component with water generates a negative control for the reaction (35).

Cross-feeding of siderophore-deficient strains. Strains 90-166 and 90-166-2882 were tested in assays using *Escherichia coli* (21) and *Salmonella typhimurium* (32) with mutations in specific enterobactin biosynthesis genes as indicator strains (9). The Ent⁻ (*entB*-1, AN93) or DHBA⁻ (*entB*-7, AN193) strains cannot produce the siderophore but can utilize iron in the ferric-enterobactin complex. The indicator strains will grow on iron-deficient medium only in the presence of siderophores that are recognized by the receptor for ferric-enterobactin in the bacterial outer membrane. Therefore, the growth of indicator strains can be induced only by siderophores with functional similarity to enterobactin. Indicator strains were grown overnight in Tris minimal salts (TMS) broth (44) supplemented with tryptophan (0.003%, wt/vol), thiamine (0.0002%, wt/vol), deferrated Casamino acids (0.3%, wt/vol), FeCl₃ (0.1 µM), and streptomycin (100 µg/ml). All chemicals were purchased from Sigma Chemical. TMS agar amended with 150 µM 2,2'-dipyridyl was seeded with 10⁵ CFU/ml of the indicator strains. Test strains were spotted onto seeded, solidified TMS medium, and the plates were incubated for 24 to 48 h at 28°C. After incubation, plates were examined for growth of the indicator strains surrounding colonies producing siderophores with functional similarity to enterobactin. *P. fluorescens* strain 89B-61 and *Escherichia coli* strain K12 were included to provide negative and positive controls, respectively.

Cucumber ISR assays. Strains 90-166 and 90-166-2882 were compared to determine if siderophore production was involved in induction of systemic disease resistance in cucumber. Strains were grown in either KMB or KMB amended with 50 µg/ml of kanamycin and 100 µg/ml of tetracycline, respectively, for 48 h at 25°C with shaking at 200 rpm. Strains were centrifuged and resuspended in physiological saline (0.14 M NaCl). Cucumber seeds (cv. SMR58) were either soaked in bacterial suspensions or saline for 30 min, and planted in pots containing Promix (Premier Peat, Rivière-du-Loup, Quebec). Seeds were covered, and pots were placed on a greenhouse bench for the duration of the experiment. Plants were fertilized with 15-16-17 (N-P-K; Peter's Professional, The Scott's Co., Marysville, OH; 2.5 g/liter) at 14 days after planting (DAP) and were challenged with the pathogen, *C. orbiculare*, at 28 DAP. Conidial suspensions of *C. orbiculare* were maintained in 50% glycerol (vol/vol) at -80°C for long-term storage. *C. orbiculare* was cultured by spread-planting a conidial suspension on green bean agar plates (17), incubating plates for 7 days at 28°C, and suspending conidia in water (10⁵ conidia per ml). Plants were sprayed to runoff, placed in a humidity chamber for 24 h to facilitate infection, and returned to the greenhouse bench. Pots were arranged in a randomized complete block design (RCBD) with 10 replications. Control treatments included non-bacterized (disease) control and a pathogen-induced (positive) control. For the positive control, cucumbers were induced with the pathogen at the cotyledon stage by pricking the underside of the cotyledon with a pipette tip and inoculating the wounds with 10⁶ conidia per ml. For these experiments, each leaf of each plant was rated according to the disease rating scale of Thompson and Jenkins (45), where 1 = 0 to 1% infected leaf area, 2 = 1 to 3%, 3 = 3 to 6%, 4 = 6 to 12%, 5 = 12 to 25%, 6 = 25 to 50%, 7 = 50 to 75%, and 8 = 75 to 100% of the leaf being infected. Rates were averaged for each plant, and treatment differences were compared using single-degree-of-freedom contrasts with SAS and JMP software (SAS Institute, Cary, NC). Experiments were repeated at least four times.

Experiments were designed to determine if lowered iron concentrations in the potting medium influenced ISR. Cucumber plants were treated biweekly with 10 µM ethylenediamine di(o-hydroxyphenylacetic acid) (EDDHA) solution as a root drench. Fe-EDDHA has a formation constant of K^{33.9} and decreases iron available to bacteria in soil. The experiment was a 3 × 2 factorial arranged as a RCBD. Factors were three levels of cucumber treatment (no treatment [disease control], pathogen-induced [ISR con-

TABLE 1. Bacterial strains and plasmids^z

Species and strain	Description	Source
<i>Escherichia</i>		
<i>E. coli</i> AN93	<i>entE405</i> , <i>fluA</i> derivative of AN194(20)	(31)
<i>E. coli</i> AN193	<i>entA403</i> , <i>fluA</i>	(47)
<i>E. coli</i> DH5α	<i>F</i> ⁻ <i>endA1 hsdR17</i> ($\tau_K^- m_K^+$) <i>supE44 thi-1 recA1 gyrA96 relA1</i> ϕ 80 <i>dlacZ</i> M15 λ^-	(24,51)
<i>E. coli</i> K12	<i>thi-1</i> , <i>thr</i> , <i>leu</i> , <i>tonA</i> , <i>lacY</i> , <i>supE</i>	ATCC25290
<i>E. coli</i> SM10 λ pir	<i>recA</i> ::RP4-2-Tc::Mu, λ pir	(13)
<i>E. coli</i> XL1-BlueMR	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB</i> - <i>hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i>	Stratagene
<i>Pseudomonas</i>		
<i>P. fluorescens</i> 89B-61	Field isolate	J. W. Kloepper
<i>Salmonella</i>		
<i>S. typhimurium</i> <i>entB</i> 1	Ent ⁻ Sm ^R	(32)
<i>S. typhimurium</i> <i>entB</i> 7	Ent ⁻ DHBA ⁻ Sm ^R	(32)
<i>Serratia</i>		
<i>S. marcescens</i> 90-166	Tc ^R , Ap ^R (intrinsic)	J. W. Kloepper
<i>S. marcescens</i> 90-166-2882	<i>entA</i> ::mini-Tn5- <i>phoA</i> derivative of 90-166, Tc ^R , Ap ^R , Km ^R , Cat ⁻	C. M. Press
Plasmids		
pUT mini-Tn5- <i>phoA</i>	Ap ^R Km ^R ; derived from pGP704	(13)
SuperCos 1	Ap ^R Km ^R	Stratagene

^z Cat⁻ = catechol siderophore deficient, Ent⁻ = enterobactin deficient, DHBA⁻ = 2,3-dihydroxybenzoic acid deficient, Tc^R, Ap^R, Km^R, or Sm^R = tetracycline, ampicillin, kanamycin, or streptomycin resistant, respectively.

tol], and bacterial treatment [90-166]) and two levels of EDDHA treatment (0 and 10 μ M). All other aspects of the experiment were described previously.

Root colonization assays. Strains 90-166 and 90-166-2882 were evaluated for total (internal and external) and internal root colonization of cucumber. The cucumber assay was as described previously, except seeds were planted in a 2:1 mixture of sand/clay. The experiment was a 2×5 factorial arranged in a RCBD with seven replications. In order to determine the total root population sizes, the following were used: two levels of bacterial treatment (90-166 or 90-166-2882) and six levels of sampling time (0, 1, 4, 7, and 21 DAP). In order to determine the internal root population sizes, roots were harvested at 4, 8, and 10 DAP, rinsed under running water to remove the potting medium, and weighed. Total root population sizes were determined by triturating roots in 0.02 M phosphate buffer (pH 7) and plating on tryptic soy agar amended with either 100 μ g/ml of ampicillin (for 90-166) or ampicillin and 50 μ g/ml of kanamycin (for 90-166-2882). Internal root population sizes were determined after surface-disinfecting roots for 1 min in 30% hydrogen peroxide and 1 min in 1.05% sodium hypochlorite (pH 10.4) followed by five rinses in sterile distilled water. Roots were treated subsequently as previously described for total root population determinations. All other aspects of the experiment were completed as described previously, and the experiment was repeated three times.

Southern analysis. Total genomic DNA of 90-166 or 90-166-2882 was isolated by the cetyltrimethylammoniumbromide (CTAB) method (5), digested with *Bam*HI (Gibco BRL Life Technologies, Gaithersburg, MD), and restriction fragments were separated by agarose gel electrophoresis. DNA was transferred from agarose gels to Nytran filters according to the manufacturer's directions (Schleicher and Schuell, Keene, NH). pUT-mini-Tn5-*phoA* (13), used as a probe, was labeled with biotinylated dATP by nick-translation (Blue-Gene Kit; Gibco BRL Life Technologies) and purified with a D50 column (International Biotechnologies Inc., New Haven, CT). Blots were visualized according to the manufacturer's directions.

Genetic analysis of the region surrounding Tn5-*phoA* insertion. A 4.8-kb *Bam*HI fragment containing the mini-Tn5-*phoA* and 1.3 kb of genomic DNA flanking the insertion site was cloned into pUC19 and transformed into *Escherichia coli* DH5 α (24,40). Transformants were selected based on kanamycin resistance conferred by mini-Tn5-*phoA*. The genomic DNA flanking the transposon insertion site was sequenced with primers complementary to pUC19 sequences flanking the cloning site. DNA sequencing was conducted by the Center for Gene Research and Biotechnology (Oregon State University, Corvallis). Subsequent sequencing was performed with primers designed from previous sequence determinations. A contiguous sequence was compiled using the SeqLab software program (version 10, Genetics Computer Group, Madison, WI). DNA sequence results were compared with known sequences in the GenBank and EMBL databases using BLAST (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD). The accession number of the nucleotide sequence of the *entC-entA* region of *Serratia marcescens* 90-166 is AF358745.

Genomic library preparation. A genomic library of DNA fragments from 90-166 was created using a cosmid vector kit (SuperCos1; Stratagene, La Jolla, CA). Total genomic DNA of 90-166 was extracted and partially digested with *Sau*3AI. Resulting fragments were dephosphorylated, ligated into SuperCos 1 DNA, and packaged into *Escherichia coli* XL1-Blue MR using the Gigapack III Gold packaging extract (Stratagene) according to manufacturer's directions. Cosmid clones were screened by colony hybridization with a 400-bp polymerase chain reaction amplification product of the *entA* homolog adjacent to the mini-Tn5-*phoA* insertion of 90-166-2882 as a probe. One of three colonies that hybridized to the probe was subjected to Southern analysis,

and a 2.0-kb *Pst*I-*Eco*RI restriction fragment hybridizing to the *entA* probe was cloned into pUC19 and sequenced.

RESULTS

The effect of low iron concentration on bacterial-mediated ISR. To characterize the effect of iron concentration on 90-166-mediated ISR, iron concentration of the planting mix was decreased by addition of an iron chelator. Root drenches with 10 μ M EDDHA applied every 4 days did not suppress cucumber anthracnose nor did they enhance ISR achieved by pre-inoculation with pathogen *C. orbiculare* (Table 2). In association with the 90-166 seed treatment, however, EDDHA root drenches significantly decreased disease ($P = 0.05$) compared with 90-166 treatment alone.

Siderophore determination. Strain 90-166 produced 101 ± 43 mg/liter of catechol siderophores in MMDN and 465 ± 70 mg/liter in deferrated KMB after 48 h. Lower catechol siderophore production in MMDN was attributed in part to the reduced growth of strain 90-166 in this medium than in deferrated KMB. Strain 90-166-2882, a mini-Tn5-*phoA* insertion mutant of 90-166, did not produce detectable catechol siderophores in MMDN or deferrated KMB as determined by the Rioux assay.

Cross-feeding of siderophore-deficient strains. Ent⁻ (*enb-1*, AN93) and DHBA⁻ (*enb-7*, AN193), indicator strains of *Salmonella typhimurium* and *Escherichia coli*, were used to detect siderophore production by 90-166. Wild-type strain 90-166 induced growth of all the indicator strains, suggesting that 90-166 produces a siderophore with functional similarity to enterobactin. The Cat⁻ derivative 90-166-2882 did not induce growth of any of the indicator strains, suggesting that the transposon insertion disrupted a gene required for production of DHBA.

Genetic analysis of the Cat⁻ mutant. Southern analysis detected mini-Tn5-*phoA* on a single 4.8-kb *Bam*HI fragment in the genome of 90-166-2882, indicating that the catechol-deficient mutant has a single transposon insertion. The *Bam*HI fragment was cloned and DNA flanking the mini-Tn5-*phoA* insertion was sequenced. The nucleotide sequence of 176 bp, adjacent to the transposon insertion, was 71% identical to nucleotides 1665-1841 of *entA* of *Escherichia coli*, which encodes 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, an enzyme in the enterobactin

TABLE 2. The role of iron on induced systemic resistance (ISR) mediated by *Serratia marcescens* strain 90-166 against anthracnose of cucumber caused by *Colletotrichum orbiculare*

Treatment ^w	Mean disease rating ^x	
	Ambient iron availability ^y	Low iron availability ^z
Nonbacterized	8.00 a	7.89 a
SAR control	6.75 d	6.85 d
<i>Serratia marcescens</i> 90-166	7.60 b	7.25 c

^w Cucumber seeds were soaked in 0.14 M NaCl (nonbacterized [disease] control and systemic acquired resistance (SAR) [pathogen-induced] control), or bacterial suspensions (10 log CFU/ml). The experiment was a 3×2 factorial (three bacterization levels: disease, pathogen-induced control, and 90-166) and two iron levels (0 and 10 μ M ethylenediamine di(o-hydroxyphenylacetic acid [EDDHA]) arranged as a randomized complete block design with 10 replications.

^x Values describe the mean disease rating per plant as determined from a disease rating scale from 1 (0 to 1% necrotic leaf area) to 8 (75 to 100% necrotic leaf area) (45). Means followed by the same letter are not significantly different ($P = 0.05$) and were compared using single-degree-of-freedom contrasts. The experiment was repeated six times. Data are from a representative experiment.

^y Cucumber plants were grown in a potting mix that was not amended with an iron chelator.

^z Cucumber plants were grown in potting mix that was drenched twice weekly with 10 μ M EDDHA solution to lower the concentration of available iron in the rhizosphere.

biosynthesis pathway. Genomic DNA adjacent to the opposite side of the mini-Tn5-*phoA* insertion was also similar to the enterobactin biosynthesis gene cluster of *Escherichia coli*. A 698-bp region located 283 bp from the mini-Tn5-*phoA* insertion exhibited nucleotide similarity to a number of homologs of *entC*, which encodes isochorismate synthase, another enterobactin biosynthetic enzyme. These data provide convincing evidence that the mini-Tn5-*phoA* insertion is in the catechol siderophore biosynthesis gene cluster of *Serratia marcescens*.

A 2-kb *PstI-EcoRI* fragment containing the wild-type *entC-entA* region of strain 90-166 was subcloned from a cosmid that hybridized to DNA adjacent to the mini-Tn5-*phoA* insertion of mutant 90-166-2882. Sequence analysis indicated the presence of *entC* upstream of *entA*, with a 284-bp intergenic region separating the two genes. *entC* of *Serratia marcescens* 90-166 was 956 bp compared with 1,175 bp in *Escherichia coli*, and the sequences of the homologs were 57 and 31% identical at the nucleotide and predicted amino acid levels, respectively. Only 617 bp of the 5' portion of *entA* was present on the 2-kb fragment, and the sequence of this was 57% identical to the corresponding 5' portion of *entA* of *Escherichia coli* at both the nucleotide and predicted amino acid levels. *entA* contains a characteristic 2Fe-2S iron-sulfur cluster motif present in all known dehydrogenases (7,19), further supporting the role of the *entA* homologue as the gene encoding 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. The organization of the enterobactin gene cluster in *Serratia marcescens* clearly differs from that published for *Escherichia coli* K12 where *entA* and *entC* are separated by *entE* and *entB* (8). Based on these data, strain 90-166-2882 is hereafter referred to as an *entA* mutant of 90-166.

Influence of catechol siderophore production by *Serratia marcescens* on ISR. Severity of anthracnose was reduced in plants induced by prior inoculation with the pathogen *C. orbiculare* or in plants induced by inoculation with *Serratia marcescens* 90-166. In contrast, disease severity of cucumbers treated with 90-166-2882 (*entA*) was not significantly different ($P = 0.05$) from the disease control (plants inoculated with the pathogen only) (Table 3).

Root colonization assays. The population sizes of wild-type strain 90-166 and *entA* mutant 90-166-2882 were compared in order to determine if lack of ISR could be attributed to a reduction in root population size of the *entA* derivative. Total (internal plus external) population sizes of 90-166 and 90-166-2882 on roots did not differ significantly ($P = 0.05$) at any sample times (Fig. 1A). Internal population sizes of the *entA* strain were significantly lower ($P = 0.05$) than those of the wild-type strain at two sampling times (Fig. 1B). Internal population sizes of 90-166 were 7.42 and 2.97 log (CFU per gram) at 4 and 10 DAP, respectively, compared with 90-166-2882 populations, which were 6.27 and 2.56 log (CFU per gram) on those days. There was no significant difference between population sizes of the strains at 8 DAP.

TABLE 3. Effect of *Serratia marcescens* strain 90-166 and its catechol siderophore deficient (*Cat*⁻) mutant on anthracnose of cucumber caused by *Colletotrichum orbiculare*

Treatment ^y	Mean disease rating ^z
Disease control	8.00
SAR control	7.04*
90-166 wild type	7.57*
90-166-2882 <i>Cat</i> ⁻	7.97

^y Cucumber seeds were soaked in 0.14 M NaCl (nonbacterized [disease] control and (systemic acquired resistance [SAR; pathogen-induced] control), or bacterial suspensions (10 log CFU/ml).

^z Data are means of four experiments combined after performing Bartlett's test for homogeneity of variance ($\chi^2 = 30.0$; $\alpha = 0.000001$). * Indicates a significant reduction in disease compared with the disease control at $P \leq 0.05$ using single-degree-of-freedom contrasts.

DISCUSSION

The level of ISR mediated by *Serratia marcescens* strain 90-166 varied with iron concentration of the potting mix in which cucumber was grown. ISR mediated by strain 90-166 was improved significantly when external iron availability to the host plant was reduced through addition of iron chelator EDDHA. These results complement those of an earlier study demonstrating that ISR by strain 90-166 is reduced significantly when iron availability is increased (34). Together, these studies indicate that 90-166-mediated ISR is dependent on iron concentration. These observations agree with those reported by Leeman et al. (22); in that study, treatment of radish with *P. putida* strain WCS358 did not result in ISR under conditions of high iron availability. Similarly, another rhizobacterial strain, *P. fluorescens* WCS374, was more effective in suppressing disease through ISR under conditions of low iron availability (22).

ISR by strain 90-166 was affected by external iron concentration, indicating that iron-regulated production of components such as siderophores might be important in ISR by this strain. Bacteria commonly produce siderophores (iron-chelating agents) in response to iron deficit during their growth (6,49), and siderophores can contribute to biological control of certain soilborne plant pathogens (2,20). A mutagenesis approach was employed to

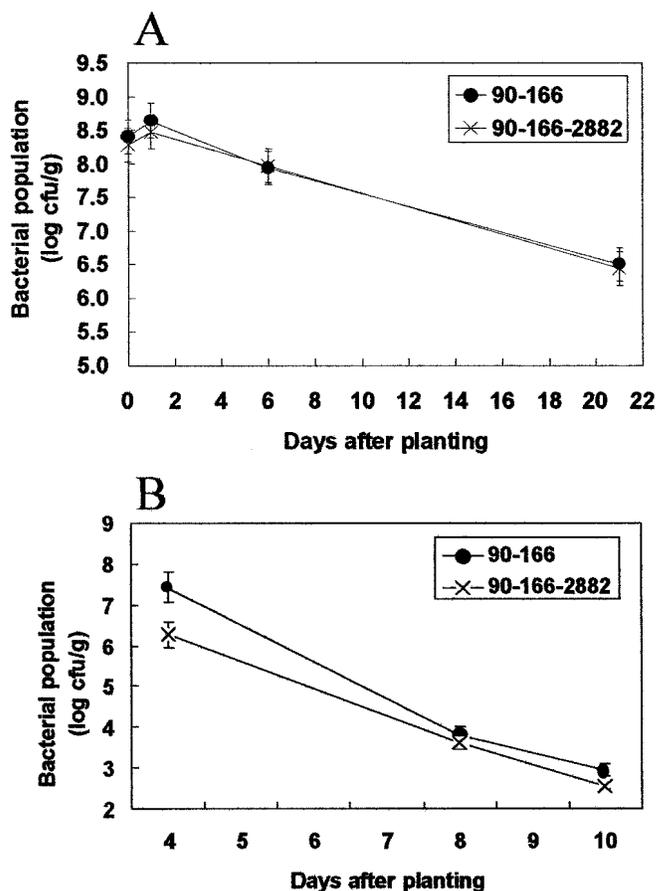


Fig. 1. Population sizes of *Serratia marcescens* 90-166 (●) and the *entA*⁻ mutant 90-166-2882 (×) on cucumber. **A**, Total root population sizes were determined by triturating roots in 0.02 M phosphate buffer and plating on tryptic soy agar amended with either 100 μg/ml of ampicillin (for 90-166) or ampicillin and 50 μg/ml of kanamycin (for 90-166-2882). The experiment was a 2 × 5 (2 bacterial treatments [90-166 or 90-166-2882] and 5 sample times; 0, 1, 4, 7, and 21 days after planting [DAP]) factorial arranged in a random complete block design (RCBD) with seven replications. **B**, Internal root population sizes were determined by triturating surface-disinfested roots as described previously. The experiment was a 2 × 3 (2 bacterial treatments [90-166 or 90-166-2882] and 3 sample times; 4, 8, and 10 DAP) factorial arranged in a RCBD with seven replications.

LITERATURE CITED

evaluate the role of siderophore production by 90-166 in ISR. One mutant (90-166-2882) lacking catechol siderophore production had a transposon insertion in an *entA* homolog. Based on known pathways of enterobactin production in *Escherichia coli* (18,39), a disruption of *entA* would predict a loss of 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase activity and the inability to form the product 2,3-DHBA. This prediction is supported by the result that 90-166-2882 did not cross-feed mutants (*enb-7*, AN193) that were deficient in 2,3-DHBA production. Because the DNA flanking the insertion site was homologous to several other genes involved in iron acquisition in different bacterial species (*entC*, and its homologues *dhbC*, *pmsC*, and *amoA*; *entA* and its homologue *dhbA*), it is likely that this genetic region also functions in catechol production in 90-166.

The *entA* mutant 90-166-2882 no longer induced resistance in cucumber to *C. orbiculare* supporting the hypothesis that the catechol siderophore produced by the wild-type strain 90-166 is necessary for ISR. A role for siderophores in ISR was also reported by Maurhofer et al. (28) who observed that a pyoverdinin-deficient strain of *P. fluorescens* CHA0 (CHA400) no longer induced resistance against *Tobacco necrosis virus* in tobacco. Clearly, siderophores are only one determinant of bacterial-mediated ISR, because pyoverdinin-deficient mutants of *P. putida* WCS374 retain their capacity to induce resistance at high iron concentrations (22) and other factors, such as lipopolysaccharide or SA, can contribute to ISR. For *Serratia marcescens* 90-166, however, the *entA* mutant that does not induce resistance retains SA production, indicating that catechol rather than SA is required for ISR. Taken together, results from various studies suggest that siderophores differ in their influence on plant resistance responses or that some bacterial strains have additional characteristics involved in ISR that can compensate for lack of siderophore production.

The loss of ISR capacity by mutant strain 90-166-2882 is unlikely to be attributable to lack of rhizosphere colonization, because total root population sizes of the *entA* mutant and its parental strain were similar. These results agree with those of many other scientists who have observed that siderophore-deficient mutants establish rhizosphere population sizes similar to those of parental siderophore-producing strains (10,11,28). In our experiments, there was a significant decrease ($P = 0.05$) in internal root population sizes of 90-166-2882 compared with wild-type strain 90-166 (Fig. 1B) at two sample times. It remains to be determined whether changes in internal colonization by the *entA* mutant contributed to changes in the ISR phenotype of this strain. It is possible that siderophore production by strain 90-166 serves to detoxify the active oxygen species (AOS) produced by the plant in response to the bacterium as was reported for *Erwinia amylovora* siderophores (12). The lack of enterobactin production by 90-166-2882 may render this strain more susceptible to AOS and result in lowered internal populations.

In conclusion, the reduction in disease severity associated with rhizobacterial treatment under conditions of reduced iron concentration and the lack of ISR by the *entA*⁻ mutant disrupted in catechol-siderophore biosynthetic genes support the hypothesis that a catechol siderophore produced by 90-166 under iron limited conditions in vivo contributes to ISR. Future experiments evaluating the influence of iron on production of bacterial determinants necessary for ISR and the activity of the ferric-catechol complex on plant disease resistance promise to further develop an understanding of iron in plant-microbe interactions.

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