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Impact of Siderophore Production by *Pseudomonas syringae* pv. syringae 22d/93 on Epiphytic Fitness and Biocontrol Activity against *Pseudomonas syringae* pv. glycinea 1a/96[▽]

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The use of naturally occurring microbial antagonists to suppress plant diseases offers a favorable alternative to classical methods of plant protection. The soybean epiphyte *Pseudomonas syringae* pv. syringae strain 22d/93 shows great potential for controlling *P. syringae* pv. glycinea, the causal agent of bacterial blight of soybean. Its activity against *P. syringae* pv. glycinea is highly reproducible even in field trials, and the suppression mechanisms involved are of special interest. In this work we demonstrated that *P. syringae* pv. syringae 22d/93 produced a significantly larger amount of siderophores than the pathogen *P. syringae* pv. glycinea produced. While *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea produce the same siderophores, achromobactin and pyoverdin, the regulation of siderophore biosynthesis in the former organism is very different from that in the latter organism. The epiphytic fitness of *P. syringae* pv. syringae 22d/93 mutants defective in siderophore biosynthesis was determined following spray inoculation of soybean leaves. The population size of the siderophore-negative mutant *P. syringae* pv. syringae strain 22d/93ΔSid was 2 orders of magnitude lower than that of the wild type 10 days after inoculation. The growth deficiency was compensated for when wound inoculation was used, indicating the availability of iron in the presence of small lesions on the leaves. Our results suggest that siderophore production has an indirect effect on the biocontrol activity of *P. syringae* pv. syringae 22d/93. Although siderophore-defective mutants of *P. syringae* pv. syringae 22d/93 still suppressed development of bacterial blight caused by *P. syringae* pv. glycinea, siderophore production enhanced the epiphytic fitness and thus the competitiveness of the antagonist.

Application of epiphytic bacteria as control agents is considered a nonpolluting approach for alternative plant protection, and a number of potential antagonistic isolates have been described. However, only a few of these isolates have proven to be as effective under field conditions as they are in laboratory setups (1, 39). It has been proposed that several attributes contribute to biocontrol, including competition for nutrients, antibiosis, niche exclusion, and interference with cell signaling systems (13, 36).

Many potential antagonists have been selected from the fluorescent pseudomonad group, as this group includes various nonpathogenic species that are adapted to plant colonization and well known for their competitiveness (11, 19, 20). *Pseudomonas fluorescens* CHA0 has been proposed as biocontrol organism that can be used against several soilborne plant diseases (13). It has been suggested that secondary metabolites, such as 2,4-diacetylphloroglucinol, hydrogen cyanide, pyoverdin, and salicylate, are active principles in this isolate (11, 13).

P. fluorescens Pf-5 is a rhizosphere bacterium that suppresses seedling emergence diseases and produces a spectrum of antibiotics toxic to plant-pathogenic fungi (34). *Pseudomonas putida* WCS358, a plant growth-promoting rhizobacterium, is thought to protect its host plants by induction of induced systemic resistance (ISR) (46).

Screening for antagonistic epiphytes that can be used against *Pseudomonas syringae* plant pathogens identified *P. syringae* pv. syringae strain 22d/93 as a promising biocontrol agent. *P. syringae* pv. syringae 22d/93 was isolated from a soybean leaf that did not show any disease symptoms (48). It has a high level of antagonistic activity against the closely related pathogen *P. syringae* pv. glycinea, the causative agent of bacterial blight of soybean (48). *P. syringae* pv. syringae 22d/93 provided effective protection against *P. syringae* pv. glycinea in laboratory experiments, as well as in field trials (36, 47).

Supernatants of *P. syringae* pv. syringae 22d/93 had a direct inhibitory effect on the growth of *P. syringae* pv. glycinea isolate 1a/96, implying that antibiosis is involved in the antagonism (36). Production of the following three toxins by *P. syringae* pv. syringae 22d/93 has been demonstrated: the lipodepsipeptides syringomycin and syringopeptin and the amino acid derivative 3-methylarginine (5, 48). However, a comparison of different toxin-negative mutants with the wild type revealed that there were no differences in biocontrol activity against *P. syringae* pv. glycinea, suggesting that none of these toxins was essential for *P. syringae* pv. syringae 22d/93's antagonistic activity *in planta* (4).

Despite its great abundance, Fe^{3+} is considered a limiting nutrient in most microbial habitats due to its low solubility at

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neutral pH (33). The bioavailability of iron on leaf surfaces has been described as varying between low and limiting (32). Direct suppression of pathogen development by competition for iron has been proposed as a potential biocontrol trait (20, 31, 42). The hypothesis that iron uptake systems have an impact on biocontrol was proven for the control of *Gaeumannomyces graminis* by strains of *P. fluorescens* and for the control of *Pythium*-induced damping-off of tomato by *Pseudomonas aeruginosa* 7NSK2 (8, 21).

Recent studies have indicated that the iron uptake systems in *P. syringae* are more complex than previously supposed. In addition to the well-described peptide-type siderophore pyoverdin, some isolates produce a citrate-based siderophore called achromobactin (3). Other strains are able to produce yersiniabactin (6).

To determine the relevance of individual siderophore systems for the epiphytic fitness and biocontrol activity of *P. syringae* pv. syringae 22d/93, mutants defective in single or multiple siderophore biosynthesis systems were generated. The siderophore production of *P. syringae* pv. syringae 22d/93 and derivatives of this strain was analyzed and compared to that of the pathogen *P. syringae* pv. glycinea 1a/96.

MATERIALS AND METHODS

Bacterial strains, cultivation conditions, and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were routinely grown in King's B (KB) medium at 28°C (28). *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium (43). Antibiotics were used at final concentrations of 50 mg/liter for ampicillin and 25 mg/liter for chloramphenicol, kanamycin, and spectinomycin.

DNA manipulation techniques, plasmids, and primers. Isolation and manipulation of DNA were performed using standard techniques (43). All chemicals and enzymes were commercial preparations and were used as specified by the supplier (Fermentas, St. Leon-Roth, Germany). All primers used in this study are listed in Table 1. Synthesis of oligonucleotides and DNA sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany).

Growth under iron-limiting conditions and assays for siderophore detection. Siderophore production was visualized on CAS agar plates (2). Bacterial concentrations were adjusted so that the optical density at 600 nm (OD_{600}) was 1.0 in sterile water, and 5 μl of each suspension was spotted on a plate. Plates were incubated for 48 h at 28°C.

Different minimal media were used to investigate siderophore production by *P. syringae* pv. glycinea 1a/96 and *P. syringae* pv. syringae 22d/93 under iron-limiting conditions. The compositions of the media were as follows. Casamino Acids medium (CAA) (10) contained (per liter of demineralized water) 5 g of Difco Bacto Casamino Acids (nondesferrated), 0.9 g of $K_2HPO_4 \cdot 3H_2O$, and 0.25 g of $MgSO_4 \cdot 7H_2O$. Succinate medium (SM) (37) contained (per liter of demineralized water) 4 g of succinic acid, 6 g of K_2HPO_4, 3 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ (pH 7.0). 5b medium contained (per liter of demineralized water) 2.6 g of KH_2PO_4, 5.5 g of Na_2HPO_4, 2.5 g of NH_4Cl, and 1 g of Na_2SO_4 (solution A), as well as 10 g of glucose, 0.1 g of $MgCl_2 \cdot 6H_2O$, and 0.01 g of $MnSO_4 \cdot 4H_2O$ (solution B). PIPES medium (44) contained (per liter of demineralized water) 30.24 g of piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 0.3 g of KH_2PO_4, 1 g of NH_4Cl, and 1 g of Na_2SO_4 (pH 7.0) (solution A), as well as solution B of 5b medium. Solutions A and B of 5b and PIPES media were autoclaved separately and mixed 1:1 prior to use.

Siderophore content was quantified using the CAS assay (44). Briefly, 500 μl of CAS indicator solution containing 4 mM sulfosalicylic acid was mixed with the same volume of supernatant. The reaction mixtures were incubated for 60 min at room temperature to allow complex formation, and the siderophore-dependent color change was determined at a wavelength of 630 nm. For quantification, deferoxamin mesylate (DFOM) was used as the standard, and there was a linear relationship between decolorization and the DFOM concentration in the range from 0 to 20 μM.

Separation and identification of siderophores were performed by isoelectric focusing (IEF) using a Multiphore II electrophoresis unit (Amersham Pharmacia Biotech) as described by Koedam et al. (29). Purified siderophores of reference strains were used as standards. Siderophores were desalted and concentrated with XAD-4 resin (Sigma-Aldrich, Taufkirchen, Germany) prior to IEF separation (17). For binding of pyoverdin and achromobactin to XAD-4 resin, supernatants were acidified to pH 3.0, applied to water-equilibrated XAD-4 resin, washed twice with distilled water, and eluted with 50% methanol. The eluate was dried under a vacuum and resuspended in water.

Achromobactin cross-feeding assay. The production of achromobactin by *P. syringae* pv. syringae 22d/93 was confirmed by performing cross-feeding assays under iron-depleted conditions as described previously (16). Briefly, 15 ml of L agar (38) supplemented with the iron chelator ethylenediamine-*N*,*N*′-bis(2-hydroxy-phenylacetic acid) (EDDHA) (40 μM; Sigma-Aldrich, Taufkirchen, Germany) was poured into plates, which were seeded with 10 μl of an overnight L broth culture of the indicator strain *Dickeya dadantii* 3937 *cbsE-1 acsA-37* or 3937 *acr-1 fct34*. Under these conditions, the indicator strains were not able to grow unless an iron source, such as ferric siderophores, was provided. Sterile filter disks (diameter, 6 mm) were placed on the agar surface. Then 15-μl portions of filter-sterilized culture supernatants of the strains to be tested grown in KB medium for 24 h at 28°C were added to the filter disks. For reference, supernatant of the achromobactin producer *D. dadantii* 3937 *cbsE-1 tonB60* grown overnight in Tris medium (16) was applied. Fe^{2+} (20 μM) was used as a control. The diameters of the zones of growth of the indicator strains were measured after 24 h.

Generation of siderophore-deficient mutants of *P. syringae* pv. syringae 22d/93. For inactivation of pyoverdin biosynthesis, *pvsA* encoding a nonribosomal peptide synthase which is responsible for the formation of the pyoverdin chromophore was disrupted by marker exchange mutagenesis. Two fragments were PCR amplified using primer pairs pvsA_fwd1_Spe/pvsA_rev6_Kpn and pvsA_fwd7_Kpn/pvsA_rev4_Bam (Table 1). Primer sequences were derived from the genome sequence of *P. syringae* pv. syringae B728a. PCR products were cloned into pGEM-T Easy (Promega, Mannheim, Germany), yielding plasmids pPVSA1 and pPVSA2. A 1.4-kb KpnI-SpeI fragment cut from pPVSA1 was ligated into KpnI-SpeI-digested pPVSA2, yielding plasmid pPVSA3. A 1.8-kb KpnI kanamycin resistance cassette cut from pMKm (41) was ligated into KpnI-digested pPVSA3, yielding plasmid pPVSA4.

An achromobactin-negative mutant was constructed by disrupting the achromobactin biosynthesis gene *acsD*. A 1.3-kb fragment located in the *acsF* gene was PCR amplified using the primer pair Achr1_fwd/Achr2_KpnI_rev. A second 2-kb fragment located in the *acsE* and *yhcA* genes was amplified using the primer pair Achr5_EcoRV_fwd/Achr6_rev (Table 1). PCR products were ligated into pGEM-T Easy, yielding plasmids pACS1 and pACS2. A 1.8-kb PstI kanamycin resistance cassette cut from pMKm was blunt ended and ligated into SpeI-digested and blunt-ended pACS1, yielding pACS3. A 3-kb ScaI-EcoRI fragment cut from pACS3 was blunt ended and ligated into EcoRV-digested pACS2, yielding plasmid pACS4. Plasmids pPVSA4 and pACS4 were transformed into electrocompetent *P. syringae* pv. syringae 22d/93, and recombinants were selected on kanamycin plates. Putative mutants were screened for double homologous recombination events by PCR analysis. Mutants with the correct genotype were designated *P. syringae* pv. syringae 22d/93ΔPvd and *P. syringae* pv. syringae 22d/93ΔAch, respectively.

A siderophore-negative double mutant was constructed by Tn*5* mutagenesis of *P. syringae* pv. syringae 22d/93ΔPvd. Plasmid pUT/mini-Tn*5* Sm/Sp (12) was mobilized into *P. syringae* pv. syringae 22d/93ΔPvd by triparental mating. Mutants were selected on MG agar (27) with spectinomycin as the selection agent and screened for loss of siderophore production on CAS agar. For identification of the Tn*5* insertion site, genomic DNA of the siderophore-negative strain *P. syringae* pv. syringae 22d/93ΔSid was cut with PstI and cloned into PstI-digested pBBR1MCS. Derived plasmids were transformed into electrocompetent *E. coli* and selected on spectinomycin plates. Sequencing of plasmid pBBR-11-Sid harboring an approximately 6-kb PstI fragment revealed insertion of Tn*5* in the *yhcA* achromobactin biosynthesis gene.

Plant material and inoculation procedures. Soybean plants (*Glycine max* cv. Maple Arrow) were grown on shelves equipped with fluorescent lamps at 22 to 24°C with 50% humidity and with supplemental light using a 14-h photoperiod (350 microeinsteins m^{-2} s^{-1}).

For wound inoculation experiments, *P. syringae* strains grown on KB agar for 24 h at 28°C were suspended in distilled water, and the concentration was adjusted to 1.0×10^7 CFU per ml. For coinoculation experiments, cell suspensions of the pathogen *P. syringae* pv. glycinea 1a/96 were mixed with cell suspensions of *P. syringae* pv. syringae 22d/93 or mutants of this strain at a ratio of 1:2 (vol/vol). For single inoculations, the suspensions of *P. syringae* pv. syringae 22d/93, its mutants, or *P. syringae* pv. glycinea 1a/96 were mixed with sterile water to obtain the same number of cells that were used for coinoculation. Trifoliate leaves of 28-day-old soybean plants were inoculated by the wound inoculation

TABLE 1. Strains, plasmids, and primer used in this study

Strain, plasmid, or primer	Relevant characteristics	Reference or source
Escherichia coli strains		
DH5α	*supE44* ΔlacU169 (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	43
S17-1 λ-pir	λl-pir lysogen of S17-1 (*thi pro hsdR hsdM*$^+$ *recA* RP4:2$^-$ Tc::MU-Km::Tn*7*) Tc^r Sm^r	12
Pseudomonas syringae strains		
P. syringae pv. glycinea 1a/96	Wild type isolated from soybean; Pvd$^+$ Ach$^+$	47
P. syringae pv. syringae 22d/93	Wild type isolated from soybean; Pvd$^+$ Ach$^+$	48
P. syringae pv. syringae 22d/93ΔPvd	*pvsA* mutant carrying Km^r cassette in *pvsA* gene; Pvd$^-$ Ach$^+$ Km^r	This study
P. syringae pv. syringae 22d/93ΔAch	*acsD* mutant carrying Km^r cassette in *acsD* gene; Pvd$^+$ Ach$^-$ Km^r	This study
P. syringae pv. syringae 22d/93ΔSid	Tn*5* mutant of *P. syringae* pv. syringae 22d/93ΔPvd carrying mini-Tn*5* in *acsD* gene; Pvd$^-$ Ach$^-$ Km^r Sp^r	This study
Dickeya dadantii strains		
3937 *cbsE-1 acsA-37*	*cbsE*::Ω *acsA-37*::Mu*d*II1734; chrysobactin$^-$ Ach$^-$ Sp^r Sm^r Km^r	14
3937 *acr-1 fct-34*	*acr-1*::Ω *fct-34*::*lacZ*; chrysobactin$^-$ Ach$^-$ Sp^r Sm^r Km^r	16
3937 *cbsE-1 tonB60*	*cbsE*::Ω *tonB60*::Mu*d*II1734; chrysobactin$^-$ Ach$^+$ Sp^r Sm^r	14
Plasmids		
pGEM-T Easy	TA cloning vector; Ap^r	Promega
pPVSA1	1.4-kb PCR fragment located in *pvsA* gene of *P. syringae* pv. syringae 22d/93, cloned in pGEM-T Easy; Ap^r	This study
pPVSA2	1.6-kb PCR fragment located in *pvsA* gene of *P. syringae* pv. syringae 22d/93, cloned in pGEM-T Easy; Ap^r	This study
pPVSA3	1.4-kb SpeI-KpnI fragment from pPVSA1 cloned in SpeI-KpnI-digested pPVSA2; Ap^r	This study
pPVSA4	1.8-kb KpnI Km^r cassette from pMKm cloned in KpnI-digested pPVSA3; Ap^r Km^r	This study
pACS1	1.3-kb PCR fragment located in *acsF* gene of *P. syringae* pv. syringae 22d/93, cloned in pGEM-T Easy; Ap^r	This study
pACS2	2-kb PCR fragment located in *acsE* and *yhcA* genes of *P. syringae* pv. syringae 22d/93, cloned in pGEM-T Easy; Ap^r	This study
pACS3	1.8-kb PstI Km^r cassette from pMKm blunt ended and cloned in SpeI-digested and blunt-ended pACS1; Ap^r Km^r	This study
pACS4	3-kb ScaI-EcoRI fragment from pACS3 blunt ended and cloned in EcoRV-digested pACS2; Ap^r Km^r	This study
pBBR1MCS	Broad-host-range cloning vector; Cm^r	30
pBBR-11-Sid	6-kb PstI fragment of *P. syringae* pv. syringae 22d/93ΔSid carrying Tn*5* insertion site in pBBR1MCS; Cm^r Sp^r	This study
pMKm	Donor of kanamycin cassette; Km^r	41
pUT/mini-Tn*5* Sm/Sp	Mini-Tn*5* encoding Sm^r/Sp^r on broad-host-range suicide plasmid pUT; Ap^r Sm^r Sp^r	12
Primers		
pvsA_fwd1_Spe	5′-AATACTAGTGGATCCTGATGCGACTGGCCTTCGATC-3′	This study
pvsA_rev6_Kpn	5′-TAAGGTACCACGTCGAGGCTGAGCGGATC-3′	This study
pvsA_fwd7_Kpn	5′-TTAGGTACCTCGAACTTGGCCTCGCGGCTG-3′	This study
pvsA_rev4_Bam	5′-TTTGGATCCGGCAGACCGTGGCTGAG-3′	This study
Achr1_fwd	5′-AGCGAGGACTCACAGATGTTG-3′	This study
Achr2_KpnI_rev	5′-GGTACCCAATGCTGCTGAATGGCAAC-3′	This study
Achr5_EcoRV_fwd	5′-GATATCAACTATGTGCGTCTTGCGTC-3′	This study
Achr6_rev	5′-ACGAATGCCACCAGACAGG-3′	This study

technique described by May et al. (36). Leaves were pricked using a sterile needle, and each wound was inoculated with 5 μl of a bacterial suspension. Bacterial populations were monitored by removing 20 disks (diameter, 7 mm) surrounding the inoculation sites and homogenizing the leaf disks in 20 ml of isotonic NaCl. Bacterial counts (CFU per wound) were determined by plating dilutions of leaf homogenates onto KB agar.

For spray inoculation, *P. syringae* pv. syringae 22d/93 or siderophore mutants of this strain grown on KB agar for 24 h at 28°C were suspended in distilled water, the concentration was adjusted to 1.0×10^7 CFU per ml, and the strains were applied to leaves of 4-week-old soybean plants with an airbrush (~8 lb/in^2) until the leaf surface was uniformly wet. Growth of bacterial strains was monitored by removing random leaf samples 10 days after inoculation. Leaves were macerated in 10 ml isotonic NaCl per g (fresh weight). Bacterial counts (CFU per g [fresh weight]) were determined by plating dilutions of leaf homogenates onto KB agar.

RESULTS

Siderophore production by *P. syringae* pv. syringae 22d/93 in different media. Screening for siderophore production on CAS agar plates showed that there was a remarkable difference between the antagonist *P. syringae* pv. syringae 22d/93 and the pathogen *P. syringae* pv. glycinea 1a/96; a significantly larger siderophore halo was produced by *P. syringae* pv. syringae

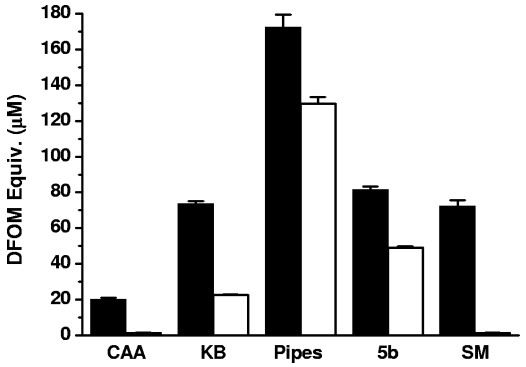

FIG. 1. Influence of culture media on siderophore production. *P. syringae* pv. syringae 22d/93 (filled bars) and *P. syringae* pv. glycinea 1a/96 (open bars) were cultivated in various low-iron media at 28°C for 48 h. The siderophore activity of supernatants was determined by the CAS assay and normalized to an OD_{600} of 1.0. The data are the means and standard deviations of three independent experiments.

TABLE 2. Cross-feeding assay using achromobactin biosynthesis and receptor mutants of *D. dadantii* as indicator strains

Culture supernatant	Diam of indicator strain growth zone (mm)[a]	
	3937 *cbsE-1 acs-37*[b]	3937 *acr-1 fct-34*[c]
No additive	0	0
3937 *cbsE-1 tonB60*[d]	11	0
P. syringae pv. syringae 22d/93	11	0
P. syringae pv. syringae B728a	8	0
Fe^{2+} control[e]	5	5

[a] The experiment was repeated three times. The data are data from a representative experiment.
[b] Achromobactin and chrysobactin biosynthesis mutant of *D. dadantii* 3937.
[c] Achromobactin and chrysobactin receptor mutant of *D. dadantii* 3937.
[d] Achromobactin-producing and chrysobactin-negative mutant of *D. dadantii* 3937.
[e] Fe^{2+} (20 μM) was used as a control.

22d/93 than by *P. syringae* pv. glycinea 1a/96 (data not shown). The same tendency was observed in various low-iron liquid media when the siderophore activities of supernatants were determined by the CAS assay and normalized using cell density. In all media tested, the siderophore production by *P. syringae* pv. syringae 22d/93 was greater than that by *P. syringae* pv. glycinea 1a/96 (Fig. 1). The most obvious difference was observed with SM medium, while the greatest siderophore production by both strains was detected with PIPES medium. Thus, PIPES medium was used in subsequent experiments.

Production of pyoverdin and achromobactin by *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96. Analysis of concentrated culture supernatants by IEF and detection of siderophore activity using CAS agar overlays revealed that *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96 produced identical siderophore patterns. The siderophores produced by *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96 were identified as pyoverdin and achromobactin by comparison with purified standards (Fig. 2). In addition to IEF analysis, cross-feeding experiments using achromobactin-deficient mutants of *D. dadantii* (formerly *Erwinia chrysanthemi*) were conducted to confirm the structural conformity of the achromobactin produced by *P. syringae* pv. syringae 22d/93 and that produced by *D. dadantii* (Table 2). Growth of the indicator strain *D. dadantii* 3937 *cbsE-1 acs-37*, which cannot produce endogenous siderophores, was restored by adding supernatant of *P. syringae* pv. syringae 22d/93, *P. syringae* pv. syringae B728a, or *D. dadantii* 3937 *cbsE-1 tonB60*, an achromobactin-producing *D. dadantii* strain. In contrast, supernatants of *P. syringae* pv. syringae 22d/93, *P. syringae* pv. syringae B728a, and *D. dadantii* 3937 *cbsE-1 tonB60* could not restore growth of the achromobactin receptor mutant *D. dadantii* 3937 *acr-1 fct34*, which cannot produce or import achromobactin. Growth of both indicator strains was restored by adding the Fe^{2+} control, which excluded free iron in the supernatants tested.

Further confirmation that achromobactin and pyoverdin are the only siderophores produced by *P. syringae* pv. syringae 22d/93 was obtained by performing a mutation analysis. Mutants defective for biosynthesis of either pyoverdin (*P. syringae* pv. syringae 22d/93ΔPvd) or achromobactin (*P. syringae* pv.

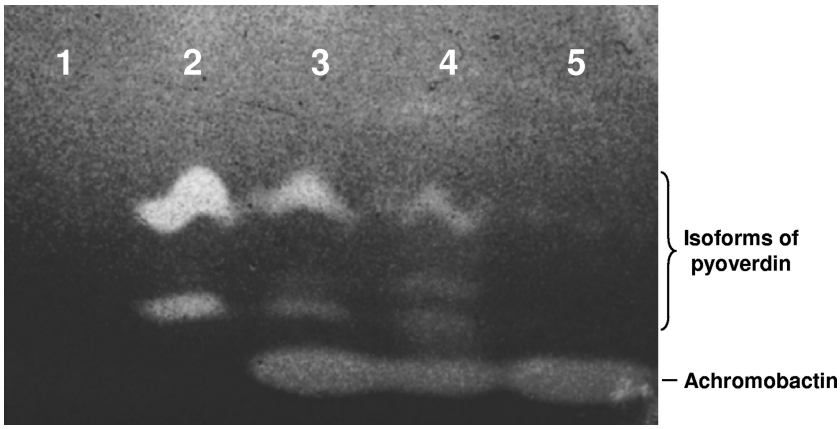

FIG. 2. Isoelectric focusing and CAS overlay for detection of siderophores in *P. syringae*. Siderophores were extracted from concentrated culture supernatants using XAD-4 resin at pH 3.0. After separation by isoelectric focusing, the polyacrylamide gel was overlaid with a thin layer of CAS agar to visualize siderophore activity. Lane 1, *P. syringae* pv. syringae 22d/93ΔSid; lane 2, purified pyoverdin of *P. syringae*; lane 3, *P. syringae* pv. syringae 22d/93; lane 4, *P. syringae* pv. glycinea 1a/96; lane 5, *P. syringae* pv. syringae 22d/93ΔPvd.

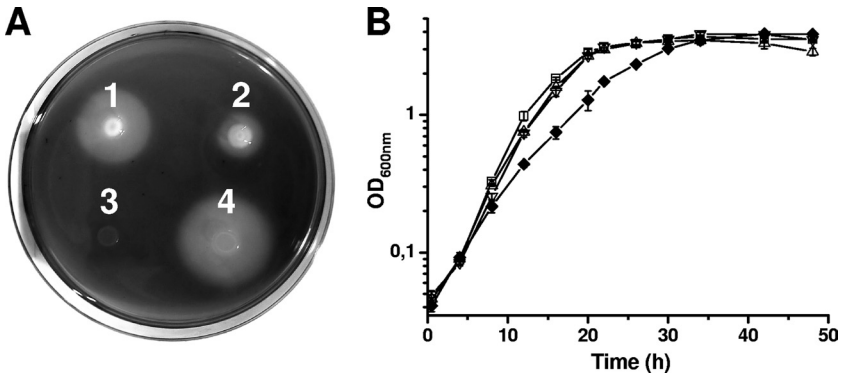

FIG. 3. (A) Siderophore production by *P. syringae* pv. syringae 22d/93 and siderophore mutants of this strain on CAS agar. CAS agar plates were inoculated with 5-μl portions of suspensions ($>10^6$ CFU/ml) of the different strains and incubated at 28°C for 48 h. Siderophore production is indicated by the formation of haloes. 1, *P. syringae* pv. syringae 22d/93; 2, *P. syringae* pv. syringae 22d/93ΔAch; 3, *P. syringae* pv. syringae 22d/93ΔSid; 4, *P. syringae* pv. syringae 22d/93ΔPvd. (B) Growth of *P. syringae* pv. syringae 22d/93 and siderophore mutants of this strain in PIPES medium at 28°C, as determined by measurement of the OD_{600}. The data are the means and standard deviations of three independent cultures. □, *P. syringae* pv. syringae 22d/93; ▽, *P. syringae* pv. syringae 22d/93ΔAch; △, *P. syringae* pv. syringae 22d/93ΔPvd; ◆, *P. syringae* pv. syringae 22d/93ΔSid.

syringae 22d/93ΔAch) still showed siderophore activity on CAS agar plates. The amounts of siderophores produced by *P. syringae* pv. syringae 22d/93ΔPvd and the wild type on CAS agar were similar, while *P. syringae* pv. syringae 22d/93ΔAch produced a significantly smaller siderophore halo (Fig. 3A). The pyoverdin- and achromobactin-negative double mutant *P. syringae* pv. syringae 22d/93ΔSid did not show any residual siderophore activity. To investigate the phenotypic difference between *P. syringae* pv. syringae 22d/93ΔPvd and *P. syringae* pv. syringae 22d/93ΔAch in more detail, the influence of siderophore production on *in vitro* growth was analyzed by culturing *P. syringae* pv. syringae 22d/93 and the siderophore mutants of this strain in PIPES medium. The optical densities of the cultures were monitored continuously until the cultures entered the late stationary growth phase (Fig. 3B). The doubling times of *P. syringae* pv. syringae 22d/93, *P. syringae* pv. syringae 22d/93ΔAch, and *P. syringae* pv. syringae 22d/93ΔPvd were comparable (2.4 to 2.8 h), while growth of *P. syringae* pv. syringae 22d/93ΔSid was significantly delayed under iron-limiting growth conditions (doubling time, about 3.8 h). Still, cultures of all of the strains reached similar optical densities in the stationary growth phase.

Regulation of siderophore biosynthesis in *P. syringae* pv. syringae 22d/93 and regulation of siderophore biosynthesis in *P. syringae* pv. glycinea 1a/96 are different. Siderophore production by the pathogen *P. syringae* pv. glycinea 1a/96 during growth in PIPES medium and siderophore production by the antagonist *P. syringae* pv. syringae 22d/93 during growth in PIPES medium were compared. During the exponential growth phase, the siderophore production by both strains increased steadily, and the maximal values were reached in the early stationary phase. A slight decrease in the late stationary phase was observed. However, *P. syringae* pv. glycinea 1a/96 and *P. syringae* pv. syringae 22d/93 responded quite differently to changes in the growth conditions, indicating that they have different regulatory mechanisms for siderophore production. Incubation with a low ratio of culture volume to flask volume resulted in a growth delay for both strains. The doubling times of *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96 were 3.5 h and 5.0 h, respectively, at a ratio of 1:5. However, the doubling times of both strains increased to 9.5 h at a ratio of 1:10. Nevertheless, cultures of both strains reached similar cell densities under these growth conditions in late stationary phase.

The siderophore activity produced by the antagonist *P. syringae* pv. syringae 22d/93 was strongly dependent on the ratio of culture volume to flask volume. The maximal CAS activity was approximately 40 μM DFOM equivalents at a ratio of 1:5, compared to a CAS activity of approximately 1.2 mM DFOM equivalents at a 1:10 ratio. The siderophore activity of *P. syringae* pv. glycinea 1a/96 was not influenced by the ratio of culture volume to flask volume and reached values of approximately 500 μM DFOM equivalents in the stationary phase under both conditions.

Impact of siderophores produced by *P. syringae* pv. syringae 22d/93 on epiphytic fitness and biocontrol efficacy. Development of bacterial populations on soybean leaves was observed for *P. syringae* pv. syringae 22d/93 and siderophore mutants of this strain 10 days after a single spray inoculation to evaluate the contribution of siderophore production to the epiphytic fitness of the antagonist (Table 3). *P. syringae* pv. syringae 22d/93 reached a population size of 6.7×10^4 CFU per g leaf

TABLE 3. Population dynamics of *P. syringae* pv. syringae 22d/93 and siderophore mutants of this strain on soybean leaves after a single spray inoculation

Strain	Concn (CFU per g leaf tissue)[a]	
	Day 1	Day 10
P. syringae pv. syringae 22d/93	$5.0 \times 10^4 \pm 2.2 \times 10^4$	$6.7 \times 10^4 \pm 3.0 \times 10^3$
P. syringae pv. syringae 22d/93ΔPvd	$1.3 \times 10^4 \pm 2.0 \times 10^3$	$5.4 \times 10^3 \pm 2.0 \times 10^3$
P. syringae pv. syringae 22d/93ΔAch	$8.9 \times 10^4 \pm 1.6 \times 10^3$	$4.6 \times 10^3 \pm 2.0 \times 10^3$
P. syringae pv. syringae 22d/93ΔSid	$9.2 \times 10^4 \pm 6.4 \times 10^3$	$4.0 \times 10^2 \pm 5.0 \times 10^1$

[a] The data are the means and standard deviations of three independent determinations.

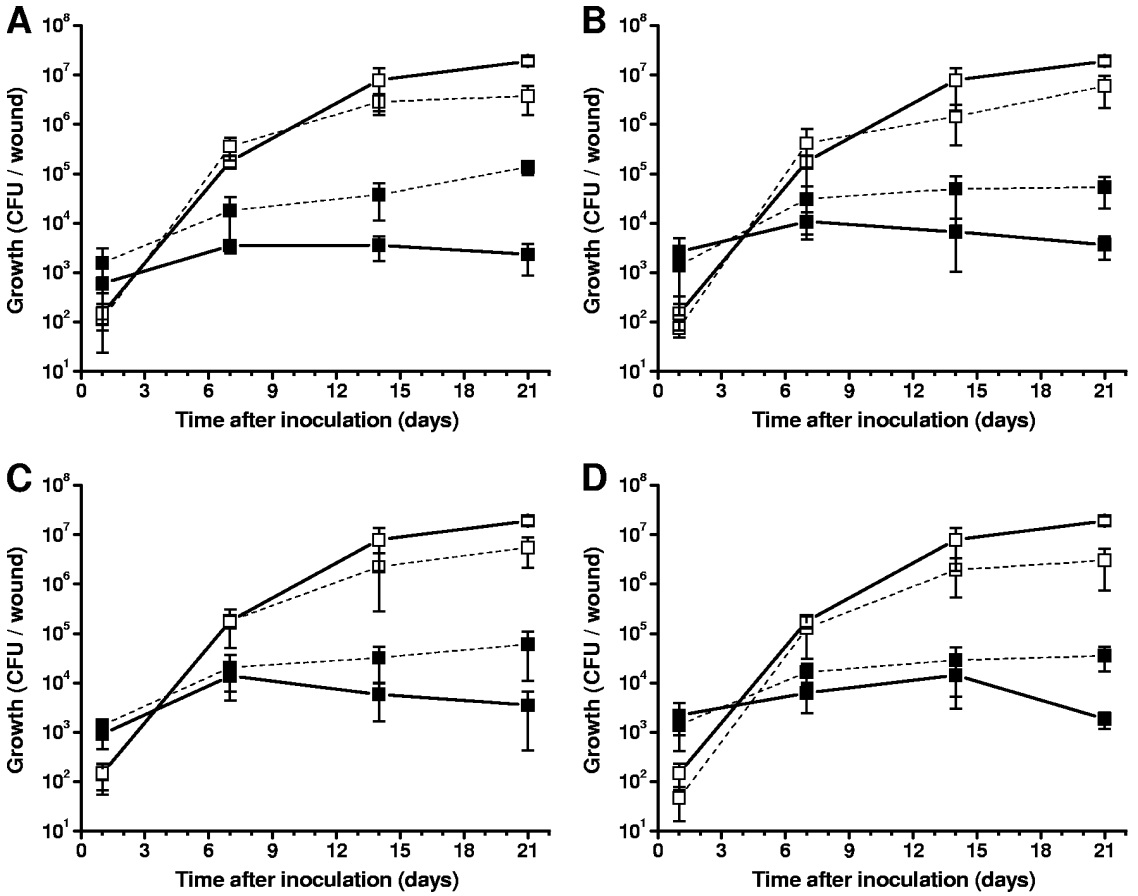

FIG. 4. Population dynamics of *P. syringae* pv. glycinea 1a/96, *P. syringae* pv. syringae 22d/93, and *P. syringae* pv. syringae 22d/93 siderophore mutants after single inoculation (solid lines) and coinoculation (dashed lines) into soybean leaves using wound inoculation. Single inoculations were used to evaluate the epiphytic fitness of the strains and as controls for comparison with the coinoculation experiment. (A) Coinoculation of *P. syringae* pv. glycinea 1a/96 (□) with *P. syringae* pv. syringae 22d/93 (■) and controls; (B) coinoculation of *P. syringae* pv. glycinea 1a/96 (□) with *P. syringae* pv. syringae 22d/93ΔPvd (■) and controls; (C) coinoculation of *P. syringae* pv. glycinea 1a/96 (□) with *P. syringae* pv. syringae 22d/93ΔAch (■) and controls; (D) coinoculation of *P. syringae* pv. glycinea 1a/96 (□) with *P. syringae* pv. syringae 22d/93ΔSid (■) and controls. The data are the means and standard deviations of four independent experiments.

tissue, whereas the population sizes of both single mutants, *P. syringae* pv. syringae 22d/93ΔAch and *P. syringae* pv. syringae 22d/93ΔPvd, were 1 order of magnitude lower (4.6×10^3 and 5.4×10^3 CFU per g leaf tissue, respectively). The greatest effect on *in planta* growth was observed for the siderophore-negative strain *P. syringae* pv. syringae 22d/93ΔSid, which reached a population size that was 2 orders of magnitude lower (4.0×10^2 CFU per g leaf tissue) than that of the wild type.

To examine if siderophores produced by *P. syringae* pv. syringae 22d/93 are responsible for the antagonistic activity against *P. syringae* pv. glycinea, the pathogen was coinoculated with either *P. syringae* pv. syringae 22d/93 or one of the siderophore mutants into wounds on pin-pricked soybean leaves. Single-inoculation experiments were conducted as a control. Following a single inoculation, *P. syringae* pv. glycinea 1a/96 caused typical symptoms (necrotic spots surrounded by chlorotic haloes) approximately 14 days after inoculation. At this time, the population size of *P. syringae* pv. glycinea 1a/96 was about 10^7 CFU per wound. *P. syringae* pv. syringae 22d/93 and the siderophore mutants developed stable populations whose sizes were about 10^4 CFU per wound 7 days after a single inoculation, and they did not cause any disease symptoms (Fig. 4).

In coinoculation experiments with *P. syringae* pv. glycinea 1a/96, the population sizes of *P. syringae* pv. syringae 22d/93 and mutants of this strain were approximately 1 order of magnitude higher than the population sizes in single-inoculation experiments, suggesting that the antagonist benefited from coinoculation with the pathogen. In contrast, the population size of the pathogen was about 1 order of magnitude lower in all coinoculation experiments than in the respective single-inoculation experiment. The smaller population was sufficient to completely suppress development of disease symptoms. Further, siderophore production by the antagonist had no effect on the population size of the pathogen, showing that siderophore production and uptake are not factors involved in the direct biocontrol activity of *P. syringae* pv. syringae 22d/93.

DISCUSSION

Siderophore production by *P. syringae* pv. syringae 22d/93. It has been known for a long time that plant-associated *P. syringae* isolates produce pyoverdin, a peptide-type siderophore that causes the green fluorescence responsible for the term "fluorescent pseudomonads" (7, 9, 25). However, the hypoth-

esis that one bacterial isolate produces one type of siderophore has been revised due to the increased availability of genome sequence information so that it is now acknowledged that there is great redundancy in iron uptake systems, which includes the presence of multiple of siderophore receptors in, and the production of several different siderophores by, a single bacterium (18). Thus, a recent study demonstrated that *P. syringae* pv. syringae B728a produces a second citrate-type siderophore, achromobactin, that was first isolated from *D. dadantii* (3, 40). In this study, we demonstrated that pyoverdin and achromobactin are also synthesized by *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96. However, a significant difference in the amounts of siderophores produced by these *P. syringae* isolates was found. Moreover, a strong correlation between siderophore production and growth conditions was observed for *P. syringae* pv. syringae 22d/93 but not for *P. syringae* pv. glycinea 1a/96. Since all other growth parameters were identical, the large amount of oxygen available was the most likely cause of the reduced growth rates of both organisms when the ratio of culture volume to flask volume was 1:10. There is an intimate relationship between iron metabolism and the oxygen concentration (22, 49). Generation of oxidative stress is an important plant defense response, and production of achromobactin has been linked to the survival of *D. dadantii* during an oxidative burst (15). The different responses of *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96 to high-oxygen conditions might be linked to adaptation to their epiphytic and pathogenic lifestyles, respectively.

Iron limitation during *in planta* growth. Several studies have addressed the question of iron availability in the phyllosphere. Elaborative reporter gene analyses used ice nucleation activity and green fluorescent protein to determine iron resources in different habitats (24, 35). Both reporter constructs were based on an iron-regulated promoter derived from the pyoverdin biosynthesis gene cluster. Heterogeneous reporter activity responses were observed in the phyllosphere, indicating that only a small subset of the *P. syringae* population analyzed experienced low iron availability (24). However, the pathogen *P. syringae* pv. tabaci showed reduced virulence after mutagenesis of the pyoverdin biosynthesis system (45). Our analysis of siderophore mutants of the epiphyte *P. syringae* pv. syringae 22d/93 showed that loss of pyoverdin production results in only a slight reduction in *in planta* growth, while loss of all siderophore production had a more severe impact. Similarly, analysis of a yersiniabactin-negative mutant of *P. syringae* pv. tomato DC3000 that was still able to produce pyoverdin showed no impairment of growth on *Arabidopsis* (23). In contrast, we observed significantly reduced growth of the siderophore-negative mutant *P. syringae* pv. syringae 22d/93ΔSid after spray inoculation. However, no differences in growth between *P. syringae* pv. syringae wild-type strain 22d/93 and siderophore mutants of this strain were found in wound inoculation experiments, which might have been due to nutrient leakage and therefore improved iron availability. A similar effect of the inoculation method was observed for *P. syringae* pv. tabaci (45). While mutants affected in pyoverdin production showed only slightly reduced virulence when they were infiltrated into tobacco leaves, the phenotype was more severe when spray inoculation was used.

Another system in which siderophore production has been shown to influence plant colonization is the achromobactin-chrysobactin system in *D. dadantii* (15, 16). Mutational analysis resulted in the conclusion that achromobactin is required for initiation of plant colonization at the onset of infection, while chrysobactin is necessary for development of a systemic infection. The reduced population sizes of the *P. syringae* pv. syringae 22d/93 siderophore mutants indicate that achromobactin production had a similar influence on epiphytic colonization of soybean leaves. Studies of the relevance of the achromobactin-pyoverdin siderophore system in *P. syringae* might increase our understanding of the role of iron availability in host-pathogen interactions. Karamanoli and Lindow (26) demonstrated that leaf surface compounds had an iron-sequestering effect. The different results for the relevance of siderophore production for plant-associated bacteria undoubtedly reflect the natural gradient from iron sequestration by the host plant to leakage of iron from the plant tissue.

Impact of siderophore production on the biocontrol activity of *P. syringae* pv. syringae 22d/93. There were no significant differences between disease suppression after coinoculation of *P. syringae* pv. syringae 22d/93 siderophore mutants and *P. syringae* pv. glycinea 1a/96 and disease suppression after coinoculation of *P. syringae* pv. syringae wild-type strain 22d/93 and *P. syringae* pv. glycinea 1a/96, suggesting that siderophores do not play an essential role in this antagonism. We demonstrated that *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea 1a/96 produce the same siderophores, precluding the possibility of direct competition for iron between *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea. Still, the foremost characteristic of a good biocontrol organism is efficient colonization of the host plant. Production of achromobactin and pyoverdin contributes significantly to the epiphytic fitness of *P. syringae* pv. syringae 22d/93, thus improving its biocontrol activity in an indirect way. Furthermore, the production of two high-affinity iron acquisition systems by *P. syringae* pv. syringae 22d/93 might increase its competitiveness with other epiphytes.

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