Analysis of Achromobactin Biosynthesis by *Pseudomonas syringae* pv. *syringae* B728a[▽]

Andrew D. Berti and Michael G. Thomas*

Department of Bacteriology and Microbiology Doctoral Training Program, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received 3 April 2009/Accepted 11 May 2009

***Pseudomonas syringae* pv. *syringae* B728a is known to produce the siderophore pyoverdine under iron-limited conditions. It has also been proposed that this pathovar has the ability to produce a second siderophore, achromobactin. Here we present genetic and biochemical evidence supporting the hypothesis that *P. syringae* pv. *syringae* B728a produces both of these siderophores. We show that strains unable to synthesize either pyoverdine or achromobactin are unable to grow under iron-limiting conditions, which is consistent with these two molecules being the only siderophores synthesized by *P. syringae* pv. *syringae* B728a. Enzymes associated with achromobactin biosynthesis were purified and analyzed for substrate recognition. We showed that AcsD, AcsA, and AcsC together are able to condense citrate, ethanolamine, 2,4-diaminobutyrate, and α -ketoglutarate into achromobactin. Replacement of ethanolamine with ethylene diamine or 1,3-diaminopropane in these reactions resulted in the formation of achromobactin analogs that were biologically active. This work provides insights into the biosynthetic steps in the formation of achromobactin and is the first in vitro reconstitution of achromobactin biosynthesis.**

Iron is a micronutrient essential for the growth and metabolism of the vast majority of microorganisms. Although iron is the fourth-most-abundant element on earth, at neutral-to-alkaline pH and in the presence of oxygen, iron spontaneously assembles into ferric oxyhydroxide complexes (44). The solubility of these ferric polymers in water is extremely low, and therefore this nutrient is not readily bioavailable. Many microbes have developed mechanisms to access this insoluble ferric iron through the secretion of high-affinity iron chelators called siderophores (12). These siderophores are secreted, solubilize ferric iron in the environment, bind to receptors on the surface of the producing organism as a ferric-siderophore complex, and are actively transported into the cell, thus providing the iron needed for cell growth and maintenance.

Production of siderophores is of particular importance for pathogenic bacteria, as they grow in or on a host where iron is not only relatively scarce but is often maintained in a host-sequestered form (23, 26). To multiply to a sufficient concentration to initiate and maintain a wild-type level of infection, pathogenic bacteria must acquire sufficient intracellular stores of iron. For example, in an in vivo urinary tract model of *Pseudomonas aeruginosa* infection, inocula grown in low-iron media are preprimed for iron-restricted growth and show significant enhancements in virulence relative to inocula grown in iron-replete media (33). This need for iron is true not only for animal pathogens; some plant pathogens require iron acquisition mechanisms to cause disease in plants. On its African violet host, the soft rot plant pathogen *Dickeya dadantii* (formerly *Erwinia chrysanthemi* or *Pectobacterium chrysanthemi*)

shows reduced pathogenicity when production of its two known siderophores is genetically inactivated (14). Iron availability is also emerging as a significant factor in the formulation of growth media to allow for the expression of virulence-associated genes in culture (24). Due to the essential nature of iron acquisition for pathogenic organisms to cause disease, iron acquisition in general and siderophore biosynthesis and transport in particular have emerged as attractive targets for therapeutic intervention against pathogenic microbes (31, 32).

Initially, a detailed understanding of the mechanism of siderophore biosynthesis was limited to the thiotemplate-based assembly mechanism of nonribosomal peptide synthetases (NRPS) and polyketide synthases (8). More recently, a different siderophore assembly mechanism based on NRPS-independent enzymes has been recognized (5). Such siderophores are assembled from dicarboxylic acid subunits and dinucleophile linkers by the action of a conserved family of adenylation/condensation enzymes structurally distinct from NRPS and polyketide synthase enzymes. These NRPS-independent siderophore (NIS) synthetases were initially described only in the context of production of the *Escherichia coli* siderophore aerobactin (10). Recently, however, genes coding for NIS synthetases have been found in over 40 species of bacteria and are associated with production of no fewer than eight structurally distinct siderophores (5, 7).

NIS synthetases activate carboxylic acid substrates as acid adenylates in a fashion that is conceptually analogous to the activation of amino acids by NRPSs but is enzymatically and mechanistically distinct (41). The NIS synthetase superfamily can be subdivided into three subclasses of functionally related enzymes. The current model is that each subclass recognizes and activates via adenylation a particular type of carboxylic acid substrate (5). Type A NIS synthetases are predicted to recognize one of the pro-chiral acid groups of citrate, type B NIS synthetases are proposed to recognize the δ -acid group of

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin—Madison, 6155 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706. Phone: (608) 263-9075. Fax: (608) 262-9865. E-mail: thomas@bact.wisc.edu.

[▽] Published ahead of print on 29 May 2009.

FIG. 1. Chemical structure of the siderophore ACR. Portions of the siderophore structure that derive from incorporation of specific precursors are shown. The ethanolamine moiety may be derived directly from ethanolamine or indirectly via decarboxylation of L-serine after condensation with citrate (41).

α -ketoglutarate, and type C NIS synthetases are proposed to recognize esterified or amidated derivatives of carboxylic acids. These classifications were developed using extensive bioinformatic analyses (5) and are supported by earlier genetic evidence from the aerobactin pathway that suggests that IucA, a type A enzyme, condenses citrate with an N^{ϵ} -acetyl- N^{ϵ} -hydroxylysine, while IucC, a type C enzyme, condenses the citryl-monoamide product of IucA with a second equivalent of N^{ϵ} -acetyl- N^{ϵ} -hydroxylysine (10). More recently, analyses of the enzymes involved in the biosynthesis pathway for the production of the siderophore petrobactin have provided biochemical support for the proposed grouping of the NIS synthetases (37, 38).

Another siderophore that has been proposed to be assembled by NIS synthetases is the siderophore achromobactin

(ACR), which is produced by the soft rot plant pathogen *D. dadantii* (36, 40). The structure of ACR consists of a citrate core that is decorated with ethanolamine and diaminobutyrate, which are both condensed with α -ketoglutarate. The α -ketoglutarate moieties each cyclize in neutral aqueous solution to form pyrrolidine rings (Fig. 1) (36). The genes for ACR biosynthesis in *D. dadantii* were identified as part of a study investigating iron acquisition mechanisms by this bacterium, and an initial biosynthetic scheme was presented (14). Separately, as part of an extensive bioinformatics analysis of multiple siderophore gene clusters, a different scheme for ACR biosynthesis was proposed. This biosynthetic scheme involves four enzymes, with three of the enzymes being NIS synthetases (5) (Fig. 2). The first step is the conversion of citrate (Fig. 2, compound [1]) to *O*-citryl-serine (compound [2]) by the type A NIS synthetase AcsD. AcsD was initially proposed to catalyze the conversion of citrate to *O*-citryl-ethanolamine (compound [3]); however, as this manuscript was being prepared, a biochemical analysis of AcsD from *D. dadantii* suggested that [2] is the most likely product of the AcsD reaction, with AcsE catalyzing the decarboxylation of [2] to generate [3] (41). The type B NIS synthetase AcsC is proposed to then convert [3] to diaminobutyryl-citryl-ethanolamine (compound [4]). A type C NIS synthetase, AcsA, is proposed to catalyze the final steps in ACR biosynthesis by adding successive α -ketoglutarate moieties to [4], generating ACR. While there is biochemical data in support of the role AcsD plays in ACR biosynthesis in *D.*

FIG. 2. Proposed scheme for the biosynthesis of ACR. The preferential use of L-serine by AcsD is based on recent kinetic analyses of AcsD from *D. dadantii* (41). However, the initial biosynthesis proposal suggested that L-serine was converted to ethanolamine prior to AcsD activity (5). As shown in this study, AcsD is also able to use ethanolamine as a cosubstrate with citrate (1). The following abbreviations are used: DAB, 2,4-diaminobutyrate; α KG, α -ketoglutarate; PLP, pyridoxal phosphate.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or description	Source or reference
<i>E. coli</i>		
S17-1	<i>recA thi pro hsdR hsdM⁺</i> RP4-2 Tc ^r ::Mu-Km::Tn7	Laboratory strain
DH5 α	$\Delta(lac)U169 \phi 80\Delta(lacZ)M15 hsdR17 endA1 gyrA96 recA1 relA1 supE44 thi-1$	Laboratory strain
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen
<i>P. syringae</i> pv. <i>syringae</i> B728a	Wild-type	1, 29
ADB1005 (PVD ⁻)	<i>pvdL::nptII</i> (Kan ^r)	This study
ADB1006 (ACR ⁻)	Psyr2583-2589:: <i>aadA</i> (Spc ^r)	This study
ADB1007 (PVD ⁻ ACR ⁻)	<i>pvdL::nptII</i> (Kan ^r), Psyr2583-2589:: <i>aadA</i> (Spc ^r)	This study
Plasmid		
pEX19-Tc	Suicide vector	20
pSRA2	Source of Spc ^r cassette (<i>aadA</i>)	15
pUC4K	Source of Kan ^r cassette (<i>nptII</i>)	45
pET28b	Overexpression vector	Novagen
pADB7	PCR amplicons containing portions of <i>pvdL</i> cloned into pEX19-Tc	This study
pADB8	Kan ^r cassette insertion into BamHI site of pADB7	This study
pADB9	PCR amplicons containing portions of Psyr2583 and Psyr2589 cloned into pEX19-Tc	This study
pADB10	Spc ^r cassette insertion into BamHI site of pADB9	This study
pADB11	Psyr2589 (<i>acsA</i>) cloned into pET28b	This study
pADB12	Psyr2587 (<i>acsC</i>) cloned into pET28b	This study
pADB13	Psyr2584 (<i>acsD</i>) cloned into pET28b	This study

dadantii, the other steps have yet to be examined experimentally.

Pseudomonas syringae is a plant-associated microbe evolutionarily and pathologically distinct from *D. dadantii*. *P. syringae* strains are separated into different pathovars based on the preferred plant host of each individual isolate. Of the six *P. syringae* pathovars for which genome sequences are currently available, five possess a cluster of genes whose gene products show high identity to proteins proposed to be involved in ACR biosynthesis by *D. dadantii*. Herein we establish that *P. syringae* pv. *syringae* B728a (B728a) does produce ACR as one of its two siderophores. We report the reconstitution in vitro of a minimal ACR synthetase consisting of AcsA, AcsC, and AcsD. We employ this in vitro siderophore synthesis system to assess the ability of these enzymes to incorporate alternative precursors and demonstrate that some of the ACR analogs thus produced are competent for biologically relevant iron transport.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are described in Table 1. The following media were used to support the growth of B728a. King's B (KB) (25) was used as a general rich medium. A minimal medium (MM) consisting of 6 mM K₂HPO₄, 5 mM KH₂PO₄, 17 mM NaCl, 30 mM (NH₄)₂SO₄, 2.8 mM MgSO₄, 1.7 mM sodium citrate, and 10 mM fructose was employed as an iron-poor medium. Sistrom's medium (43) containing 2,2'-dipyridyl at a concentration of 500 μ M was used as a defined medium for testing the biological activity of in vitro-synthesized siderophores. *E. coli* strains were grown in lysogeny broth (LB) medium (2). As needed, strains were grown on medium solidified with 1.5% (wt/vol) agar. When required, antibiotics were added at the following concentrations: spectinomycin, 100 μ g/ml; nalidixic acid, 5 μ g/ml; tetracycline, 10 μ g/ml; and kanamycin, 50 μ g/ml.

Assays of siderophore activity. For qualitative measurements, a 10- μ l sample was mixed with 10 μ l of a chrome azurol S (CAS) solution containing 4 mM of a 5-sulfosalicylate shuttle (42) (the mixture is referred to here as CAS solution) and observed for color transition from blue to pink. For quantitative measurements, overnight cultures to be tested for siderophore activity were grown in MM at 30°C until they reached an optical density at 600 nm (OD₆₀₀) of 1.5. Cellular material was removed via centrifugation, and 500 μ l of clarified supernatant was mixed with 500 μ l of CAS solution. Spectrophotometric data were recorded at

630 nm after 5 min of incubation at room temperature. Data are presented as an absolute change in absorbance at 630 nm relative to that of a control using water in lieu of clarified supernatant.

Purification of ACR from B728a. ACR was purified from the pyoverdine (PVD)-deficient mutant strain ADB1005 (PVD⁻). One hundred milliliters of MM was inoculated with 1 ml of overnight culture grown in MM and shaken at 30°C for 3 days. Cells were removed by centrifugation, and the culture supernatant was tested for iron chelation activity by the CAS-based siderophore assay. Supernatants were concentrated to a 10-ml final volume by rotary evaporation, brought to a 90% methanol concentration, and filtered (0.2- μ m diameter pore size; Nalgene). The filtrate was mixed 1:1 with ethyl acetate and chromatographed onto a column of silica resin (Selecto Scientific; Suwanee, GA). The column was washed twice with a 10:9:1 solution of ethyl acetate/methanol/water, and bound ACR was eluted 9:1 in methanol/water. Eluted ACR was concentrated to dryness in a rotary evaporator and resuspended in 1 ml of water. Both iron-repressed cultures of the PVD⁻ mutant (MM + 50 μ M FeSO₄) and cultures of the PVD⁻ ACR⁻ double mutant (strain ADB1007) were CAS negative prior to purification. No CAS reactivity was observed when the supernatants from these cultures were purified as above.

In vivo ACR activity. Assays for biologically-relevant siderophore activity were performed as follows. An overnight culture of the PVD⁻ ACR⁻ mutant strain was grown in MM and diluted 1:100 into 1 ml of iron-sequestered Sistrom's defined medium. Filter-sterilized, in vitro-synthesized ACR (500 μ l) or ACR purified from the PVD⁻ strain (100 μ l) was added, and the culture was shaken at 30°C and 200 rpm. Cultures with observable turbidity (OD₆₀₀ $\geq$ 0.2) after 24 h of growth were considered to contain a biologically relevant siderophore.

Construction of siderophore-deficient B728a strains. (i) *pvdL::nptII* (strain ADB1005 [PVD⁻]). Two 2.0-kb PCR amplicons flanking the putative *pvdL* gene (Psyr1945) from B728a were generated, using the following primer sets: *pvdL* up (F), 5'-TTCGTCGCCTGGTACCTGATGCCGACGCGACAA-3', and (R), 5'-GAGTTCAAACGGATCCATGATGGGGTTCTGCTA-3', and *pvdL* down (F), 5'-ACTTGATGGCGGATCCGGAAGGGCTCTGATCAGC-3', and (R), 5'-CCTCACGCTCAAGCTTGGGAATGCAGGTCCGCGAC-3'. The amplicon from the first primer set was cloned into the corresponding Acc65I/BamHI sites of pEX19-Tc. The amplicon from the second primer set was cloned into the BamHI/HindIII sites of the pEX19-Tc vector containing the first amplicon. The resulting plasmid, pADB7, was digested with BamHI, and the BamHI fragment of pUC4K containing the kanamycin resistance gene cassette *nptII* was ligated into this site. The resulting plasmid, pADB8, was transformed into *E. coli* S17-1 and used for conjugations with B728a. Exconjugants were grown on KB-kanamycin agar lacking tetracycline and supplemented with 10% (wt/vol) sucrose. Strains that grew were screened for tetracycline sensitivity and the inability to fluoresce under UV light. The disruption of *pvdL* was confirmed

by PCR amplification analysis. One of the resulting strains was used for further analysis.

(ii) **ACR::*aadA* (strain ADB1006 [ACR⁻]).** Two 2.0-kb PCR amplicons flanking the putative ACR cluster of the B728a genome were generated, using the following primer sets: ACR up (F), 5'-ACGGCGTGAAGAATTCCGGCGACACAGAATGGGA-3', and (R), 5'-GGTGCCTCGGGATCCGTGACCCACACGCCATGT-3', and ACR down (F), 5'-CTGACGCCAGGATCCAGCGTGCGGGCGTCCGG-3', and (R), 5'-CTGCCGGAAAAGCTTCGGTAGCGACGGTGAAA-3'. The amplicon from the first primer set was cloned into the corresponding EcoRI/BamHI sites of pEX19-Tc. The amplicon from the second primer set was cloned into the BamHI/HindIII sites of the pEX19-Tc vector containing the first amplicon. The resulting plasmid, pADB9, was digested with BamHI, and the BamHI fragment of pSRA2 containing the spectinomycin resistance gene cassette *aadA* was ligated into this site. The resulting plasmid, pADB10, was transformed into *E. coli* S17-1 and used for conjugations with B728a. Exconjugants were grown on KB-spectinomycin agar lacking tetracycline and supplemented with 10% (wt/vol) sucrose. Strains that grew were screened for tetracycline sensitivity. The disruption of the ACR cluster was confirmed by PCR amplification analysis. One of the resulting strains was used for further analysis.

(iii) ***pvdL::nptII* ACR::*aadA* (strain ADB1007 [PVD⁻ ACR⁻]).** *E. coli* S17-1 containing plasmid pADB8 was used for conjugations with strain ADB1006. Exconjugants were grown on KB-kanamycin agar lacking tetracycline and supplemented with 10% (wt/vol) sucrose. Strains that grew were screened for tetracycline sensitivity and the inability to fluoresce under UV light. All media used in the generation of this mutant were supplemented with 50 μ M FeSO₄ to reduce the stress involved due to the loss of both siderophore pathways. The disruption of *pvdL* was confirmed by PCR amplification analysis. One of the resulting strains was used for further analysis.

Construction of plasmids encoding AcsA, AcsC, and AcsD. The coding sequences for AcsA (Psr2589), AcsC (Psr2587), and AcsD (Psr2584) were amplified by PCR from B728a chromosomal DNA prepared using an Easy-DNA kit (Invitrogen). The following oligo pairs were used for PCR amplification: AcsA, 5'-AGGAAAATAACATATGAACCTCACTCACTCGCC-3' and 5'-GTACGCCGTGAAGCTTGGGGGTTTCGGTCAAGTT-3'; AcsC, 5'-TCGAGGTATGCATATGGTACCCCTTACCGCTG-3' and 5'-GTCGGCAATCAGCTTTTGGAGCGTATTGGTCTCTC-3'; and AcsD, 5'-CTGTGGAGCACATATGACTAATACCGATCGCACC-3' and 5'-CGGTCTCCGGAAGCTTATGCATAACGTGCCTCCG-3'. PCRs were performed using Herculase II DNA polymerase (Stratagene) according to the manufacturer's instructions. The resulting amplicons were digested with NdeI and EcoRI and cloned into the corresponding sites of pET28b (Novagen).

Expression and purification of AcsA, AcsC, and AcsD. Each of three 2.8-liter flasks, each containing 1 liter of LB-kanamycin medium, were inoculated with 10 ml of an overnight culture of *E. coli* BL21(DE3) containing the appropriate expression plasmid and were incubated on a rotary shaker at 200 rpm at 25°C. Once the OD₆₀₀ of the culture had reached 0.5, the temperature was shifted to 15°C. Following a 1-h incubation at 15°C, isopropyl β -D-1-thiogalactopyranoside was added to a final concentration of 500 μ M. The culture was allowed to continue incubating at 200 rpm overnight at 15°C. Cells from induced overnight cultures were harvested via centrifugation. Cell pellets were resuspended in 35 ml of column buffer (20 mM Tris [pH 8], 300 mM NaCl, 10% glycerol) and sonicated for 5 min three times at 30% power with 1-s pulses. Clarified supernatant generated by centrifugation (30 min at 15,000 rpm on a Beckman J2-21 centrifuge with a Beckman JA-25.50 rotor) was brought to a concentration of 5 mM of imidazole and incubated for 2 h with Ni-NTA agarose resin at 4°C. Resin was collected by centrifugation (5 min at 913 $\times$ g), and bound protein was eluted, using stepwise washes of increasing imidazole concentrations (20 to 250 mM). Fractions were assayed for protein yield and purity via sodium dodecyl sulfate-polyacrylamide gel electrophoresis with gels stained with Coomassie blue dye. Positive fractions of high yield and purity were dialyzed at 4°C into FPLC buffer A (50 mM Tris [pH 8], 50 mM NaCl, 10% glycerol) (molecular mass cutoff, 10 kDa) in preparation for further purification. Dialyzed protein was bound to an in-line 5-ml HiTrap Q HP column (Amersham) and washed with FPLC buffer A at a constant flow rate of 2 ml/min. Protein was eluted over a 20-min gradient from 100% FPLC buffer A to 100% FPLC buffer B (buffer B consists of 50 mM Tris [pH 8], 1 M NaCl, and 10% glycerol). One-minute samples were collected by an automated fraction collector. Samples containing strong absorption at 280 nm were assayed for purity and yield via sodium dodecyl sulfate-polyacrylamide gel electrophoresis with gels stained with Coomassie blue dye. Positive fractions of high yield and purity were dialyzed at 4°C into storage buffer (50 mM Tris [pH 8], 100 mM NaCl, 10% glycerol) (molecular weight cutoff, 10), concentrated to 100 μ M, using Amicon Centriprep Ultracel YM-10 filters (Millipore; Billerica, MA), flash-frozen as aliquots in liquid nitrogen, and stored at -80°C until

further use. The concentrations of each protein were determined using the calculated molar extinction coefficients (AcsA, 118,740 cm⁻¹ M⁻¹; AcsC, 94,150 cm⁻¹ M⁻¹; AcsD, 97,500 cm⁻¹ M⁻¹).

In vitro synthesis of ACR. All reactions were performed in 1 ml of synthesis buffer overnight at room temperature. Synthesis reaction buffer consists of 5 mM ATP, 15 mM MgSO₄, 100 mM Tris [pH 8], 400 μ M TCEP [Tris(2-carboxyethyl)-phosphine], 5 μ M AcsA, 5 μ M AcsC, and 5 μ M AcsD. The following ACR precursors were added: 1 mM sodium citrate, 1 mM ethanolamine, 1 mM diaminobutyrate, and 2 mM α -ketoglutarate. Substrate analogs were added at the same concentrations as the precursors for which they substituted.

Substrate selectivity of AcsA, AcsC, and AcsD. All reactions were performed in 300 μ l of hydroxamate buffer for 45 min at room temperature. The reaction was stopped by the addition of an equal volume of stop buffer (defined below), and the reaction mixture was centrifuged briefly and assayed at 540 nm for the presence of ferric hydroxamates. Hydroxamate reaction buffer consists of 3 mM ATP, 15 mM MgSO₄, 100 mM Tris [pH 8], 133 mM hydroxylamine, 100 mM NaCl, 400 μ M TCEP, and 10% glycerol. Stop buffer consists of 200 mM trichloroacetic acid, 370 mM FeCl₃ · 6H₂O, and 700 mM HCl. For reactions analyzing the use of citrate as a substrate, 3 mM of sodium citrate was incubated with 3 μ M of AcsA, AcsC, or AcsD in hydroxamate buffer. For reactions analyzing the use of α -ketoglutarate, 3 mM of α -ketoglutarate was incubated with 3 μ M of AcsA, AcsC, or AcsD in hydroxamate buffer. For reactions analyzing the use of citrilythanolamine as a substrate, an additional citrilythanolamine synthesis reaction was required. For this reaction, 3 mM of sodium citrate was incubated with 3 μ M of AcsD in modified hydroxamate buffer containing 133 mM ethanolamine in lieu of 133 mM hydroxylamine. After the mixture was allowed to react overnight, samples were incubated at 70°C for 15 min, briefly centrifuged to remove inactivated AcsD, and allowed to return to room temperature. Fresh ATP, TCEP, and hydroxylamine were added, and the hydroxamate formation reaction was initiated with 3 μ M of AcsA or AcsC. Reactions analyzing the use of citrilyl-2,4-diaminobutyrate were performed in an analogous fashion by substituting 2,4-diaminobutyrate for ethanolamine in the synthesis and analysis procedures outlined above.

Determination of kinetic parameters of AcsD. (i) Determination of extinction coefficient. A series of overnight incubations spanning a range of sodium citrate concentrations in hydroxamate buffer containing 5 μ M of AcsD was assayed at 540 nm. The quotient of the absorbance reading and the initial citrate concentration was 0.55 mM⁻¹ cm⁻¹ and is here reported as the extinction coefficient of citrilyl-hydroxamate. This experimentally determined coefficient is comparable to values obtained for similar carboxylic acid hydroxamates (16, 17, 19). In each case, incubation with additional fresh AcsD following overnight incubation caused no increase in absorbance at 540 nm, which suggests that the reaction had run to completion.

(ii) Determination of K_m and k_{cat} . Five hundred micromolars of citrate in hydroxamate buffer containing 1.5 μ M AcsD showed a linear increase in absorption at 540 nm for at least the first 15 min of the reaction, with less than a 10% substrate-to-product conversion. Subsequently, rates of product formation were determined at 15 min for a series of citrate concentrations from 500 μ M to 3 μ M. Nonlinear regression analysis suggested a K_m of approximately 2 mM. A more-representative series of 10 citrate concentrations centered at 2 mM was used to more accurately determine the K_m and catalytic constant (k_{cat}) values of AcsD. Nonlinear regression analyses were performed using the Kaleidagraph software package (Synergy Software). Kinetic data were fitted to the Michaelis-Menten equation.

LC-MS. Liquid chromatography-mass spectrometry (LC-MS) was performed in-line on an Agilent LC/MSD time-of-flight mass spectrometer with an RP C₁₈ column (Agilent Zorbax C₁₈ 1.8 μ m 2.1- by 50-mm column). Mobile-phase solvents for LC consisted of 50 mM ammonium acetate [pH 5] in water (solvent A) and 50 mM ammonium acetate [pH 5] in acetonitrile (solvent B). LC separation consisted of 1 min of isocratic development at 2% solvent B, a linear increase from 2% solvent B to 90% solvent B over 24 min, and 1 min of isocratic development at 90% solvent B. Flow rates were maintained at 0.25 ml/min. Spectra were acquired in negative-ion mode with an m/z range of 50 to 3,200.

RESULTS AND DISCUSSION

B728a produces two chemically distinct siderophores under iron-limiting conditions. B728a is known to produce the fluorescent siderophore PVD under iron-limiting conditions (6). Not surprisingly, analysis of the B728a genome identified a gene cluster (Psr1944-1961) coding for enzymes that show a

TABLE 2. CAS reactivity of B728a cell-free supernatants

Strain	Abs ₆₃₀ ± SD ^a
B728a	1.193 ± 0.004
ADB1005 (PVD ⁻)	1.240 ± 0.011
ADB1006 (ACR ⁻)	1.170 ± 0.003
ADB1007 (PVD ⁻ ACR ⁻)	0.122 ± 0.022

^a Values reported are the averages of the results for three independently grown cultures; standard deviations (SD) are shown. Abs₆₃₀, absorbance at 630 nm.

high level of amino acid identity to known PVD biosynthetic enzymes (13). One of these genes, Psyr1945, codes for a homolog of PvdL, a key NRPS involved in assembling the chromophore of PVD siderophores (34). Analysis of the B728a genome also identified a gene cluster (Psyr2582-2589) coding for proteins similar to those involved in ACR biosynthesis in *D. dadantii* (13, 14). ACR production by B728a, however, has not been demonstrated experimentally (13). To determine if B728a produces two or more chemically distinct siderophores, genes from both putative siderophore biosynthesis gene clusters were inactivated and the resulting strains were analyzed for iron-chelating and iron-acquisition capabilities. For the putative PVD pathway, Psyr1945 was inactivated by the insertion of a kanamycin resistance cassette (the PVD⁻ mutant strain). This strain no longer produced a metabolite with the characteristic fluorescence of PVD, confirming that Psyr1945 was involved in PVD production. For the putative ACR gene cluster, genes Psyr2582 to Psyr2589 were replaced by a spectinomycin resistance cassette (the ACR⁻ mutant strain). Additionally, a third strain containing both resistance cassettes was constructed to investigate whether B728a produces any additional siderophores (the PVD⁻ ACR⁻ mutant strain).

In order to analyze the ability of B728a and the mutant strains to produce compounds that solubilize ferric iron, cell-free supernatants of strains were assayed for reactivity with CAS, a colorimetric, weak-affinity iron chelator (42). Wild-type B728a along with the PVD⁻ and ACR⁻ mutant strains demonstrated clear CAS reactivity, indicating the presence of a biosynthesized ferric-iron chelator (Table 2). In contrast, supernatant from the PVD⁻ ACR⁻ strain was unable to acquire ferric iron from a CAS-Fe³⁺ complex. These data strongly suggest that B728a can produce and secrete two distinct siderophores and that the targeted gene clusters code for the enzymes to produce the respective siderophores.

In order to test whether the mutant strains also showed a growth phenotype consistent with a disruption in siderophore production, growth curves of wild-type and mutant strains were performed in a defined medium in which iron was sequestered by 2,2'-dipyridyl. As shown in Fig. 3A, the wild-type and mutant strains grew equally well in defined media. The wild-type and ACR⁻ strains also grew equally well when the medium contained 500 μM of the iron chelator 2,2'-dipyridyl. Interestingly, the PVD⁻ strain eventually reached the same final OD₆₀₀ but had a reduced growth rate, which suggests that PVD is the preferred siderophore of B728a under these growth conditions. Finally, disruption of both the PVD and ACR gene clusters resulted in a strain that was unable to grow in the presence of 2,2'-dipyridyl (Fig. 3B). These data confirm that the genes Psyr2582 to Psyr2589 code for enzymes involved in

the production of a siderophore. Additionally, these data support the hypothesis that B728a produces two distinct siderophores.

ACR is the siderophore associated with Psyr2582-2589. Having demonstrated that Psyr2582-2589 is associated with production of a siderophore, we next sought to determine whether the siderophore was ACR. CAS-reactive material was separated from PVD⁻ cell-free supernatants via organic extraction and silica affinity chromatography. We used the extract from the PVD⁻ strain to ensure that PVD would not be present in the cell extracts. The chromatographed product was able to restore growth of the PVD⁻ ACR⁻ strain in the presence of 2,2'-dipyridyl, indicating that it contains a biologically active siderophore. Analysis of the chromatographed product by electrospray ionization (ESI)-MS detected primary mass ions of 590.15 and 294.57, as expected for the [M-H]⁻¹ and [M-2H]⁻² of ACR (Fig. 4) (36). Siderophores such as ACR that use an α-hydroxy acid derived from citrate to bind iron are often photosensitive and can undergo light-induced decarboxylation/oxidation (27). At much lower levels, we also detected the presence of mass ions for both the ACR photodecomposition product ([M-H]⁻¹ = 544.14) and product derived from the acid-mediated conversion of α-ketoglutarate to succinate

FIG. 3. Growth of siderophore-deficient B728a strains. (A) Growth in MM. (B) Growth in MM containing 2,2'-dipyridyl. Symbols: closed squares, B728a; open circles, strain ADB1005 (PVD⁻); open squares, strain ADB1006 (ACR⁻); X's, strain ADB1007 (PVD⁻ ACR⁻). Trendlines are drawn according to data collected hourly. Data points are shown in 2-h (A) and 4-h (B) intervals for visual clarity.

FIG. 4. ESI-MS spectra of siderophore purified from PVD-deficient B728a. The observed mass ions are consistent with ACR ($[M-H]^{-1} = 590.14$; $[M-2H]^{-2} = 294.57$). amu, atomic mass unit.

($[M-H]^{-1} = 562.15$) in the mass spectra (data not shown), providing strong evidence that the parent mass ion is ACR. The very low abundance of mass ions unrelated to ACR present in the ESI-MS spectra suggested that further purification of ACR was not necessary. Consistent with this, further resolution of silica-chromatographed, CAS-reactive material from the PVD⁻ strain separated by reverse-phase high-performance liquid chromatography yielded a single CAS-reactive fraction that produced the same ESI-MS spectra (data not shown). The mass ions for ACR, the ACR photodecomposition product, and other ACR decomposition products were absent from a preparation isolated in parallel from the PVD⁻ ACR⁻ strain. The parallel preparation was also unable to restore the growth of the PVD⁻ ACR⁻ strain in media containing 2,2'-dipyridyl.

Since ACR is produced by both B728a and *D. dadantii*, a comparison of the genes associated with ACR biosynthesis in the two ACR producers could lend insight into similarities and differences in ACR biosynthesis and utilization. The gene cluster responsible for ACR biosynthesis in B728a has many similarities to the characterized ACR gene cluster from *D. dadantii* (13, 14) (Fig. 5). The encoded gene products from each of these two organisms are between 64% and 75% identical. In terms of arrangement, the two clusters are highly syntenic with the exception of two notable differences. In *D. dadantii*, *acsF* and *acsD* are separated by a gene, *acr*, which codes for the ACR receptor, while in B728a, the *acr* gene is absent and *acsF* and *acsD* are adjacent to each other. A gene present immediately upstream of *acsF* in B728a but absent in *D. dadantii* is predicted to encode a siderophore receptor, but the encoded gene product shares only 26% identity to Acr. Recent work on siderophore receptor specificity has shown that both FatB and FpuA, two siderophore receptors encoded by the same strain of *B. subtilis*, can recognize and internalize ferric-petrobactin complexes, despite sharing only 26% identity (47). Therefore, it is reasonable to propose that Psyr2582 functions as a receptor for ferric-ACR complexes analogous to the role Acr serves in *D. dadantii*.

A second difference between the two clusters associated with ACR biosynthesis may be related to the fate of the internalized ferric-ACR complex. In both systems, *cbrD* is followed by a gene of unknown function structurally similar to the *menG* family of menaquinone methyltransferases. However, in B728a there is an open reading frame (ORF) between *cbrD* and the *menG* homologue that is not present in *D. dadantii*. This ORF, Psyr2594, is predicted to encode a protein containing an atypical [2Fe-2S] cluster. These [2Fe-2S] cluster proteins are often

FIG. 5. (A) Schematic representation of the Psyr2580-2595 region of the *P. syringae* pv. *syringae* B728a chromosome. Proposed gene nomenclature is shown above the respected ORFs, followed by a four-digit Psyr genome locus tag. (B) Schematic representation of the region of the *D. dadantii* 3937 chromosome associated with ACR production and transport. Gene nomenclature used is as outlined in Franza et al. (14) and is listed above the respective ORFs. Coding regions involved in ACR biosynthesis and utilization are shown with open arrows.

associated with efficient iron reduction and release from internalized ferric-siderophore complexes, as exemplified by the *E. coli* protein PhuF (30, 35). Therefore, the cluster found in B728a may provide a mechanism for more-efficient iron recovery from internalized ferric-ACR complexes than is provided in the cluster from *D. dadantii*. Further characterization of ACR utilization will be required to test this hypothesis.

AcsA, AcsC, and AcsD encode a minimal synthetase for production of ACR. Analysis of the proteins encoded by the ACR biosynthesis gene cluster of B728a identified three enzymes (AcsA, AcsC, and AcsD) belonging to the NIS class of siderophore synthetases that have homologs in *D. dadantii* ACR biosynthesis. A substrate prediction tool for siderophore synthetases based on sequence alignment has recently been proposed by Challis (5). We decided to use in vitro analysis of each of these enzymes to experimentally test the predicted specificity of the siderophore synthetases in the B728a ACR pathway. We independently cloned the genes coding for each putative NIS synthetase into an *E. coli* expression vector, overproduced the associated hexahistidine-tagged proteins in *E. coli*, and purified each protein, using nickel-chelate chromatography (Fig. 6).

To assay for enzymatic activity, we used a method that detects the presence of hydroxamates that form when activated carboxylic acids react with hydroxylamine (28). Each enzyme was assayed for activation of a dicarboxylic acid ACR precursor, either citrate or α -ketoglutarate. We observed that hydroxamates were formed when AcsD was incubated with citrate (Fig. 7A). Using this hydroxamate assay, we determined the kinetic parameters for AcsD recognition of citrate to be $K_m = 1.9 \pm 0.3$ mM and $k_{cat} = 7.4 \pm 0.7$ min⁻¹. These values are similar to values reported for other NIS synthetases (22, 37, 38). The activation of citrate by AcsD was consistent with its initially proposed function to catalyze the condensation between citrate and ethanolamine. As this manuscript was being

FIG. 6. NuPAGE (4 to 15%) Coomassie blue-stained protein gel of N-terminal hexahistidine-tagged AcsA, AcsC, and AcsD. Approximately 4 μ g of protein was loaded in each lane. Calculated molecular masses for tagged proteins are as follows: AcsA, 73.9 kDa; AcsC, 72.6 kDa; AcsD, 69.3 kDa.

prepared, the biochemical and structural analysis of AcsD from *D. dadantii* was published (41). In that study, although ethanolamine was found to be a substrate for AcsD, it was argued that based on kinetic data, L-serine was recognized preferentially over ethanolamine substrate. Thus, the decarboxylation of L-serine would occur after the AcsD-catalyzed reaction. Investigations into the timing of this decarboxylation reaction will be discussed below.

We also detected hydroxamate formation by AcsA, but only when α -ketoglutarate was incubated with the enzyme (Fig. 7B). These data are consistent with the proposal that AcsA catalyzes the activation of α -ketoglutarate and its condensation with [4] (Fig. 2). We were unable to determine the kinetic parameters for α -ketoglutarate activation because of the limit of detection for the formation of α -ketoglutaryl- δ -hydroxamate.

The failure to detect AcsC-dependent hydroxamate formation at this stage was expected, since the substrate for this enzyme is proposed to be [3] (Fig. 2). In order to determine whether this hypothesis was correct, reaction mixtures consisting of AcsD, citrate, and either ethanolamine or 2,4-diaminobutyrate were set up. After an extended incubation at room temperature, AcsD was heat inactivated and the resulting reaction mixture was incubated with AcsC. Hydroxamate formation was detected only when the AcsC reaction mixture contained ethanolamine (Fig. 7C). These data are consistent with AcsD having the ability to catalyze the formation of [3] and

FIG. 7. Substrate specificity determination of Acs enzymes. Enzymes were incubated with hydroxylamine, Mg^{2+} -ATP, and carboxylic acid substrates and assayed for production of hydroxamates. (A) Results for reaction mixture containing citrate. (B) Results for reaction mixture containing α -ketoglutarate. (C) Results for reaction mixture containing reaction mixture of AcsD incubated with citrate and ethanolamine (left) or with citrate and 2,4-diaminobutyrate (right). mAbs₅₄₀, absorbance at 540 nm (10^3).

AcsC catalyzing the activation of [3] and with subsequent condensation with 2,4-diaminobutyrate.

To support our hypothesis that ethanolamine can be used directly in the synthesis of ACR, we incubated α -ketoglutarate, citrate, 2,4-diaminobutyrate, ethanolamine, and Mg^{2+} -ATP with combinations of one, two, or all three Acs enzymes and analyzed the products by LC-MS (Fig. 8). The data clearly show that incubation with AcsD is necessary and sufficient to produce a mass-ion product consistent with [3] (Fig. 8A, spectra iii and v). The addition of AcsC to reactions containing AcsD leads to a loss of [3] and the appearance of a new mass-ion product consistent with [4] (Fig. 8B, spectra vi and vii). Finally, incubation with all three enzymes yields new mass-ion products consistent with α -ketoglutaryl-diaminobutyryl-citryl-ethanolamine (compound [5]), diaminobutyryl-citryl-ethanolamino- α -ketoglutarate (compound [6]) (Fig. 8C, spectrum vii), and ACR (Fig. 8D, spectrum vii). The mass ion corresponding to the decarboxylated photoproduct of ACR is present only in the reaction mixture containing all three enzymes, further supporting our argument that ACR is synthesized in this reaction (data not shown). Taken together, these data strongly suggest that ethanolamine can replace serine during ACR synthesis.

To further validate that AcsA, AcsC, and AcsD constitute a minimal ACR synthetase, we investigated whether the products of this *in vitro* reaction can function as a biologically relevant siderophore *in vivo*. AcsA, AcsC, and AcsD were incubated with α -ketoglutarate, citrate, 2,4-diaminobutyrate, ethanolamine, and Mg^{2+} -ATP. Filtrates of this reaction mix-

FIG. 8. Summary of LC-MS data. "A," "C," and "D" represent AcsA, AcsC, and AcsD, respectively. A plus sign (+) indicates that the corresponding enzyme was included in the incubation. (A) MS spectra focused on mass range for detection of [3] (calc. $[M-H]^{-1} = 234.06$). (B) MS spectra focused on mass range for detection of [4] (calc. $[M-H]^{-1} = 334.13$). (C) MS spectra focused on mass range for detection of [5] or [6] (calc. $[M-H]^{-1} = 462.14$). (D) MS spectra focused on mass range for detection of ACR (calc. $[M-H]^{-1} = 590.14$). MS data were collected from LC separation at 0.74 min, 0.65 min, 1.85 min, and 4.70 min, respectively, which correspond to the optimal extracted ion signal for the desired mass ion. Each data set was normalized to the intensity of the most-prevalent mass ion present. amu, atomic mass unit.

ture were able to restore growth to the PVD^-ACR^- strain grown in defined media containing 2,2'-dipyridyl. Reaction mixtures lacking any of these enzymes, precursors, or cosubstrates were unable to restore growth (data not shown), indicating that this combination of enzymes and substrates is both necessary and sufficient to synthesize a biologically relevant siderophore.

Having established that functional ACR can be assembled in vitro, we investigated what would occur if L-serine replaced the

ethanolamine in the ACR synthesis reaction. Since the known structure of ACR contains ethanolamine as one of its subunits, a decarboxylation step must occur if L-serine is to be utilized as the biologically relevant nucleophile. This decarboxylation could occur to one of the precursors formed during ACR biosynthesis or to a fully assembled, carboxylated version of ACR. In order to investigate the timing of L-serine decarboxylation during ACR biosynthesis, we incubated α -ketoglutarate, citrate, 2,4-diaminobutyrate, L-serine, and Mg^{2+} -ATP

FIG. 9. Summary of LC-MS analysis of reactions where L-serine has replaced ethanolamine as a substrate. The AcsA-catalyzed steps and the associated LC-MS mass ion spectra are shown. A mass ion of 7 was also detected (data not shown). The MS spectra for the range of m/z appropriate for the detection of α -ketoglutaryl-diaminobutyryl-citryl-serine and carboxylated ACR are shown. amu, atomic mass unit.

with all three Acs enzymes and analyzed the products by LC-MS (Fig. 9). Mass ions corresponding to *O*-citryl-serine and diaminobutyryl-citryl-serine (compound [7]) were readily identified in the LC-MS, indicating that decarboxylation of serine to ethanolamine is not a prerequisite for recognition by either AcsD or AcsC. In contrast, while there is an abundance of mass ions corresponding to α -ketoglutaryl-diaminobutyryl-citryl-serine (compound [8]), no mass ions corresponding to carboxylated ACR (compound [9]) are identifiable (Fig. 9). This suggests that decarboxylation is not a prerequisite for recognition by AcsA, but AcsA will condense only one equivalent of α -ketoglutarate to the 2,4-diaminobutryl moiety of [7]. The second α -ketoglutarate moiety cannot be added until after the L-seryl moiety is decarboxylated. While these experiments do not preclude that serine decarboxylation can occur at earlier stages, they do show that serine decarboxylation must occur prior to the final step of ACR biosynthesis.

The in vitro siderophore synthesis system can be modified to produce structural analogs of ACR. Having demonstrated that we can synthesize a functional siderophore in vitro using purified enzymes, we probed the specificity of these enzymes by substituting substrate analogs for the normal substrates in the reaction mixtures. Filtered reaction mixtures were added to cultures of the PVD[−] ACR[−] strain grown in MM containing 2,2′-dipyridyl to determine if any analog was able to restore growth to this strain. While reaction mixtures containing analogs of citrate (*trans*-aconitate, isocitrate, and β -ketoglutarate), diaminobutyrate (L-diaminopropionate, L-ornithine, L-lysine, D-lysine, and L-homoserine), and α -ketoglutarate (oxaloacetate and succinate) were tested, all failed to generate a molecule that restored growth to the PVD[−] ACR[−] strain in the presence of 2,2′-dipyridyl (data not shown). In contrast, reaction mixtures containing certain analogs of ethanolamine were able to restore growth (Table 3). The reaction mixtures that successfully restored growth were those that had either ethylene diamine or 1,3-diaminopropane in place of ethanolamine. Since ACR analogs may be synthesized in vitro but may be incapable of biologically relevant iron transport, the ethanolamine analog reactions were separately analyzed by LC-MS to detect mass ions associated with the production of any structural analogs. As shown in Table 3, mass ions for the ACR analogs that incorporated ethylene diamine and 1,3-diaminopropane were consistent with the theoretical mass of the proposed analog. None of the other reaction mixtures resulted in the formation of products with the expected mass-ion peaks,

suggesting that the substrate analogs are not recognized by AcsD.

Variability in the incorporation of ethanolamine structural analogs appears to be tolerated, and certain ACR analogs are capable of restoring growth to a PVD[−] ACR[−] strain grown in iron-sequestered media. It is understandable that ethylene diamine can functionally replace ethanolamine during ACR synthesis, as the two substrates, once condensed with citrate, differ only in the identity of the atom involved in bond formation, i.e., an oxygen from ethanolamine or a nitrogen from ethylene diamine. Furthermore, staphyloferrin B, a siderophore produced by *Staphylococcus aureus* and *Ralstonia solanacearum* that is structurally related to ACR, contains an ethylene diamine-citrate condensate as one structural element (3, 11, 18). Interestingly, clusters of genes in *R. solanacearum* and *S. aureus* that contain homologs of *acsABCDE* have been identified and have been proposed to be involved in the production of staphyloferrin B (9). These clusters also contain homologues of *vioB* and *vioK* that are associated with the formation of 2,3-diaminopropionate in the context of viomycin biosynthesis (46). Decarboxylation of 2,3-diaminopropionate, potentially by the homologue of *acsE*, would yield a source of ethylene diamine. Therefore, incorporation of ethylene diamine into the structure of an NIS synthetase-generated siderophore analog to that of ACR has already been identified.

The ability of 1,3-diaminopropane to functionally replace ethanolamine was unexpected, as its incorporation adds an extra methylene unit between citrate and one of the equivalents of α -ketoglutarate, potentially altering the positioning of one of the α -hydroxy acids involved in ferric iron coordination. Given the results of 1,3-diaminopropane incorporation, it is

TABLE 3. Summary of ACR analog data

Ethanolamine analog	Mass [M-H] ^{−1} observed ^a (theoretical mass)	Restores growth ^b
Ethylene diamine	589.16 (589.17)	+
Ethylene glycol	ND (591.14)	−
β -Mercaptoethanol	ND (607.12)	−
3-Amino-1-propanol	ND (604.17)	−
1,3-Diaminopropane	603.18 (603.19)	+
L-Serine	ND (634.14)	−

^a ND, a mass was not detected.

^b A plus sign indicates a sample capable of restoring growth to a PVD[−] ACR[−] strain grown in iron-sequestered defined media to an OD₆₀₀ reading of at least 0.2 within 24 h. A negative sign indicates that no growth was observed after 24 h.

surprising that 3-amino-1-propanol, which more closely resembles ethanolamine than does 1,3-diaminopropane, does not appear capable of functionally replacing ethanolamine. Mass ions corresponding to 3-amino-1-propanol-containing analogs of [4] and [5] were not found in the mass spectra, suggesting that ACR analog intermediates are not formed in reactions containing 3-amino-1-propanol. In contrast, for reaction mixtures containing the precursor 1,3-diaminopropane, mass ions corresponding to 1,3-diaminopropane-containing analogs of [4] and [5] were present.

Summary. In this work, we presented genetic and biochemical evidence that demonstrates that B728a can produce two siderophores, PVD and ACR, and that possession of at least one of these pathways is required for growth under iron-limiting conditions. We showed that purified enzymes associated with ACR biosynthesis, AcsA, AcsC, and AcsD, condense citrate, ethanolamine, 2,4-diaminobutyrate, and α -ketoglutarate in vitro into ACR. This work establishes the enzymatic roles AcsA and AcsC play in ACR biosynthesis and support the finding that AcsD catalyzes the first condensation step in ACR biosynthesis in *D. dadantii*. Replacement of ethanolamine with L-serine resulted in the formation of carboxylated ACR intermediates but did not result in the formation of carboxylated ACR or any biologically active siderophore. Thus, decarboxylation of L-serine must occur prior to the final step in ACR biosynthesis. Interestingly, the replacement of ethanolamine with ethylene diamine or 1,3-diaminopropane for in vitro synthesis reactions resulted in the formation of ACR analogs that were biologically active.

It is not clear at this time what benefit the possession of independent siderophore biosynthesis pathways confers to *P. syringae* pv. *syringae* B728a. In an African violet model, *D. dadantii* requires production of both ACR and chrysobactin for full virulence (14). Furthermore, it appears that each siderophore is preferentially employed at a different stage of infection. *D. dadantii* seems to rely on ACR for iron acquisition while growing as an epiphyte and shifts toward production of chrysobactin once infection has been established and symptoms develop. A similar situation may occur with *P. syringae* pv. *syringae* B728a, for which it was observed that in situ production of PVD did not occur with bacteria growing epiphytically (and asymptotically) on plant leaves (29). One can imagine that the epiphytic bacteria are acquiring iron via an ACR-dependent pathway and reserve production of PVD for iron acquisition during active infection. Alternatively, siderophore production in general may not confer a significant advantage to growth in planta. Indeed, a strain of *D. dadantii* that is unable to produce either ACR or chrysobactin is still competent to initiate and maintain infection, albeit with a reduction in efficiency (14). Metabolic intermediates such as citrate and nicotinamide are relatively prevalent in the phyllosphere. These compounds are able to mobilize ferric iron directly and may be sufficient to provide biologically relevant amounts of iron in a siderophore-independent fashion (14).

To date, the genomes of six strains of *P. syringae* have been sequenced and are available for analysis (B728a [13], *P. syringae* pv. *tomato* DC3000 [4], *P. syringae* pv. *tomato* T1 [1], *P. syringae* pv. *phaseolicola* 1448A [21], *P. syringae* pv. *oryzae* 1_6 [39], and *P. syringae* pv. *tabaci* ATCC 11528 (accession no. ACHU01000000). Based on comparative genomics, it appears

that each of these strains has the metabolic potential to produce at least two siderophores. All six strains have the potential to produce PVD, five have the potential to produce ACR, and half have the potential to produce yersiniabactin. This redundancy suggests a competitive benefit to the production of lower-affinity siderophores at a lower metabolic cost during asymptomatic growth while maintaining the potential to produce higher-affinity, higher-cost siderophores during infection.

ACKNOWLEDGMENTS

This work was supported by the Wisconsin Agricultural Experiment Station (project WIS04976 from the United States Department of Agriculture) and the Alfred Toepfer Faculty Fellow Award from the College of Agriculture and Life Sciences of the University of Wisconsin—Madison (M.G.T). A.D.B. was supported, in part, by a Jerome J. Stefaniak Predoctoral Fellowship.

We thank Amy C. Harms of the University of Wisconsin Biotechnology Center for her expert assistance in the mass spectrometry analyses.

REFERENCES

- Almeida, N. F., S. Yan, M. Lindeberg, D. J. Studholme, D. J. Schneider, B. Condon, H. Liu, C. J. Viana, A. Warren, C. Evans, E. Kemen, D. Maclean, A. Angot, G. B. Martin, J. D. Jones, A. Collmer, J. C. Setubal, and B. A. Vinatzer. 2009. A draft genome sequence of *Pseudomonas syringae* pv. *tomato* T1 reveals a type III effector repertoire significantly divergent from that of *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant-Microbe Interact.* **22**:52–62.
- Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* **62**:293–300.
- Bhatt, G., and T. P. Denny. 2004. *Ralstonia solanacearum* iron scavenging by the siderophore staphyloferrin B is controlled by PhcA, the global virulence regulator. *J. Bacteriol.* **186**:7896–7904.
- Buell, C. R., V. Joardar, M. Lindeberg, J. Selengut, I. T. Paulsen, M. L. Gwinn, R. J. Dodson, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, S. Daugherty, L. Brinkac, M. J. Beanan, D. H. Haft, W. C. Nelson, T. Daviden, N. Zafar, L. Zhou, J. Liu, Q. Yuan, H. Khouri, N. Fedorova, B. Tran, D. Russell, K. Berry, T. Utterback, S. E. Van Aken, T. V. Feldblyum, M. D'Ascenzo, W. L. Deng, A. R. Ramos, J. R. Alfano, S. Cartinhour, A. K. Chatterjee, T. P. Delaney, S. G. Lazarowitz, G. B. Martin, D. J. Schneider, X. Tang, C. L. Bender, O. White, C. M. Fraser, and A. Collmer. 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA* **100**:10181–10186.
- Challis, G. L. 2005. A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases. *ChemBiochem* **6**:601–611.
- Cody, Y. S., and D. C. Gross. 1987. Characterization of pyoverdinin_{PSB}, the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. *Appl. Environ. Microbiol.* **53**:928–934.
- Cotton, J. L., J. Tao, and C. J. Balibar. 2009. Identification and characterization of the *Staphylococcus aureus* gene cluster coding for staphyloferrin A. *Biochemistry* **48**:1025–1035.
- Crosa, J. H., and C. T. Walsh. 2002. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiol. Mol. Biol. Rev.* **66**:223–249.
- Dale, S. E., A. Doherty-Kirby, G. Lajoie, and D. E. Heinrichs. 2004. Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. *Infect. Immun.* **72**:29–37.
- de Lorenzo, V., and J. B. Neilands. 1986. Characterization of *iucA* and *iucC* genes of the aerobactin system of plasmid ColV-K30 in *Escherichia coli*. *J. Bacteriol.* **167**:350–355.
- Drechsel, H., S. Freund, G. Nicholson, H. Haag, O. Jung, H. Zahner, and G. Jung. 1993. Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci. *Biochim. Biophys. Acta* **1185**:185–192.
- Drechsel, H., and G. Jung. 1998. Peptide siderophores. *J. Pept. Sci.* **4**:147–181.
- Feil, H., W. S. Feil, P. Chain, F. Larimer, G. DiBartolo, A. Copeland, A. Lykidis, S. Trong, M. Nolan, E. Goltsman, J. Thiel, S. Malfatti, J. E. Loper, A. Lapidus, J. C. Detter, M. Land, P. M. Richardson, N. C. Kyrpides, N. Ivanova, and S. E. Lindow. 2005. Comparison of the complete genome sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA* **102**:11064–11069.
- Franza, T., B. Mahe, and D. Expert. 2005. *Erwinia chrysanthemi* requires a second iron transport route dependent of the siderophore achromobactin for extracellular growth and plant infection. *Mol. Microbiol.* **55**:261–275.

15. Frigaard, N.-U., H. Li, K. J. Milks, and D. A. Bryant. 2004. Nine mutants of *Chlorobium tepidum* each unable to synthesize a different chlorosome protein still assemble functional chlorosomes. *J. Bacteriol.* **186**:646–653.
16. Gibson, J., C. D. Upper, and I. C. Gunsalus. 1967. Succinyl coenzyme A synthetase from *Escherichia coli*. I. Purification and properties. *J. Biol. Chem.* **242**:2474–2477.
17. Gupta, S. C., and E. E. Dekker. 1984. Maly-CoA formation in the NAD⁺, CoASH⁻, and α -ketoglutarate dehydrogenase-dependent oxidation of 2-keto-4-hydroxyglutarate. Possible coupled role of this reaction with 2-keto-4-hydroxyglutarate aldolase activity in a pyruvate-catalyzed cyclic oxidation of glyoxylate. *J. Biol. Chem.* **259**:10012–10019.
18. Haag, H., H. P. Fiedler, J. Meiwes, H. Drechsel, G. Jung, and H. Zahner. 1994. Isolation and biological characterization of staphyloferrin B, a compound with siderophore activity from staphylococci. *FEMS Microbiol. Lett.* **115**:125–130.
19. Hersh, L. B. 1973. Malate adenosine triphosphate lyase. Separation of the reaction into a malate thiokinase and maly coenzyme A lyase. *J. Biol. Chem.* **248**:7295–7303.
20. Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**:77–86.
21. Joardar, V., M. Lindeberg, R. W. Jackson, J. Selengut, R. Dodson, L. M. Brinkac, S. C. Daugherty, R. Deboy, A. S. Durkin, M. G. Giglio, R. Madupu, W. C. Nelson, M. J. Rosovitz, S. Sullivan, J. Crabtree, T. Creasy, T. Davidson, D. H. Haft, N. Zafar, L. Zhou, R. Halpin, T. Holley, H. Khouri, T. Feldblyum, O. White, C. M. Fraser, A. K. Chatterjee, S. Cartinhour, D. J. Schneider, J. Mansfield, A. Collmer, and C. R. Buell. 2005. Whole-genome sequence analysis of *Pseudomonas syringae* pv. phaseolicola 1448A reveals divergence among pathovars in genes involved in virulence and transposition. *J. Bacteriol.* **187**:6488–6498.
22. Kadi, N., D. Oves-Costales, F. Barona-Gomez, and G. L. Challis. 2007. A new family of ATP-dependent oligomerization-macrocyclization biocatalysts. *Nat. Chem. Biol.* **3**:652–656.
23. Karamanoli, K., and S. E. Lindow. 2006. Disruption of *N*-acyl homoserine lactone-mediated cell signaling and iron acquisition in epiphytic bacteria by leaf surface compounds. *Appl. Environ. Microbiol.* **72**:7678–7686.
24. Kim, B. J., J. H. Park, T. H. Park, P. A. Bronstein, D. J. Schneider, S. W. Cartinhour, and M. L. Shuler. 2009. Effect of iron concentration on the growth rate of *Pseudomonas syringae* and the expression of virulence factors in *hrp*-inducing minimal medium. *Appl. Environ. Microbiol.* **75**:2720–2726.
25. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44**:301–307.
26. Koorts, A. M., and M. Viljoen. 2007. Ferritin and ferritin isoforms I structure-function relationships, synthesis, degradation and secretion. *Arch. Physiol. Biochem.* **113**:30–54.
27. Küpper, F. C., C. J. Carrano, J. U. Kuhn, and A. Butler. 2006. Photoreactivity of iron(III)-aerobactin: photoproduct structure and iron(III) coordination. *Inorg. Chem.* **45**:6028–6033.
28. Lipmann, F., and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl phosphates. *J. Biol. Chem.* **159**:21–28.
29. Loper, J. E., and S. E. Lindow. 1987. Lack of evidence for in situ fluorescent pigment production by *Pseudomonas syringae* pv. *syringae* on bean leaf surfaces. *Phytopathology* **77**:1449–1454.
30. Matzanke, B. F., S. Anemuller, V. Schunemann, A. X. Trautwein, and K. Hantke. 2004. FhuF, part of a siderophore-reductase system. *Biochemistry* **43**:1386–1392.
31. Miethke, M., and M. A. Marahiel. 2007. Siderophore-based iron acquisition and pathogen control. *Microbiol. Mol. Biol. Rev.* **71**:413–451.
32. Miller, M. J., H. Zhu, Y. Xu, C. Wu, A. J. Walz, A. Vergne, J. M. Roosenberg, G. Moraski, A. A. Minnick, J. McKee-Dolence, J. Hu, K. Fennell, E. Kurt Dolence, L. Dong, S. Franzblau, F. Malouin, and U. Mollmann. 2009. Utilization of microbial iron assimilation processes for the development of new antibiotics and inspiration for the design of new anticancer agents. *Biometals* **22**:61–75.
33. Mittal, R., S. Sharma, S. Chhibber, and K. Harjai. 2008. Iron dictates the virulence of *Pseudomonas aeruginosa* in urinary tract infections. *J. Biomed. Sci.* **15**:731–741.
34. Mossialos, D., U. Ochsner, C. Baysse, P. Chablain, J. P. Pirnay, N. Koedam, H. Budzikiewicz, D. U. Fernandez, M. Schafer, J. Ravel, and P. Cornelis. 2002. Identification of new, conserved, non-ribosomal peptide synthetases from fluorescent pseudomonads involved in the biosynthesis of the siderophore pyoverdine. *Mol. Microbiol.* **45**:1673–1685.
35. Müller, K., B. F. Matzanke, V. Schunemann, A. X. Trautwein, and K. Hantke. 1998. FhuF, an iron-regulated protein of *Escherichia coli* with a new type of [2Fe-2S] center. *Eur. J. Biochem.* **258**:1001–1008.
36. Munzinger, M., H. Budzikiewicz, D. Expert, C. Enard, and J. M. Meyer. 2000. Achromobactin, a new citrate siderophore of *Erwinia chrysanthemi*. *Z. Naturforsch. Teil C* **55**:328–332.
37. Oves-Costales, D., N. Kadi, M. J. Fogg, L. Song, K. S. Wilson, and G. L. Challis. 2007. Enzymatic logic of anthrax stealth siderophore biosynthesis: AsbA catalyzes ATP-dependent condensation of citric acid and spermidine. *J. Am. Chem. Soc.* **129**:8416–8417.
38. Oves-Costales, D., N. Kadi, M. J. Fogg, L. Song, K. S. Wilson, and G. L. Challis. 2008. Petrobactin biosynthesis: AsbB catalyzes condensation of spermidine with *N*⁸-citryl-spermidine and its *N*¹-(3,4-dihydroxybenzoyl) derivative. *Chem. Comm.* **34**:4034–4036.
39. Reinhardt, J. A., D. A. Baltrus, M. T. Nishimura, W. R. Jeck, C. D. Jones, and J. L. Dangl. 2009. De novo assembly using low-coverage short read sequence data from the rice pathogen *Pseudomonas syringae* pv. *oryzae*. *Genome Res.* **19**:294–305.
40. Samson, R., J. B. Legendre, R. Christen, M. Fischer-Le Saux, W. Achouak, and L. Gardan. 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder *et al.* 1953) Brenner *et al.* 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *Int. J. Syst. Evol. Microbiol.* **55**:1415–1427.
41. Schmelz, S., N. Kadi, S. A. McMahon, L. Song, D. Oves-Costales, M. Oke, H. Liu, K. A. Johnson, L. G. Carter, C. H. Botting, M. F. White, G. L. Challis, and J. H. Naismith. 2009. AcsD catalyzes enantioselective citrate desymmetrization in siderophore biosynthesis. *Nat. Chem. Biol.* **5**:174–182.
42. Schwyn, B., and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**:47–56.
43. Siström, W. R. 1960. A requirement for sodium in the growth of *Rhodopseudomonas spheroides*. *J. Gen. Microbiol.* **22**:778–785.
44. Spiro, T. G., S. F. Allerton, J. Renner, A. Terzis, R. Bils, and P. Saltman. 1966. The hydrolytic polymerization of iron(III). *J. Am. Chem. Soc.* **88**:2721–2725.
45. Taylor, L. A., and R. E. Rose. 1988. A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. *Nucleic Acids Res.* **16**:358.
46. Thomas, M. G., Y. A. Chan, and S. G. Ozanick. 2003. Deciphering tuberactinomycin biosynthesis: isolation, sequencing, and annotation of the viomycin biosynthetic gene cluster. *Antimicrob. Agents Chemother.* **47**:2823–2830.
47. Zawadzka, A., R. Abergel, R. Nichiporuk, U. Andersen, and K. Raymond. 2009. Siderophore-mediated iron acquisition systems in *Bacillus cereus*: identification of receptors for anthrax virulence-associated petrobactin. *Biochemistry* **48**:3645–3657.