The Phytopathogen \textit{Pseudomonas syringae} pv. tomato DC3000 Has Three High-Affinity Iron-Scavenging Systems Functional under Iron Limitation Conditions but Dispensable for Pathogenesis\textsuperscript{†¶}

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High-affinity iron scavenging through the use of siderophores is a well-established virulence determinant in mammalian pathogenesis. However, few examples have been reported for plant pathogens. Here, we use a genetic approach to investigate the role of siderophores in \textit{Pseudomonas syringae} pv. tomato DC3000 (DC3000) virulence in tomato. DC3000, an agronomically important pathogen, has two known siderophores for high-affinity iron scavenging, yersiniabactin and pyoverdin, and we uncover a third siderophore, citrate, required for growth when iron is limiting. Though growth of a DC3000 triple mutant unable to either synthesize or import these siderophores is severely restricted in iron-limited culture, it is fully pathogenic. One explanation for this phenotype is that the DC3000 triple mutant is able to directly pirate plant iron compounds such as heme/hemin or iron-nicotianamine, and our data indicate that DC3000 can import iron-nicotianamine with high affinity. However, an alternative explanation, supported by data from others, is that the pathogenic environment of DC3000 (i.e., leaf apoplast) is not iron limited but is iron replete, with available iron of >1 μM. Growth of the triple mutant in culture is restored to wild-type levels by supplementation with a variety of iron chelates at >1 μM, including iron(III) dicitrate, a dominant chelate of the leaf apoplast. This suggests that lower-affinity iron import would be sufficient for DC3000 iron nutrition \textit{in planta} and is in sharp contrast to the high-affinity iron-scavenging mechanisms required in mammalian pathogenesis.

Pathogenic microorganisms must acquire all critical nutrients from the host environment in order to survive. Iron nutrition in particular presents a fundamental challenge due to its extremely low solubility in aerobic environments at moderate pHs (\(5 \times 10^{-19}\) M) and further host limitation of available free iron (e.g., \(5 \times 10^{-18}\) M in mammalian fluids [22, 44]). Most bacteria require much higher levels (i.e., \(10^{-6}\) to \(10^{-7}\) M of bioavailable iron) for optimal growth (24) and therefore must solve an iron supply problem for survival. There is abundant evidence that competition for iron is a key virulence determinant in mammalian pathosystems. Iron in human serum is predominantly complexed with transferrin protein (pFe = 10\(^{22}\) M [1]) and is therefore limited from invading pathogens. The human immune system can limit iron further through release of the iron-binding protein lactoferrin at the site of infection (57). Evading these defenses, several mammalian pathogens pirate host-synthesized heme for iron nutrition directly from host fluids (20). Alternatively, pathogenic bacteria can scavenge iron from host fluids using very high-affinity iron carriers termed siderophores that can effectively compete with host iron chelates for iron. Siderophores are low-molecular-weight compounds that are synthesized, exported from the bacterial cell, and then imported once bound to iron (43). As this strategy carries metabolic costs associated with siderophore synthesis and transport, siderophores are typically repressed in iron-replete environments where less-costly and lower-affinity iron import is effective (55). It has long been recognized that siderophores act as key virulence factors for many mammalian pathogens (43, 49). For example, the siderophores yersiniabactin (Ybt) and pyoverdin (Pvd) are required for full virulence of \textit{Yersinia pestis} (3) and \textit{Pseudomonas aeruginosa} (42) infections of mice, respectively.

In contrast to the situation in mammalian pathosystems, the role of microbial iron nutrition in plant pathogenesis is less well understood. Although microbial infection is known to alter plant iron homeostasis at several sites (37), it is not known whether these alterations significantly limit the iron available to invading microorganisms. Furthermore, though siderophore production is critical for full virulence of specific phytopathogens on their hosts, these examples are limited. For instance, fungal siderophores are virulence determinants of pathogenic ascomycetes (e.g., \textit{Cochliobolus heterostrophus} on maize [46]) but not of basidiomycetes such as \textit{Ustilago maydis} (41) and \textit{Microbotryum violaceum} (6). For phytopathogenic bacteria, the importance of siderophores in pathogenesis has been documented for \textit{Erwinia chrysanthemi} (also known as \textit{Dickeya dadantii}) and \textit{Erwinia amylovora} (18), but siderophores do not play a significant role in \textit{Erwinia carotovora} infection of potato (10). In addition, Pvd biosynthetic mutants in distinct \textit{Pseudomonas syringae} pathovars tested on different hosts yield very different results (e.g., [14, 30, 51, 53]). One possible explanation for a lack of virulence phenotype in single-siderophore mutants is the compensatory action of other high-affinity siderophores. However, an alternative hypothesis

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is that siderophore-based high-affinity iron scavenging is not a dominant mechanism of iron nutrition in these systems. DC3000 is an important pathogen of tomato (Solanum lycopersicum) crops that also infects the model plant Arabidopsis thaliana. In a susceptible plant, DC3000 enters the leaf tissue through wounds or natural openings such as the stomata and multiplies in the intercellular spaces (apoplast) to high levels (31). Extensive genetic and genomic resources are available for DC3000, and it has been the subject of highly productive research centered on the mechanisms of plant host susceptibility/resistance and pathogen virulence and avirulence determinants (31). However, little is known regarding its means of iron acquisition in planta. Herein, we build on our previous work (30) showing Ybt is dispensable for DC3000 growth both in iron-limited culture and in planta to determine the complement of DC3000 siderophores required for bacterial iron nutrition in iron-limited culture and whether these siderophores, singly or in combination, may be virulence determinants.

MATERIALS AND METHODS

Bacterial strains and culture. Liquid cultures of P. syringae pv. tomato isolate DC3000 (15), the DC3000 pchA mutant (30), and all other DC3000 strains (this study) were incubated in 100 μl of a defined low-iron MOPS1 medium described previously (30). Cultures were initiated at an optical density at 600 nm (OD600) of 0.1 (5 × 10⁷ CFU/ml) with log-phase cells that were cultured overnight in Luria-Bertani medium supplemented with 20 μM FeCl3 (LB + Fe) and washed three times in MOPS1 medium. Cultures were then incubated in 100 μl MOPS1 at 30°C with shaking at 225 rpm in Falcon Microtest 96-well plates for the times indicated. Chrome azurol S (CAS) assays were carried out as described by Schwyn and Neillands (52). As notated, cultures were supplemented with 2.2'-dipyridyl, citric acid, iron(II) Cl₂, iron(III) Cl₃, salicylic acid, pyoverdin (this study), iron(III) hydroxide, iron(III) citrate, iron(II) diacite, hemin, hemoglobin, or nicotinamide. Iron(II) Cl₃, iron(III) Cl₄, and iron(III) citrate were prepared fresh. Iron(III) hydroxide precipitates were obtained from 1-year-old MOPS1 medium. Iron(III) citrate was quantified in culture supernatants using an enzymatic citrate assay (51). Bacterial culture supernatants were prepared by centrifugation at 20,000 g for 10 min and evaporated to dryness under a vacuum. This sample was then resuspended in 1-ml water, and a dilution was analyzed by HPLC to verify purity and reproducibility of results from experiment to experiment.

Construction of Pch DC3000 mutants. Removal of the pchl gene (PSPTO_2147) in order to create the Pch mutant was accomplished by marker exchange selection. A 5,315-bp genomic fragment containing the pchl gene was amplified using Turbo Pfu DNA polymerase (Strategene) and the primers 5'-CACCCACAGCAGCCAGCTC13-3' and 5'-ACCTGTGTCTGCTGTCG C13-3'. The product was ligated into the pENTR/DTOPO vector (Invitrogen) and confirmed by sequencing. A kanamycin (Kan) resistance cassette was amplified from pCR-BluntII-TOPO vector using Turbo Pfu DNA polymerase (Strategene) and the primers 5'-AGCGCGCCGCGTGTCAAGGG-3' and 5'-ACCTGTGTCTGCTGTCGGC13-3'. The product was ligated into the pENTR/D-TOPO vector (Invitrogen) and confirmed by sequencing. Next, the vector containing the pchl genomic fragment was digested with MluI to remove a 2,671-bp fragment of the pchl gene and then ligated with the MluI-digested Kan cassette. The product was recombined with the pLVC-D vector destination gene (pLVC18 [DNA Plant Technologies, Oakland, CA] with a gateway cassette cloned into the EcoRI site) in a gateway LR reaction (Invitrogen). The resulting pLVC-D vector containing DC3000 genomic DNA flanking the Kan cassette was then mated into DC3000 from Escherichia coli DH5α in a triparental mating. Mated cells were grown on LB + Fe agar with 100 μg/ml rifampin (Rif), 25 μg/ml Kan, and 5 μg/ml tetracycline (Tet), and single colonies were isolated. These colonies retain the pLVC-D vector containing a Tet resistance marker in their genomic DNA, and thus the cultures grown from these colonies were spread onto plates containing Rif and Kan but lacking Tet to allow for loss of plasmid. Replica plating onto plates with Rif, Kan, and Tet revealed Tet-sensitive colonies that had lost the pLVC-D vector and the pchl gene. The absence of the pchl gene was confirmed by PCR using primers 5'-TGTAGATT ATCGTGGCTGCG-3' and 5'-ACCGTTTGCTGTTGAGGC-3'. Removal of the fecA gene (PSPTO_1207) to create the FecA mutant proceeded similarly to removal of the pchl gene. A 4,121-bp genomic fragment containing fecA was amplified with the primers 5'-ACCACCATGCTAAGACCAGTAGC3' and 5'-ACGCGCCGCGTGTCAAGGG-3' and ligated into the pENTR/D-TOPO vector. An EcoRI- and ScaI-digested 1,798-bp fragment of the fecA gene was replaced with a spectinomycin resistance cassette amplified from the pE8R vector (59) with the primers 5'-GAATTCAGCGACCGCAACG-3' and 5'-AGTACTTATTTTGGCAGTAC3-3'. Further steps were as for Pch mutant creation except with spectinomycin in place of Kan. The absence of the fecA gene was confirmed by PCR using primers 5'-ACCACCATGCTAAGACCAGTAGC3' and 5'-ACCGTGCGGCTGTTGAGGC-3'. Higher-order mutants were created in the same manner as single mutants, with mutation of the pchl gene first. Creation of the Ybt mutant through mutation of the pchl gene was described previously (30). The wt fecA gene was restored to FecA mutants on the broad-host-range, gentamicin (Gen)-resistant pBBR1MCS-5 vector (34). The same 4,121-bp genomic fragment containing fecA described above was ligated into the pCR-BluntII-TOPO vector and confirmed by sequencing. A HindIII- and XhoI-digested fragment from this vector was ligated into HindIII-XhoI vector ligated into HindIII-XhoI vector to generate pBBR1MCS-5. The resulting vector carries the full-length fecA gene and promoter and was mobilized into DC3000 mutant strains through triparental mating and selection on the strain-appropriate antibiotics plus Gen.

Siderophore quantification and purification. Initial confirmation of loss of pyoverdin in the Pch mutant was by high-performance liquid chromatography (HPLC) analysis (30). Briefly, 1 ml of low-iron liquid cultures were collected and 1 mM FeCl₃ was added. The mixture was then centrifuged at 20,000 × g for 2 min at 4°C, and 50 μl of supernatant was analyzed by HPLC. The buffer used was 17 mM formic acid (pH 3.35) with an acetonitrile gradient of 0 to 2% (5 to 7 min), 2% to 70% (7 to 20 min), 70% to 80% (20 to 22 min), and 80% to 0% (22 to 27 min). The flow rate was 1 ml/min. Consistent with published work on the atypical pyoverdin (13, 30), multiple distinct peaks were detectable as Pvd by absorbance spectrum in the wt but not in the Pch mutant. For all experiments, a Prevail C₅, 5-μm, particle-size, 150 by 4.6-mm column (Alltech) maintained at 27°C was used on a Shimadzu SCL-10A VP series HPLC system equipped with a Shimadzu SPD-10A VP photodiode array detector. HPLC traces are representative of results of at least three independent samples. For Pvd purification, a wt, very low iron culture grown at 30°C for 3 days was centrifuged at 5,000 × g for 20 min. The supernatant was combined with an equal volume of 0.5 M EDTA, pH 8, in order to chelate free iron from the sample. Three ml of this solution was run through a Supelco SPE C₅ column, washed with water, and eluted with 100% methanol. Fifty μl of the eluate was then analyzed by HPLC, and the Pvd-containing fraction was collected from 10 to 11.5 min and evaporated to dryness under a vacuum. This sample was then resuspended in 100 μl water, and a dilution was analyzed by HPLC to verify purity and quantify Pvd. Pvd was quantified using absorbance at 400 nm and a reported molar extinction coefficient of 22,000 at 400 nm (13).

Citrato was quantified in culture supernatants using an enzymatic citrate assay kit (BioVision) according to the manufacturer’s instructions. DC3000 culture supernatant samples were collected after centrifugation at 20,000 × g for 2 min and then filtered through 10-kDa molecular-size cutoff Microcon spin columns (Millipore) to remove potential protein contaminants. Data are reported as means and standard deviations of results for four replicates and were highly reproducible from experiment to experiment.

Bacterial growth in planta. DC3000 strains for in planta assays were prepared from log-phase LB + Fe cultures washed three times and suspended in 10 mM MgSO₄ solution. Tomato variety VF-36 plants were grown for 5 weeks in a greenhouse and were either vacuum infiltrated or dipped in a solution containing DC3000 wt or mutant strains (three plants per strain). For infiltration, tomato plants were submerged in a 10 mM MgSO₄ solution containing bacteria at a final OD₆₀₀ of 0.0002. A vacuum of 20 to 30 torr for 60 s was used to ensure all leaves were properly infiltrated. For dipping, plants were submerged for 5 s in a 10 mM MgSO₄ solution containing bacteria at a final OD₆₀₀ of 1.0 and supplemented with 0.02% Silwet L-77 (Crompton). At the times indicated, two leaflets from the first leaf with five leaflets were collected from infected tomato plants as well as uninoculated and mock-inoculated control plants. Three leaf discs of a 3-mm radius were collected from each leaflet and ground together in 1 ml of 10 mM MgSO₄ solution with a plastic pestle. Appropriate dilutions were then plated using a spiral plater (Model D: Spiral Systems Instruments, Inc., Bethesda, MD) on LB + Fe agar plates with 100 μg/ml Rif, and bacterial colonies were counted using a laser colony counter (model 500A; Spiral System Instruments, Inc., Bethesda, MD).
Ybt and Pvd play partially overlapping roles in iron acquisition under high-iron conditions (e.g., high-iron plates [Fig. 2B]). Indeed, in contrast to the single mutants, the Ybt mutant displays a significant growth defect in low-iron cultures and plates (Fig. 2). However, in very low iron conditions, the Y− P− double mutant grows similarly to the P− single mutant, indicating that Pvd plays a greater role under these conditions. To assess the iron-scavenging activities of the wt and mutant strains, we used the chrome azurol S (CAS) assay, in which removal of iron from blue-pigmented medium results in a color change to yellow. The high-affinity iron-scavenging activity of a bacterial colony grown on this medium creates a yellow halo proportional in size to the amount of bacterial siderophore. As shown in Fig. 2C, the P− and Y− P− mutants, but not Y−, are severely reduced in iron-scavenging activity, further supporting Pvd as the more important siderophore. Interestingly, some iron-scavenging activity remains in the Y− P− mutant (Fig. 2C), suggesting that DC3000 synthesizes a third, as yet unknown siderophore.
Both Pvd and Ybt siderophores are dispensable for DC3000 pathogenesis. We were previously unable to reveal a role for Ybt in DC3000 pathogenesis (30) and postulated that Pvd compensated for loss of Ybt in pathogenic growth. Here we examine whether loss of Pvd or both Ybt and Pvd impacts DC3000 growth and virulence in a susceptible tomato host. We found that DC3000 growth and virulence in tomato are not significantly altered for Y−, P−, or Y−P− strains tested in leaf pathogenesis assays. At 1 and 3 days postinoculation of susceptible tomato plants by vacuum infiltration with these strains, all strains exhibited similar growth (Fig. 3A), and produced similar disease symptoms (Fig. 3C). Although DC3000 is an intercellular pathogen, it must colonize the apoplast from the leaf surface in the field. It is therefore possible that the epiphytic environment is more iron limited than the apoplast and that Ybt and Pvd have roles in DC3000 epiphytic fitness and leaf colonization. To address this, we also examined growth of these bacterial strains in tomato using a standard leaf dipping assay. Using this assay, we again found that the Y−P− mutant grows similarly to wt DC3000 (Fig. 3B). Thus, though the Ybt and Pvd siderophores are important for growth in iron-limited cultures, they are dispensable for pathogenic growth. This result, along with our finding that siderophore activity is retained by Y−P− DC3000 (Fig. 2C), prompted us to hypothesize and investigate a third mechanism for DC3000 pathogenic iron nutrition, high-affinity scavenging of iron bound to citrate.

**Citrate is a third DC3000 siderophore.** Studies of iron compounds in plant apoplastic fluid have shown that most iron is chelated by citrate, either as iron(III) citrate hydroxide or iron(III) dicitrate chelates (9, 38, 39). In *P. aeruginosa*, a Ybt−Pvd− mutant allowed for identification of the FecA outer membrane transporter and its role in citrate-mediated iron import (40), and high-affinity import of iron(III) dicitrate through FecA is well established in *Escherichia coli* and several other bacteria (7). As the DC3000 genome contains a fecA homolog (PSPTO_1207), DC3000 might pirate iron(III) dicitrate from host fluids. In addition, DC3000 may itself export the metabolite citrate as a siderophore, as does the plant symbiont *Bradyrhizobium japonicum* (25). Though citrate has a lower affinity for iron at neutral pH than siderophores such as Ybt and Pvd (Table 1), it is still considered a high-affinity iron carrier, and it creates a halo when applied to CAS agar plates at sufficient concentrations (data not shown and reference 25). Whether iron-bound citrate is host or DC3000 derived, its high-affinity import would require an outer membrane transporter such as FecA. Therefore, we created a marker exchange deletion of the fecA gene to create a FecA− (F−) mutant.

We found that the F− mutant did not display any significant growth defects in either low-iron or very low iron cultures and plates (Fig. 2). However, the triple Y−P−F− mutant does display more severe growth defects in low-iron and very low iron cultures and plates than the Y−P− double mutant that retains fecA (Fig. 2A and B). As citrate should not be present in our media, this *in vitro* result suggests that DC3000 exports endogenously synthesized citrate for extracellular high-affinity iron scavenging. To address this directly, we measured citrate levels in DC3000 culture supernatant grown under low-, very low, and high-iron conditions. Consistent with its role as a siderophore, citrate levels were dramatically elevated in the supernatant of DC3000 low-iron and very low iron cultures and were undetectable in supernatants from high-iron cultures or culture medium without DC3000 (Fig. 4A). Loss of the FecA importer did not significantly impact citrate accumulation in the culture supernatant (Fig. 4A). Thus, exported citrate is likely responsible for the remaining siderophore activity of the Y−P−F− mutant on CAS agar plates (Fig. 2C).

In order to confirm that DC3000 FecA is functional in iron-citrate import, we supplemented very low iron cultures with 1

**Table 1. Comparison of siderophore effectiveness in binding ferric iron**

| Siderophore          | Source     | Ligand pKa*  | pM (pH 7.4) | Reference |
|----------------------|------------|--------------|-------------|-----------|
| Yersiniabactin       | DC3000     | 11.3, 9.9, 4.8, 3.1, 3.1 | 36.6        | 47        |
| Pyoverdin*<sub>pos</sub> | DC3000     | 12.2, 10.8, −8.5, −7.5, 5.7, 4.8 | 27.1        | 2         |
| Nicotianamine        | Plant      | 10.1, 9.1, 6.9, 2.9 | 20.6        | 56        |
| Citrate              | DC3000, plant | 5.7, 4.35, 2.8 | 16.7        | 26        |

*Fe<sup>3+</sup> binding is expected to be most relevant; Fe<sup>2+</sup> values are also available in most of the cited references.

*<sup>1</sup>Ligand pKa values provide insight into the operational range of the siderophore as deprotonated ligands are readily available for metal binding.

<sup>2</sup>pM, proton-independent equilibrium constant, pM = −log [free metal ion] allows for relative comparison of siderophores under standard conditions (pH 7.4, Fe<sup>3+</sup> = 10⁻⁶ M, ligand = 10⁻⁵ M). A larger pM denotes a more effective iron-complexing agent. This calculation takes into account the effects of ligand protonation, chelate protonation/hydrolysis, and the formation of a mixture of complexes with differing metal:ligand stoichiometry (26).
mM citrate, a condition that would result in most iron being complexed in the iron(III) dicitrate form. This treatment improves iron-limited growth of the $Y^-P^-F^-$ triple mutant but not the $Y^-P^-F$ triple mutant at early time points when DC3000 exported citrate is still limited (Fig. 4B). Taken together, our results indicate that citrate is secreted by DC3000 to form iron(III) dicitrate chelates for high-affinity import by FecA. Comparison of bacterial growth for the single $Y^-$, $P^-$, and $F^-$ mutants, the double $Y^-P^-$, $Y^-F^-$, $P^-F^-$ mutants, and the triple mutant indicates the relative importance of these siderophores for iron-limited growth in culture with Pvd > Ybt and citrate (via FecA). However, high-affinity iron scavenging via Ybt and citrate can play an important role, as only the triple $Y^-P^-F^-$ mutant has a severe growth defect in vitro when iron is limiting (Fig. 2A and B). Thus, high-affinity iron scavenging by Ybt, Pvd, or citrate is required for DC3000 growth in vitro when iron is limiting.

**Mutant complementation.** In order to confirm that the low-iron growth defects of the $Y^-P^-F^-$ triple mutant are due to loss of Ybt, Pvd, and FecA and the resultant reduction in iron acquisition, we complemented the growth defect of the triple mutant in several different ways. We previously showed that exogenous salicylic acid (SA) restores Ybt synthesis in the $Y$ mutant (the $Y$ mutation abrogates the synthesis of the SA intermediate in Ybt synthesis) (30). Thus, SA should restore Ybt to the triple mutant and allow for improved growth in low-iron culture. Indeed, growth of the $Y^-P^-F^-$ triple mutant can be complemented to the level of the $P^-F^-$ double mutant that retains Ybt through the addition of 10 $\mu$M SA (Fig. 5A), demonstrating Ybt function in very low iron growth. Figure 5B shows that supplementation with exogenous Pvd rescues the growth defect of the $Y^-P^-F^-$ triple mutant compared to the $Y^-F^-$ double mutant that retains Pvd (Fig. 5B), demonstrating Pvd function in very low iron growth. Finally, a wt copy of the fecA gene under the control of the native promoter was delivered to the triple mutant on the pBBR1MCS-5 broad-host-range vector. This resulted in a triple $Y^-P^-F^-$ mutant complemented for fecA ($Y^-P^-F^-$). Growth of this strain is fully complemented in low-iron and very low iron cultures (Fig. 5C), confirming that FecA function is also important for iron-limited growth. Iron supplied as 10 $\mu$M FeCl$_3$ rescues the growth defects of all strains, demonstrating that the phenotypes are due to iron deficiencies (Fig. 5).

DC3000 does not require high-affinity iron scavenging through use of Ybt, Pvd, or citrate siderophores for DC3000 growth in planta. If high-affinity iron acquisition of iron(III) dicitrate through the FecA importer occurred, it could mask
the functions of Ybt and Pvd in pathogenic iron nutrition and explain the lack of pathogenesis phenotypes of the Y                                           

P F mutant and the double Y F P F DC3000 strains. We found the triple mutant grows similarly in planta to the wt, the single mutant, and the double Y F P F DC3000 strains. We found the triple mutant grows similarly in planta to the wild-type, the single mutant, and the double Y F P F DC3000 strains. 

We previously determined that DC3000 synthesizes the siderophores yersiniabactin and pyoverdin when grown under iron-limiting conditions (30), and we now show that DC3000 also synthesizes and exports citrate under iron-limiting conditions (Fig. 4). Furthermore, we reveal that DC3000 utilizes these functionally overlapping siderophores for high-affinity iron scavenging when iron is limiting in culture (Fig. 2). At lower pHs, such as those of the tomato leaf apoplast (pH 5.0 to 6.0 [29]), citrate is an effective iron carrier and could be a preferred DC3000 siderophore (Table 1). In addition, apoplastic leaf iron is mostly in the form of iron-citrate chelates (9, 38, 39). Therefore, we anticipated that DC3000 high-affinity iron import of iron(III) citrate through FecA would be important to DC3000 iron nutrition, growth, and virulence. We were initially surprised to find that this was not the case. In planta growth of and visible disease symptoms caused by the Y F P F triple mutant were similar to those of the DC3000 Y F P F mutant retains further iron import mechanisms. Our finding that three functional high-affinity iron-scavenging systems are dispensable for growth in planta led us to consider alternative possibilities for the mechanism(s) of pathogenic iron nutrition. It is possible that DC3000 scavenes plant iron carriers with high affinity other than citrate, for example heme/hemin and the plant siderophore nicotianamine. We found that the DC3000 genome encodes the components of a possible high-affinity heme/hemin scavenging system (PSPTO_1283-1284 and PSPTO_3256–3258). However, when Y F P F and Y F P F mutants are supplemented with 5 mM iron as either of two heme/hemin sources, growth remains iron limited and the Y F P F mutant growth is not complemented to Y F P levels (Fig. 6A). Similarly, 5 mM nicotianamine (supplied without additional iron) did not complement the growth defect of the triple high-affinity acquisition mutant in culture. However, 100 mM nicotianamine did complement growth of the triple Y F P F mutant to double Y F P levels (Fig. 6A). This suggests that citrate-bound iron can be scavenged by nicotianamine and imported with high affinity by DC3000. In contrast, even 100 mM hemin did not restore Y F P F growth to Y F P levels (data not shown). 

Alternatively, low-affinity iron import may be sufficient for DC3000 pathogenic iron acquisition. To assess whether DC3000 lower-affinity import systems are competent to utilize a variety of physiologically relevant iron sources, we supplemented the Y F P F and Y F P F mutants grown in low-iron cultures with 5 mM iron(III) Cl₃, iron(III) hydroxide precipitates, iron(II) Cl₂, iron(III) citrate hydroxide, and iron(III) citrate dicitrate (Fig. 6B). In all cases, both strains are rescued for iron limitation and Y F P F mutant growth is complemented to Y F P mutant levels. To further define the range of supplemented iron required to complement DC3000 mutant growth in low-iron cultures to wild-type levels, we added 0.5, 1, 2.5, and 5 mM iron(III) dicitrate, a dominant iron chelate of the apoplast. Supplementation with 0.5 mM iron(III) dicitrate stimulates growth of all strains but is not sufficient to complement growth of the triple mutant (Fig. 6C). This indicates that high-affinity import of iron(III) dicitrate through FecA contributes significantly to iron acquisition at this iron concentration and that it is more effective than remaining lower-affinity import systems. With 1 mM supplemented iron, the triple mutant grows almost as well as the double mutant. At 2.5 mM added iron(III) dicitrate, both strains grow equally well (Fig. 6C) and at levels similar to that of the wt. Taken together, these results indicate that lower-affinity import system(s) present in DC3000, including those for iron-citrate chelates, are able to efficiently acquire iron for growth if available iron is present in low-micromolar amounts (>1 mM).

**DISCUSSION**

**DC3000 iron acquisition.** We previously determined that DC3000 synthesizes the siderophores yersiniabactin and pyoverdin when grown under iron-limiting conditions (30), and we now show that DC3000 also synthesizes and exports citrate under iron-limiting conditions (Fig. 4). Furthermore, we reveal that DC3000 utilizes these functionally overlapping siderophores for high-affinity iron scavenging when iron is limiting in culture (Fig. 2). At lower pHs, such as those of the tomato leaf apoplast (pH 5.0 to 6.0 [29]), citrate is an effective iron carrier and could be a preferred DC3000 siderophore (Table 1). In addition, apoplastic leaf iron is mostly in the form of iron-citrate chelates (9, 38, 39). Therefore, we anticipated that DC3000 high-affinity iron import of iron(III) dicitrate through FecA would be important to DC3000 iron nutrition, growth, and virulence. We were initially surprised to find that this was not the case. In planta growth of and visible disease symptoms caused by the Y F P F triple mutant were similar to those of...
wt DC3000 using both vacuum infiltration and dipping inoculation methods (Fig. 3). This result cannot be readily explained by the presence of an additional DC3000 siderophore, as the DC3000 genome does not include additional siderophore biosynthetic pathways, including recently elucidated pathways (e.g., staphyloferrin B [12], achromobactin [4]). Furthermore, it is unlikely that this lack of an in planta growth defect is due to compensatory synthesis of Ybt or Pvd. We previously established that plant-produced SA was unable to complement the Y⁻ mutant in planta: Ybt could be only be detected by liquid chromatography-mass spectrometry (LC-MS) in plant extracts from wild-type DC3000-infected leaves and not Y⁻-infected leaves (30). As for Pvd, we have never observed Pvd to be produced in P⁻ mutants under any condition, and the few DC3000 orphan nonribosomal peptide synthetases not involved in the synthesis of known products are not predicted to have the functionality of Pvd. However, there remain two other explanations for the lack of in planta phenotype observed for the triple mutant. First, DC3000 could specifically pirate other plant-produced iron chelates such as heme/hemin or iron-nicotianamine from the leaf apoplast through high-affinity import systems. Second, DC3000 could acquire iron or iron chelates through low-affinity import systems from the iron-replete leaf apoplast.

High-affinity iron scavenging of plant-produced iron chelates other than iron-citrate. Although the DC3000 genome appears to contain the necessary genetic components for high-affinity heme/hemin import, even high levels of iron as heme/hemin neither rescue Y⁻ P⁻ F⁻ iron-limited growth nor complement Y⁻ P⁻ F⁻ growth to levels of other strains in iron-limited culture (Fig. 6A). A heme/hemin import system has been described for the nitrogen-fixing symbiont of soybeans Bradyrhizobium japonicum (45). In vitro, the TonB-dependent heme receptor HmuR was expressed under iron-limiting but not replete conditions and required for heme/hemin import; however, it was not essential for symbiotic nitrogen fixation (45). Though we might have expected the DC3000 heme/hemin import system to be expressed, and therefore functional, under our iron-limiting conditions in vitro, we cannot exclude its potential involvement in pathogenic iron acquisition. Importantly, 100 μM nicotianamine did complement growth of the triple Y⁻ P⁻ F⁻ mutant to double Y⁻ P⁻ mutant levels (Fig. 6A). This indicates that DC3000 can acquire iron-nicotianamine with high affinity and could allow for the future identification of bacterial systems for import and utilization of iron-nicotianamine.

As DC3000 exports mM levels of citrate (~1.2 mM) under iron limitation, high-affinity iron scavenging through nicotianamine would compete with citrate, and this could explain the relatively high level of nicotianamine (100 μM) required to complement growth of the Y⁻ P⁻ F⁻ mutant to Y⁻ P⁻ levels. These concentrations of citrate and nicotianamine have been reported in plant fluids (e.g., in reference 56), but citrate is predicted to be greatly favored over nicotianamine as the iron ligand at leaf apoplastic pH (50, 56). Though iron acquisition via nicotianamine is not likely to be critical for bacteria such as DC3000 that colonize the acidic leaf apoplast, a localized, more-alkaline pH could make iron nicotianamine import a viable option for iron acquisition from host plants. Also, bacterial acquisition of iron via phytosiderophores may be important for other plant-associated bacteria. For example, phytosiderophores very similar to nicotianamine are secreted from the roots of iron-deficient grasses (56) and thus could be an important source of iron for rhizospheric bacteria. In any case, high-affinity iron acquisition through Ybt, Pvd, citrate, heme/hemin, or nicotianamine is not required for DC3000 growth when iron concentrations are >1 μM (Fig. 6B and C).

Leaf apoplastic iron and low-affinity iron acquisition. A variety of direct and indirect measurements agree that apoplastic iron is over 1 μM (11, 19, 35, 38, 39, 48). For example, iron in tomato apoplastic fluids was measured to be 2.2 to 4.2 μM after collection by direct centrifugation (19). At these concentrations of iron, less-well-understood lower-affinity iron import systems are responsible for importing nutritional iron, potentially at a lower energetic cost (21, 23). We show that DC3000 could readily utilize a number of different iron sources supplied at these levels (Fig. 6B). One likely mode of low-affinity iron import would involve transport of iron or low-molecular-weight iron chelates (e.g., iron-citrate chelates) into the periplasm through an outer membrane porin. This step would rely on diffusion and thus would require higher extracellular iron concentrations to function efficiently. Once in the periplasm, specific inner membrane transporters could then import free iron ions or an iron chelate (e.g., iron-citrate complexes). The DC3000 genome contains several inner membrane transporters that might function as part of a low affinity iron import system, including those that import free iron ions (PSPTO_0314, PSPTO_0141, PSPTO_3596, PSPTO_2053, PSPTO_2464, and PSPTO_2499) and iron-citrate chelates (PSPTO_0760-0763 and PSPTO_0062).

Furthermore, expression analyses in DC3000 cultures indicate that high iron [50 μM iron(III) citrate] both represses high-affinity iron-scavenging system expression and induces expression of the type III secretion system and virulence genes in culture (8). It was expected that abundant iron would repress high-affinity iron scavenging, but the induction of virulence genes was surprising. In a follow-up study, Kim et al. found that expression of virulence factors in hhp-inducing minimal medium was limited by iron availability and that higher iron to well above 10 μM continued to induce higher virulence gene expression (32, 33). Taken together, these results indicate not only that the environment in which DC3000 causes disease is relatively iron rich, but also that iron—as opposed to the lack of it—may act as a signal for DC3000 pathogenesis.

Other pathogens that colonize similar plant environments (i.e., the leaf apoplast and vasculature) might be expected to behave similarly. The addition of bean leaf apoplastic fluid to Pseudomonas syringae pv. phaseolicola NPS3121 grown in minimal medium resulted in the expression of virulence genes and the repression of high-affinity iron import systems, including Ybt and Pvd (27). Furthermore, similar to our findings with DC3000, the hemibiotrophRalstonia solanacearum, a soil-borne pathogen that causes lethal wilt plant diseases by entry through the plant root, invasion of the xylem vessels, and systemic colonization via the vasculature, does not require siderophores for pathogenesis on tomato but does for growth in iron-limited culture (5).

Siderophores in plant-pathogen interactions. If iron is replete in the DC3000 pathogenic environment, past virulence phenotypes observed for select siderophore mutants in specific
plant pathosystems could perhaps be explained either by differences in their growth environment and mode of infection or by siderophore functions other than in iron nutrition. For example, abrogated intracellular and extracellular siderophore synthesis impacts pathogenicity of the hemibiotroph Magnaporthe grisea on rice, likely through alteration of localized reactive oxygen species (28). Similarly, siderophore biosynthetic mutants of filamentous ascomycetes exhibit reduced virulence and hypersensitivity to hydrogen peroxide (46). Alternatively, siderophores such as Pvd may act as signaling molecules that regulate bacterial growth form (e.g., biofilm formation) and the production of virulence factors, as they do for the human pathogen Pseudomonas aeruginosa (36). Indeed, a similar finding was recently reported for Pvd synthesized by the phytopathogen P. syringae pv. tabaci 6605, and 6605 Pvd biosynthetic mutants exhibited clearly reduced virulence on tobacco leaves (53). This contrasts with the lack of virulence phenotype observed for the DC3000 Pvd− mutant on tomato reported here and for the P. syringae phenotype observed for the DC3000 Pvd biosynthetic mutants exhibited clearly reduced virulence on plant leaf tissue by means of magnetic resonance imaging. Three-dimensional mapping of the iron distribution in the apoplastic fluid of tomato DC3000 continuous cultures: the role of iron in cell growth and virulence. Anal. Bioanal. Chem. 392: 7072–7077.

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