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Relationship of Siderophore-Mediated Iron Assimilation to Virulence in Crown Gall Disease

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Three classes of mutants defective in the biosynthesis of the siderophore agrobactin were isolated from *Agrobacterium tumefaciens* A217 after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Class I mutants produced uniquely the catechol 2,3-dihydroxybenzoic acid, whereas classes II and III produced no detectable catechol. Class II differed from class III mutants in that exogenous 2,3-dihydroxybenzoic acid was utilized only by the former to synthesize agrobactin. Growth of strains B6 and A217, under iron starvation, led to enhanced production of several envelope proteins migrating in the 80,000-dalton range upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One mutant, defective in agrobactin iron utilization, lacked one of these proteins. This protein may represent a siderophore receptor or fragment or subunit thereof. With a single exception, all of the mutants obtained in this work were capable of initiating tumorous growth in sunflower plants and on carrot root disks, provided pTiB6806 was present. Comparison of the catechols produced by strain B6806 and its nononcogenic, Ti-plasmid-deficient derivative A217, indicated that the genes encoding agrobactin synthesis are not associated with the virulence plasmid of *A. tumefaciens* B6806. Analysis of gall tissue for agrobactin did not reveal the presence of this siderophore. Finally, citrate, an iron-carrier in plants, enhanced significantly the growth of the agrobactin-deficient mutants in a low-iron medium. These results suggest that the production of agrobactin in planta is not requisite to infection and that citrate may serve as an alternative carrier of iron for *A. tumefaciens* within the host.

During the past decade much research has centered upon iron as one of the prime determinants of virulence in those microorganisms which infect animal species. Iron is essential for microbial growth; however, in mammals, this metal is inaccessible to microorganisms (6). In humans, the majority of the total body iron is intracellular, and the small amounts of iron in the body fluids potentially available to invading pathogens are bound by the high-affinity, iron-binding proteins transferrin (serum) and lactoferrin (secretions). During infection, the mammalian host further reduces the amount of iron in the blood plasma and increases its stores of the metal in the liver (50). This capacity of the host to deny iron to invading microbes has been termed "nutritional immunity" (26). The ability of microbial pathogens to compete successfully for this vital nutrient is considered to be a prerequisite to infection.

Although the importance of iron in mammalian infections is well established, the role of this metal in the infection of plants by phytopathogens remains an unexplored field. In a recent report (39), we described a new catechol siderophore, agrobactin, from the bacterial plant pathogen *Agrobacterium tumefaciens* B6, an organism which initiates tumorous growth in a variety of dicotyledonous plants. In this communication, we present our findings on the isolation and characterization of mutants defective in the biosynthesis and utilization of agrobactin and the relationship of agrobactin to the pathogenicity of *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* strains employed in this study are listed in Table 1. *Agrobacterium*-specific phages PS8 and S3 were obtained from R. Sung.

Media. L broth (LB; 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl) and Tris-buffered medium (46) modified to contain 1 μM $MnSO_4$ and no added iron were routinely employed. All strains were stored at 4°C on nutrient slants containing (per liter) 17.5 g of Difco antibiotic medium 2, 50 mg of thymine, 5 mg of thiamine, and 15 g of agar and were transferred every 3 months.

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TABLE 1. *Bacterial strains*

Strain	Derivation	Genotype	Relevant Ti plasmid-coded properties	Source	Reference
A. tumefaciens					
B6			Octopine utilization	N. Panopoulos	(23)
B6806	B6		Octopine utilization	E. Nester	(45)
A217	B6806		Cured of pTiB6806	E. Nester	(45)
A6			Octopine utilization	N. Panopoulos via L. Moore	(9)
$XVII_{19}$	A6	*agb*[a]	Octopine utilization	R. Sung	
agb-1 – *agb-18*	A217	Agrobactin-deficient		This study	
E. coli K-12					
RW193	AN193	F⁻ */purE*⁺ *tonA*⁺ *entA* Thi⁻ *proC leu trpE strA*		Laboratory stock	(49)

[a] Agrobactin-deficient mutants are designated *agb*.

Chemicals and reagents. All chemicals were reagent grade. Ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) and syringic acid were from K & K Laboratories, and cycloheximide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), octopine, streptomycin (Sm), and other phenolic compounds found in plants were from Sigma Chemical Co. Rifampin (Rif) was from Calbiochem-Behringer Corp. Aldehyde-free glacial acetic acid (Baker Chemical Co.) and glass-distilled, UV-grade solvents (Burdick and Jackson) were employed for high-performance liquid chromatography (HPLC) and for the preparation of plant extracts for HPLC analysis. Bis-(3-aminopropyl)amine and 2,3-dihydroxybenzoic acid (DHBA) were from Aldrich Chemical Co. Tyrosinase was obtained from Worthington Diagnostics. Itoic acid and *N,N'*-di(2,3-dihydroxybenzoyl)ethylenediamine were obtained from T. Peterson. Deferriferrichrome A was prepared as previously described (49).

Labeled ferric agrobactin was prepared by mixing 0.14 mg (0.22 μmol) of agrobactin (39), 0.5 ml of ethanol, 0.5 ml of 0.4 mM [^{55}Fe]ferric chloride (0.2 μmol; 4.7×10^8 cpm/μmol) in methanol, 1.0 ml of water, and dilute NaOH to pH 7.4. The wine-colored solution was lyophilized, and the residue was dissolved in water to give 2.0 ml of 100 μM [^{55}Fe]ferric agrobactin. Upon paper electrophoresis in phosphate buffer (pH 6.6), 95% of the counts moved with a mobility 0.85 times that of ferrichrome A, i.e., the expected behavior of ferric agrobactin (38).

Chemical analyses. Protein was determined by the method of Lowry et al. (31). Catechol was determined as described by Arnow (1). Hydroxamates were assayed by methods 1 and 2 previously described (submitted for publication).

Analytical chromatography and paper electrophoresis. The following solvent systems (vol/vol) were used for thin-layer chromatography (TLC) and paper chromatography: (i) benzene-acetic acid-water, 2:2:1, organic phase; (ii) benzene-acetic acid-water, 78:45:2, (iii) methanol-tetrahydrofuran-5% (wt/vol) ammonium acetate, 1:1:1. Paper electrophoresis was performed on a flat-bed device with 0.1 M phosphate buffer (pH 6.6).

We employed 1.0% ferric chloride in 0.1 N HCl and UV illumination as sprays and detection aids.

Thin-layer plates of cellulose were obtained from EM Laboratories. Thin-layer sheets of silica gel on plastic were obtained from Brinkmann Instruments Inc. Polyamide-11_{254} plastic-backed, thin-layer plates were obtained from Macherey-Nagel.

HPLC was performed with a Waters Associates model 6000A instrument equipped with a model 660 solvent programmer and an Altex column (1 by 25 cm) packed with a 5-μm of C_{18} reverse-phase adsorbent.

Spectroscopy. UV and visible spectra were recorded with a Beckman model 25 instrument. Optical density measurements were made on a Bausch and Lomb Spectronic-710 spectrophotometer.

Growth factor assay. Reversal of iron starvation of *A. tumefaciens* was determined in EDDA-containing agar as previously described (39).

Isolation of membrane. *A. tumefaciens* was cultured in 1 liter of Tris-glucose medium (39) supplemented with either 0.1 or 10 μM $FeSO_4$ as described previously. Cells were harvested in late log phase by centrifugation at 12,000 × *g* for 30 min. The cell pellet was washed in 50 mM Tris-hydrochloride (pH 8), resuspended in 10 ml of 0.1 M Tris-hydrochloride (pH 8), and sonicated in 10-s pulses for a total of 3 min. Whole cells were removed by centrifugation at 12,000 × *g* for 10 min, and membranes were pelleted from the supernatant fluid by centrifugation for 30 min at 175,000 × *g*. Membranes were resuspended in a 100-μl volume of 10 mM Tris-hydrochloride (pH 8), either by standing in buffer overnight at 4°C or immediately with no. 18 and no. 23 gauge needles and syringe.

Polyacrylamide gel electrophoresis. Samples of 10 to 25 μl containing 10 to 20 μg of protein were solubilized and electrophoresed as described by Lugtenberg et al. (32). Gels were stained by the procedure of Fairbanks et al. (13). The slab gel device was custom made and was employed with a Hofer 2000 constant voltage-constant current power source.

Bacterial conjugation. Mating was carried out on filters (16) in the presence of octopine (22). Cells were resuspended in λ buffer (1 mM Tris-hydrochloride, (pH 7.2), 10 mM $MgSO_4$) and spread on octopine agar plates containing Rif (100 μg/ml) and Sm (300 μg/ml). Single colonies resulting from this selection were purified on LB containing Rif and Sm and retested for their ability to utilize octopine.

Isolation and characterization of siderophore auxotrophs. *A. tumefaciens* A217 was confirmed to

produce agrobactin and was subjected to NG mutagenesis by the method of Klapwijk et al. (24), with the following modifications: an LB overnight culture was employed, and after mutagenesis, cells were washed twice in 0.1 M sodium phosphate buffer (pH 7.0) and then resuspended in LB. After incubation overnight at 30°C with shaking, the mutagenized culture was diluted onto Tris-glucose agar medium supplemented with 10 μM $FeSO_4$ and 100 μg of Difco Casamino Acids per ml.

Individual colonies were patched onto LB plates, then onto LB plates containing EDDA (1 mg/ml) and seeded with 2 drops of an overnight LB culture of *A. tumefaciens* $XVII_{19}$ per 100 ml of agar. Incubation was done at 30°C. Colonies which grew poorly or did not promote the growth of the indicator strain or both were further tested for production of agrobactin and catechol in 5 ml of Tris-glucose medium. At neutral pH, agrobactin can be selectively partitioned into ethyl acetate, leaving DHBA and other charged catechols in the aqueous layer. This provided the basis for an assay to distinguish agrobactin from other catechols in the culture. Levels of agrobactin were determined by vigorously agitating the cell-containing culture or cell-free supernatant fluid from low-iron cultures with an equal volume of ethyl acetate. The organic layer was washed with 0.5 M citrate buffer (pH 5.5) and the absorbance of the ethyl acetate extract was measured at 316 nm.

Spontaneous Rif (100 μg/ml)- and Sm (300 μg/ml)-resistant variants of the agrobactin auxotrophs were selected on LB medium by standard methods (36). The resulting antibiotic-resistant mutants were mated with *A. tumefaciens* B6806 and pTiB6806$^+$ transconjugants selected as described above. All further characterization of the mutants was carried out on the antibiotic-resistant transconjugants.

Siderophore production. Mutants were cultured in 100 ml of Tris-glucose medium with and without the addition of 0.1 mM DHBA and inoculated at 0.5% with an overnight LB culture. After 48 h of growth at 30°C and with shaking, cells were removed by centrifugation in the usual way. Twenty milliliters of supernatant fluid was removed and lyophilized to test for growth factor activity, catechol, and hydroxamate. The pH of the remaining cell-free supernatant was lowered to 2 with concentrated HCl and extracted with three 35-ml portions of ethyl acetate. The extracts were pooled, concentrated in vacuo to ca. 1 to 2 ml, and examined for DHBA-type absorbance in UV spectrum from 300 to 350 nm, and analyzed for catechol by TLC, paper electrophoresis, and paper chromatography.

Temperature-sensitive production of catechol. Strains were cultured in Tris-glucose medium with or without the addition of 0.1 mM DHBA as described above at room temperature (22 to 24°C) and 30°C, and the supernatant fluids were examined for catechol.

Uptake experiments. *A. tumefaciens agb-2* and *agb-3* were cultured overnight at 30°C in nutrient broth and then inoculated (1%) into Tris-glucose medium without iron and with 10 μM of added $FeSO_4$. After two serial transfers in these media, the cells were harvested, washed with sterile Tris medium (no glucose), and resuspended in low-iron Tris medium to give an absorbance of 0.9 at 650 nm, corresponding to approximately 10^9 colony-forming units per ml. Exactly 1.85 ml of each of the four cell suspensions was shaken in a water bath at 30°C for 30 min, at which time 0.15 ml of the labeled 100 μM solution of ferric agrobactin was added to each tube. Samples of 0.1 ml were withdrawn at timed intervals, and the cells were collected on 0.45-μm Millipore filters which had been soaked overnight in 10 μM of unlabeled ferric agrobactin. After the filters were washed with 10 ml of low-iron Tris medium containing 1.0 mM nitrilotriacetate, they were dried and counted in 10 ml of fluid in a Delta 300 scintillation detector.

Pathogenicity tests. (i) *Carrot assay.* Carrots, purchased at local supermarkets, were soaked in 10% bleach (0.5% sodium hypochlorite) for 20 min, cut aseptically into 0.5-cm rounds, and placed in sterile petri dishes on filter paper which had been previously moistened with sterile water. A loopful of bacteria from an LB plate was spread onto the center of each carrot slice. Incubation was at room temperature (22 to 24°C) for 2 to 3 weeks.

(ii) *Sunflower assay.* Routinely, 15- to 20-day-old sunflower (*Helianthus annus* variety Giant Grey Stipe, Northrup King Seeds, Co.) plants (15 to 20 cm in height), cultivated in a mixture of 50% sand–50% peat moss and watered with half-strength Hoagland solution on alternate days (19), were inoculated in the stem with a sterile toothpick which had touched a single colony. In dose-response experiments, inocula were 5-μl injections of serial decimal dilution containing 10^5 to 10 colony-forming units of *A. tumefaciens* in λ buffer (1 mM Tris-hydrochloride (pH 7.2), 10 mM $MgSO_4$). For inoculation, the stem was first wounded with the pointed end of a sterile toothpick, and the injection was delivered from the sterilized tip of an Ependorff micropipette. Inocula were prepared from buffer-washed, early log phase Tris-glucose cultures.

Reisolation of bacteria from gall tissue. A 2- to 3-cm section of stem containing tumorous growth was removed, surface sterilized for 2 to 4 min in 10% bleach (0.5% sodium hypochlorite), rinsed with sterile water, and then macerated aseptically in a mortar and pestle with sea sand and ca. 5 ml of water. The fluid was serially diluted in sterile water onto LB plates containing Rif (100 μg/ml), Sm (300 μg/ml), and cycloheximide (250 μg/ml). For strains not carrying antibiotic resistance markers, Rif and Sm were excluded.

Polyphenol oxidase assay. Mushroom polyphenol oxidase activity was measured as described in the *Worthington Enzyme Manual* (10). The increase in absorbance at 390 or 370 nm was followed for 1 to 12 min and recorded at 15-s or 1-min intervals. The abilities of various catechols to inhibit the oxidation of tyrosine were investigated by incorporating in the standard reaction 0.05 or 0.1 ml of a 1 or 10 mM ethanolic solution of agrobactin or DHBA, respectively.

Agrobactin in plant tissues. Uninoculated stem (50 g) and tumor tissue obtained from sunflower plants which had been inoculated with *A. tumefaciens B6 (39 g), B6806 (28 g), and agb-12* (29 g) were quick frozen in liquid nitrogen and then macerated with twofold (wt/vol) 50% methanol for 5 min at low speed in a

Waring blender. The resulting slurry was clarified by centrifugation at 10,000 × *g* for 20 min, and the supernatant fluid was extracted with two 10-ml volumes of n-hexane. The aqueous phase was concentrated on a rotary evaporator at 30°C to remove the solvent. After adjusting the pH of the solution to 6 with 0.1 N NaOH, the resulting solution was extracted with five 10-ml volumes of ethyl acetate. The combined ethyl acetate extract was washed with three 10-ml aliquots of 0.5 M citrate buffer (pH 5.5) and water and then taken to dryness in vacuo at 30°C. The residue was dissolved in 50 to 100 μl of methanol, and samples were examined for the presence of agrobactin by HPLC and for biological activity for strain A217 by the growth factor assay.

RESULTS

Isolation and characterization of mutants defective in the biosynthesis of agrobactin. To test whether the synthesis of agrobactin was important to the ability of *A. tumefaciens* to infect plants, mutants defective in the biosynthesis of agrobactin were sought. To eliminate the possibility of mutagenizing the virulence plasmid, a strain cured of the Ti-plasmid was employed.

Mutagenesis and selection. *A. tumefaciens* A217 was mutagenized with NG to give a killing of 99.9%, and single colonies were screened for agrobactin production by patching on EDDA-LB agar seeded with the agrobactin-deficient mutant $XVII_{19}$. Of the 1,950 colonies tested, 18 (0.9%) were found to be defective in agrobactin synthesis and were further characterized as described below.

Biochemical characterization. With the exception of the agrobactin synthesis defect, the pTiB6806$^+$ transconjugantes of the mutant strains *agb-1* to *agb-18* and $XVII_{19}$ behaved as did their respective parent strains A217 and A6 in regard to the following chromosomal traits: growth on sodium selenite (18), ferric ammonium citrate (18), Tris-buffered medium containing 0.4% (wt/vol) sodium citrate as carbon source, and Clark medium (7); growth at 37°C in LB medium; utilization of asparagine; production of ketolactose (2); production of hydrogen sulfide and hydrolysis of gelatin (8); catalase activity (41); sensitivity to phages PS8 and S3 (48), as well as the pTi-associated trait, utilization of octopine (22). All strains grew in LB, forming an extracellular slime typical of *A. tumefaciens*, with an approximate doubling time of 2 h. In Tris-glucose medium without added iron, the majority of the mutants grew equally well as the wild type with an average doubling time of 5 h. However, they did not usually attain as high a final cell density as the wild type. Growth in Tris medium was enhanced by supplementation with either 10 μM $FeSO_4$ or 1 mM sodium citrate.

The mutants could be divided into 3 classes based on their ability to make catechols and to be complemented with DHBA to produce agrobactin (Table 2). Class I mutants were found to hyperexcrete DHBA. As much as 900 μM catechol was detected in the spent medium of Tris-glucose cultures of these strains. This was the only catechol found in either neutral or low pH ethyl acetate extracts of these mutants (Table 3). Class II and III mutants did not produce any detectable catechol or hydroxamate. Class II mutants differed from class III mutants in that exogenously added DHBA could be utilized only by class II organisms to synthesize agrobactin. Only small quantities of agrobactin were found in these cultures. Strains *agb-11* and *agb-10* gave variable results from experiment to experiment, indicating possibly that a revertant had been picked. With the exception of strain $XVII_{19}$,

TABLE 2. *Catechol production by A. tumefaciens parental and mutant strains*

Strain	Class	Relative amount of catechol excreted (%)
A217 group		
A217	wild type	100
agb-1 – *agb-6*, and *agb-18*	I	140–400
agb-7	I	50–150
agb-8, agb-10 – *agb-12, agb-14*	II	0[a]
agb-9, agb-13, agb-15 – *agb-17*	III	0
A6 group		
A6	wild type	100
$XVII_{19}$	II	0

[a] In seven separate experiments, strains *agb-10* and *agb-11* produced less than 10^{-5} M catechol; in two other experiments, less than 10^{-4} M catechol was produced. Both strains exhibited slight activity in growth factor assays, which are more sensitive.

TABLE 3. *Separation of catechols of A. tumefaciens A217 class I mutants*

System	R_f		
	DHBA	Agrobactin	Catechol of class I mutants
Solvent			
iii[a]	0.60	0.37	0.60–0.61
ii[b]	0.75	1.0	0.71–0.76
i[c]	0.37	0.46	0.35–0.38
Buffer[d]	1.0[e]	1.0[e]	1.0–1.1[e]

[a] TLC on polyamide.
[b] TLC on cellulose.
[c] Descending-paper chromatography.
[d] Paper electrophoresis.
[e] Mobility relative to DHBA.

all of the mutants exhibited the same phenotype when cultured at room temperature or at 30°C. $XVII_{19}$ was only complemented with DHBA at lower temperature.

The ability of the 10-fold-concentrated spent media of the mutants to support the growth of strain A217 in the growth factor assay was investigated. With the exception of strains *agb-10* and *agb-11*, which gave variable results, none of the supernatant fluids from class II and III mutants was capable of feeding A217. In contrast, spent media from class I mutants were able to support the growth of A217, but the response was usually delayed. This behavior was similar to that observed for high concentrations of DHBA (10 mM). The 10-fold-concentrated supernatant fluids of these mutants contained quantities of DHBA of this order of magnitude.

One class I mutant, *agb-3*, was also defective in agrobactin utilization. This strain, whether it was precultured in the presence or absence of iron, was unable to accumulate [^{55}Fe]ferric agrobactin (Fig. 1). Iron was assimilated by another class I mutant, *agb-2*, and as expected, the starved cells were slightly more active in this regard. These data confirm the nutritional experiments wherein *agb-2*, but not *agb-3*, was shown to respond to agrobactin. Finally, comparison of total membrane proteins isolated from *A. tumefaciens* B6 or A217 grown in Tris-glucose medium containing either 0.1 or 10 μM added iron revealed the presence of at least three high-molecular-weight (80,000-dalton range) proteins which appeared to be iron regulated (Fig. 2). In contrast, membranes obtained from *agb-3* grown at a low-iron level lacked the lower of these

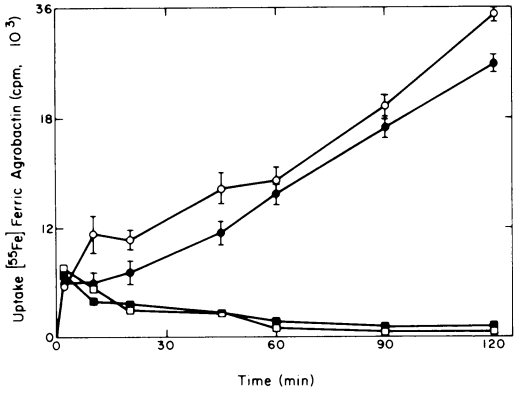

FIG. 1. *Uptake of [^{55}Fe]ferric agrobactin by A. tumefaciens agb-2 and agb-3 grown in the presence and absence of iron. Cells were shaken at 30°C in 7.5 μM [^{55}Fe]ferric agrobactin. Agb-2 plus Fe (●); agb-2 minus Fe, (○); agb-3 plus Fe, (■); and agb-3 minus Fe, (□) are shown.*

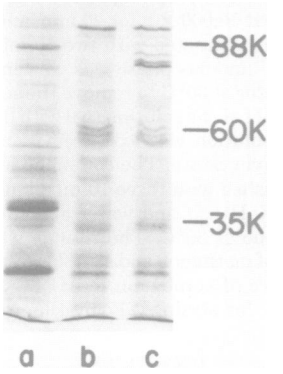

FIG. 2. *Protein composition of the membrane of A. tumefaciens B6 at low- and high-iron levels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% acrylamide was carried out as described in the text on 10-μl (20 μg of protein) samples of membranes prepared from A. tumefaciens B6 cultured in Tris-glucose medium containing either 10^{-7} M or 10^{-5} M $FeSO_4$. Membranes from A. tumefaciens B6 at 10^{-7} M (c) and 10^{-5} M $FeSO_4$ (b) are shown. For comparison, a sample of membrane obtained from E. coli RW193 grown at a low-iron level is shown in (a). 88K, 60K, and 35K are 80,000, 60,000, and 35,000, respectively.*

induced proteins (Fig. 3). This protein may serve as a receptor for agrobactin in *A. tumefaciens*.

With the exception of strain *agb-18*, all mutants were found to be pathogenic on carrot root disks and in sunflower plants. Strain A217 (pTiB6806$^-$) and mutants lacking the Ti-plasmid, as well as sham inoculations, did not give rise to tumorous growth. In dose-response experiments, all mutants were capable of forming galls at the lowest dilution which contained an average of 10 to 100 colony-forming units per inoculation. Finally, gall weight could be roughly correlated with inoculum size and was independent of the agrobactin synthesis defect.

Attempts were made to measure reversion frequency by serially diluting cells onto Tris or LB medium containing varying amounts of EDDA or deferriferrichrome A to render the media iron deficient. The latter did not retard the growth of the mutants. However, their multiplication was greatly inhibited in the presence of EDDA. In contrast to the parental strains, which produced colonies after 5 days, colonies appeared only after 1 to 3 weeks. Enumeration of these latter colonies was difficult due to the continued appearance of new colonies after very long periods of incubation.

To ascertain whether or not the mutants had reverted in planta, bacteria were reisolated from gall tissue and tested for their ability to produce

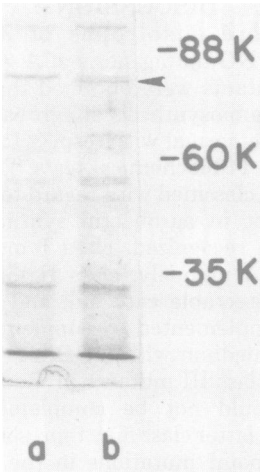

FIG. 3. *Protein composition of the membrane of A. tumefaciens A217 and agb-3 at a low-iron level. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 9% acrylamide of 10 μl (10 μg protein) sample of whole membrane prepared from A. tumefaciens. A217 and agb-3 cultured in Tris-glucose medium containing no added $FeSO_4$. Membranes from agb-3 (a) and A. tumefaciens A217 (b) are shown. 88K, 60K, and 35K are 80,000, 60,000, and 35,000, respectively.*

growth factor activity and agrobactin. Examination of a large number of isolates from each gall revealed none with these traits; all produced ketolactose.

Effect of citrate on the growth and production of siderophores by *A. tumefaciens*. Since citrate is an iron transport agent in plants (5), it was of interest to determine its effect on the growth and production of siderophores by *A. tumefaciens*. Citrate, unless complexed with iron as ferric ammonium citrate, does not act as a carbon source for biotype 1 *A. tumefaciens* (21; this study). This suggested that citrate might serve as an iron transport agent in this organism. Cells were grown in the presence of varying concentrations of sodium citrate ranging from zero to 1×10^{-2} M. After 48 h, the optical density at 650 nm and the yield of catechol were determined. The results of this experiment are recorded in Table 4. Citrate improved cell growth and appeared to enhance catechol synthesis.

The possibility that citrate might promote iron transport was further investigated by examining the effect of citrate on growth and siderophore production in *A. tumefaciens* A217 *agb* mutants. Pollack et al. (40) demonstrated that citrate (1 mM) could severely inhibit the growth of enterobactin-deficient mutants of *Salmonella typhimurium* which lack an iron-citrate transport system. In contrast, Frost and Rosenberg (15) found that (1 to 10 mM) citrate vastly improved the growth of enterobactin-deficient strains of *Escherichia coli*. *E. coli* is known to have an inducible iron-citrate transport system (15). Several agrobactin-deficient mutants were grown in Tris-glucose medium with or without added citrate (1 mM) as described above. In all cases, citrate was found to improve both the yield of cells and, in the case in which catechol was still synthesized (class I mutants), levels of catechol (data not shown). Therefore, it would appear that citrate does serve as an iron transport agent in *A. tumefaciens*. This conclusion will have to be confirmed by comparing rates of uptake of radioactive iron in the presence and absence of citrate (15).

Catechol synthesis was probably not depressed in these experiments because the growth medium did not contain an adequate level of iron to satisfy cellular needs, even though trace amounts were being efficiently transported into the bacteria.

Effect of catechol-type siderophores on the activity of polyphenol oxidase. The enzyme polyphenol oxidase (tyrosinase, phenol oxidase) is thought to play a role in the resistance of plants to infection (14). Its activity generally increases during microbial invasion of plants, and the oxidized phenolic products have been shown to be antibiotic to some pathogens (17, 34).

Since agrobactin and DHBA are phenolic compounds, it was of interest to see whether or not they might act as substrates or inhibitors of the enzyme. One important role of phenol oxidase might be to destroy these vital compounds and thus starve the pathogen for iron. Conversely, the pathogen might have evolved siderophores which could inactivate this enzyme

TABLE 4. *Effect of citrate on cell growth of A. tumefaciens*[a] *in Tris-glucose medium*

Sodium citrate (M)	Optical density (650 nm)
0	1.71
1.0×10^{-6}	1.69
1.0×10^{-5}	1.77
1.0×10^{-4}	1.86
1.0×10^{-3}	1.95
5.0×10^{-3}	2.06
1.0×10^{-2}	2.11

[a] *A. tumefaciens* A217 was inoculated to give 1% into 25-ml volumes of Tris-glucose medium containing varying amounts of added sodium citrate. After 48 h of growth at 30°C, the cell density was checked, and total catechol was determined. For no added citrate, catechol was 2.9×10^{-4} M; at the six levels of citrate, the concentration ranged between 4.0×10^{-4} and 5.3×10^{-4} M.

and thereby curtail the production of compounds toxic to itself.

The interaction of mushroom polyphenol oxidase and the catechol siderophores was examined. Catechol at 0.1, 1.0, and 10 mM gave a change of absorbance at 390 nm per min of 0.032, 0.106, and 0.154, respectively. With the same level of enzyme, itoic acid, *N,N'*-di-(2,3-dihydroxybenzoyl)ethylenediamine, DHBA, and agrobactin at 1.0, 0.1, and 0.025 mM, respectively, gave no significant change in absorbance at 370 nm. None of the siderophores acted as good substrates; agrobactin and *N,N'*-di-2,3-(dihydroxybenzoyl)ethylenediamine were tested only at low concentration because of their insolubility in aqueous solution. The ability of agrobactin and DHBA to act as enzyme inhibitors was also investigated. Rates of oxidation of 1 mM catechol in terms of the change of absorbance at 390 nm per min were: 0.29 without added inhibitor, 0.28 in the presence of 25 μM agrobactin, and 0.24 with DHBA at 500 μM. Thus, agrobactin and DHBA showed no marked inhibition of enzyme activity.

Search for agrobactin in planta. The possibility that plant tissues might provide a low-iron environment which would induce *A. tumefaciens* to synthesize agrobactin in planta was investigated. Uninoculated stem and gall tissue of sunflower plants were examined for growth factor activity and for agrobactin. Methanol-water extracts of these tissues revealed the presence of large quantities, 2 to 4 μmol/g of tissue, of catechol substances as measured by the Arnow assay. However, these extracts did not demonstrate growth factor activity for *A. tumefaciens* A217. Attempts were made to identify agrobactin in these extracts by HPLC. The HPLC column was injected with 5 to 100 μl of methanolic extract of galls. Chromatography was initiated in 10% methanol in 0.1 M acetic acid brought to pH 7.5 with concentrated ammonium hydroxide, and elution was effected over 50 min with a linear gradient to 50% methanol in the same buffer at a flow rate of 3 ml/min. Although authentic agrobactin eluted as a sharp peak with a retention time of 48 min, there was no evidence for the presence of the siderophore in the gall extracts.

The ability of a number of phenolic compounds that are commonly found in plants to feed *A. tumefaciens* was examined. No growth factor activity was detected in 20-μl volumes of 10 mM 50% methanolic solutions of caffeic acid, protocatechuic acid, gentisic acid, chlorogenic acid, vanillic acid, *trans*-cinnamic acid, benzoic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, or salicylic acid. Salicylic acid was inhibitory.

DISCUSSION

Siderophore auxotrophs of *A. tumefaciens*. After NG mutagenesis of *A. tumefaciens* A217, 18 mutants were obtained that were defective in the biosynthesis of agrobactin. These mutants were normal with respect to the parental strains in 14 biochemical tests. The mutants were further classified with regard to the nature of the defect in agrobactin synthesis. Three classes were recognized: class I mutants produced DHBA uniquely; class II mutants produced no detectable catechol; and this defect could be complemented by supplementation of the growth medium with DHBA to yield agrobactin; and class III mutants produced no catechol and could not be complemented with DHBA. This latter class may represent deletions or multiple-point mutations in the agrobactin biosynthetic genes. One mutant, *agb-3*, was also found to be incapable of utilizing agrobactin in the growth factor assay. Comparison of membranes obtained from low-iron cultures of this mutant with those from the parental strain, revealed that one of the low-iron inducible proteins was absent. This protein may be involved in transport of agrobactin iron into the cell.

With the exception of one strain, all pTi$^+$ transconjugants of the mutants were found to be pathogenic on carrot root disks and in sunflower plants, even when inoculated in small numbers. Furthermore, when reisolated from galls, these mutants appeared to have retained their iron-auxotrophic phenotype.

Because of the lack of an effective selection method, reversion frequency was not determined. Deferriferrichrome A was not inhibitory to the growth of the mutants. EDDA was toxic at a high concentration, and at lower levels it did not give rise to colonies which could be enumerated by titration. Putative revertant colonies which did grow on EDDA-containing agar did not produce agrobactin. These clones may be similar to the "pseudorevertants" of *E. coli* enterobactin mutants described by McIntosh et al. (35). These revertants were not restored in their ability to make or utilize enterobactin, but rather became capable of growing on iron-deficient agar by loss of certain major outer membrane proteins, thus altering their permeability properties. By analogy, pseudorevertants of the *A. tumefaciens* agrobactin auxotrophs might be capable of utilizing EDDA as an iron carrier and thus growing on EDDA-containing agar.

Production of siderophores in planta. Neither agrobactin nor DHBA were found to act as substrates or inhibitors of mushroom polyphenol oxidase. However, in many plant tissues this enzyme exists in a number of multiple forms or isozymes, all of which may have distinctive

specificites. Thus, the possible interaction of the catechols with phenol oxidases of broader specificity and the relation of this event to virulence remains an open question.

Attempts were made to identify agrobactin in infected plants. However, due to the large number of interfering substances with a similar retention time, the presence of agrobactin could not be demonstrated by HPLC. Moreover, the plant extracts had no capacity to enhance the growth of *A. tumefaciens* in EDDA-containing agar. The limit of detection by these two analyses is estimated at 80 and 3 pmol of agrobactin per g of gall tissue, respectively. The possibility that agrobactin was destroyed in the isolation procedure cannot be eliminated.

Twelve phenolic acids commonly found in plants (17) did not enhance the growth of A217 in EDDA-containing media. However, in plants the compounds may be glycosylated or esterified and have different biological activities. Whether or not *A. tumefaciens* might encounter these substances in planta is not certain.

Role of siderophores in the pathogenicity of *A. tumefaciens*. The role of siderophores in the pathogenicity of *A. tumefaciens* is still unclear. With the exception of *agb-18*, the agrobactin auxotrophs showed no impaired ability to initiate galls on carrot root disks or in sunflower plants. These results suggest that the synthesis of siderophores is not requisite to successful infection by this organism. However, the inability to measure reversion frequency or to demonstrate conclusively that no revertants were present in galls clouds this result. In experiments which involved small inocula, the mutants were still able to form galls at the same rate and frequency as the parental strains. Since attachment of the bacteria to the wound site and the subsequent transfer of the Ti-plasmid (T DNA) must occur within a few days after infection (3, 11, 29, 42), the doubling time of the bacteria in planta is slow (estimated 10 to 12 h) (3, 20, 27), and the number of bacteria in the infection site stabilizes or decreases after a few days (3, 33), it seems unlikely that revertants could have arisen in a small inoculum (10 to 100 colony-forming units) and have initiated gall formation with the same kinetics as the wild type.

The marked effect of citrate on the growth of *A. tumefaciens* may explain why the mutants were as capable of forming galls as the wild type. Inoculations were made in the stem of sunflower plants and on carrot root slices. In both cases, the bacteria may have been in contact with citrate. Stem wounds were extensive and may have damaged the xylem. Often liquid was exuded from the wound. This fluid may have contained adequate iron to allow the *A. tumefaciens* agrobactin-deficient mutants to initiate an infection.

Perhaps the use of a different host such as pinto bean leaves would have yielded different results (28). Studies on the infectivity of auxotrophs employing this system have shown significant differences in the number of certain auxotrophs and wild-type organisms that are required to give a certain number of tumors (30).

In another study, the production of siderophores by phytopathogenic bacteria was found to be a common trait (submitted for publication). However, with the exception of the octopine-utilizing strains of *A. tumefaciens*, such as B6, the quantities of siderophore produced were not usually very large. For comparison, among the fluorescent pseudomonads the saprophytic bacterium, *Pseudomonas* B10 (*fluorescens-putida* group) makes as much as 10 to 20 times more siderophore than the pathogen *Pseudomonas syringae* pv. *phaseolicola* in the same growth medium (J. Leong, personal communication). This discrepancy may reflect the different life styles of these two organisms: one is a parasite and one is a saprophyte. *P. s. phaseolicola*, as well as other pathogens, are well adapted to taking advantage of the specific nutrients that are available in the host, upon which their parasitic existence vitally depends. Thus citrate, endogenous phenolic compounds, heme, or iron proteins in planta, rather than siderophores, may be the major iron source for many pathogenic bacteria in planta. Indeed, citrate was found to improve significantly the growth of *A. tumefaciens* in low-iron media.

The biosynthesis of siderophores by phytopathogens may be more important at other times, such as during overwintering, when the bacteria are not associated with a viable host (44). During such periods these organisms would have to scavenge iron from the environment and compete for iron with common soil organisms such as *P. fluorescens*. Fluorescent pseudomonads have long been known to promote the growth of certain species of plants (4). This effect has recently been shown to be due, at least in part, to the production of siderophores by these bacteria which have the capacity to deny iron and thus inhibit the growth of detrimental soil-borne pathogens (25). In addition, the purified siderophore of *P. fluorescens-putida* B10 was found to be bacteriostatic for *A. tumefaciens* A6, *Erwinia carotovora* subsp. *carotovora*, and *P. s. phaseolicola* G50 (J. Leong, personal communication). However, these bacteria can eventually overcome this growth antagonism, presumably, by producing their own siderophores. A similar phenomenon has been shown to occur in algal blooms in which certain species thrive

over others by excreting siderophores which cannot be utilized by other organisms (37).

In conclusion, it is likely that the production of siderophores plays a marginal role in the infection of plants by *A. tumefaciens* and that a more probable function for these chelates is for the general survival of the pathogen ex planta. The capacity to synthesize siderophores was probably widespread among the procaryotes long before the appearance of plants and animals (hosts). Hence the prime function of these chelates may be the solubilization of environmental iron. *A. tumefaciens* is known to be a successful soil inhabitant (12, 43). Whether these conclusions apply to other phytopathogens remains to be established. Organisms which cannot utilize citrate as an iron transport agent or which infect tissues in which citrate is absent or not abundant may show impaired virulence if unable to make siderophores. Siderophore auxotrophs of *Erwinia* spp. that infect corn plants may be inhibited in planta by endogenous iron chelators such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one (47). The siderophores of *P. s. phaseolicola* might induce a localized iron deficiency in areas of the plant in which the bacteria are living and may, in part, be responsible for the chlorosis symptoms of halo blight disease. Finally, diseases which require the continued and active growth of the causative organisms for disease maintenance may depend on siderophore production by the disease-causing agent. In short, the production of siderophores by plant pathogenic bacteria and the role of these compounds in the infection of plants are likely to provide unique answers for each host-parasite relationship.

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LITERATURE CITED

1. **Arnow, E. L.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531-537.
2. **Bernaerts, M. J., and J. DeLey.** 1963. A biochemical test for crown gall bacteria. Nature (London) **197**:406-407.
3. **Braun, A. C.** 1947. Thermal studies on the factors responsible for tumor initiation in crown gall. Am. J. Bot. **34**:234-240.
4. **Brown, M. E.** 1974. Seed and root bacterization. Annu. Rev. Phytopathol. **12**:181-197.
5. **Brown, J. C., and R. L. Chaney.** 1971. Effect of iron on the transport of citrate into the xylem of soybean and tomatoes. Plant Physiol. **47**:836-840.
6. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. **80**:1-36.
7. **Clark, A. G.** 1969. A selective medium for the isolation of *Agrobacterium* species. J. Appl. Bacteriol. **32**:348-351.
8. **Conn, H. J., and M. W. Jennison (eds.).** 1957. Manual of microbiological methods. McGraw-Hill Book Co., New York.
9. **Currier, T. C., and E. W. Nester.** 1976. Evidence for diverse types of large plasmids in tumor-inducing strains of *Agrobacterium*. J. Bacteriol. **126**:157-165.
10. **Decker, L. A.** 1977. Worthington enzyme manual, p. 74. Worthington Biochemical Corp., Freehold, N.J.
11. **DeRopp, R. S.** 1949. The action of antibacterial substances on the growth of *Phytomonas tumefaciens* and of crown gall tumor tissues. Phytopathology **39**:822-828.
12. **Dickey, R. S.** 1961. Relation of some edaphic factors to *Agrobacterium tumefaciens*. Phytopathology **51**:607-614.
13. **Fairbanks, G., T. L. Steck, and D. F. H. Wallace.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**: 2606-2617.
14. **Farkas, G. L., and Z. Király.** 1962. Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopathol. Z. **44**:8-150.
15. **Frost, G. E., and H. Rosenberg.** 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K-12. Biochem. Biophys. Acta **330**:90-101.
16. **Genetello, C., N. Van Larebeke, M. Holsters, A. De Picker, M. Van Montagu, and J. Schell.** 1977. Ti plasmids of *Agrobacterium* as conjugative plasmids. Nature (London) **265**:561-563.
17. **Harborne, J. B.** 1964. Biochemistry of phenolic compounds. Academic Press, Inc., New York.
18. **Hendrickson, A. A., I. L. Baldwin, and A. J. Riker.** 1934. Studies on certain physiological characteristics of *Phytomonas tumefaciens*, *Phytomonas rhizogenes*, and *Bacillus radiobacter*. J. Bacteriol. **28**:597-618.
19. **Hoagland, D. R., and D. I. Arnon.** 1950. The water-culture method for growing plants without soil. California Agricultural Experimental Station, Berkeley.
20. **Kado, C. I.** 1976. The tumor-inducing substance of *Agrobacterium tumefaciens*. Annu. Rev. Phytopathol. **14**: 265-308.
21. **Keane, R. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. **23**:585-595.
22. **Kerr, A., P. Manigault, and J. Tempe.** 1977. Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. Nature (London) **265**:560-561.
23. **Kersters, K., J. DeLey, P. H. A. Sneath, and M. Sackin.** 1973. Numerical taxonomic analysis of *Agrobacterium*. J. Gen. Microbiol. **78**:227-239.
24. **Klapwijk, P. M., A. J. R., deJonge, R. A. Schilperoort, and A. Rorsch.** 1975. An enrichment technique for auxotrophs of *Agrobacterium tumefaciens* using a combination of carbenicillin and lysozyme. J. Gen. Microbiol. **91**:177-182.
25. **Kloepper, J. W., J. Leong, M. Teintze, and M. N. Schroth.** 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature (London) **286**:885-886.
26. **Kochan, I.** 1973. The role of iron in bacterial infections, with special consideration of host-tubercle *Bacillus* interactions. Curr. Top. Microbiol. Immunol. **60**:1-30.
27. **Langley, R. A., and C. I. Kado.** 1972. Conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine mutants to crown-gall tumor induction. Mutation Res. **14**:277-286.
28. **Lippincott, J. A., and G. T. Heberlein.** 1965. The quantitative determination of the infectivity of *Agrobacterium tumefaciens*. Am. J. Bot. **52**:856-863.
29. **Lippincott, J. A., and B. B. Lippincott.** 1965. Timing events in crown-gall tumor development on pinto bean

leaves. Dev. Biol. **12**:309-327.
30. **Lippincott, J. A., J. H. Webb, and B. B. Lippincott.** 1965. Auxotrophic mutation and infectivity of *Agrobacterium tumefaciens*. J. Bacteriol. **90**:1155-1156.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
32. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli*. Mol. Gen. Genet. **147**:251-262.
33. **Manigault, P., and G. Beaud.** 1967. Expression de l'efficacité de la bactérie *Agrobacterium tumefaciens* (Smith et Town) Conn. dans l'induction tumorale [*Datura stramonium* L.]. Ann. Inst. Pasteur (Paris) **112**: 445-457.
34. **Mayer, A. M., and E. Harel.** 1979. Polyphenol oxidases in plants. Phytochemistry **18**:193-215.
35. **McIntosh, M. A., C. L. Pickett, S. A. Chenault, and C. F. Earhart.** 1978. Suppression of iron uptake deficiency in *Escherichia coli* K-12 by loss of two major outer membrane proteins. Biochem. Biophys. Res. Commun. **81**:1106-1112.
36. **Miller, J. H.** 1972. Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor New York.
37. **Murphy, T. P., D. R. S. Lean, and C. Nalewajko.** 1976. Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. Science **192**:900-902.
38. **Neilands, J. B., T. Peterson, and S. A. Leong.** 1980. High affinity iron transport in microorganisms. Iron(III) coordination compounds of the siderophores agrobactin and parabactin p. 263-278. *In* A. Martell (ed.), Inorganic chemistry in biology and medicine. American Chemical Society, Washington, D.C.
39. **Ong, S. A., T. Peterson, and J. B. Neilands.** 1979. Agrobactin, a siderophore from *Agrobacterium tumefaciens*. J. Biol. Chem. **254**:1860-1865.
40. **Pollack, J. R., B. N. Ames, and J. B. Neilands.** 1970. Iron transport in *Salmonella typhimurium*: mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104**:635-639.
41. **Rhodes, M. E.** 1959. The characterization of *Pseudomonas fluorescens*. J. Gen. Microbiol. **21**:221-263.
42. **Riker, A. J.** 1923. Some relations of the crown gall organism to its host tissue. J. Agric. Res. **25**:119-132.
43. **Schroth, M. N., S. V. Tomson, and A. R. Weinhold.** 1979. Behavior of plant pathogenic bacteria in rhizosphere and non-rhizosphere soils, p. 105-156. *In* S. V. Krupa and Y. R. Dommergues (ed.), Ecology of root pathogens. Elsevier-North Holland Publishing Co., New York.
44. **Schuster, M. L., and D. P. Coyne.** 1974. Survival mechanisms of phytopathogenic bacteria. Annu. Rev. Phytopathol. **12**:199-221.
45. **Sciaky, D., A. L. Montoya, and M. Chilton.** 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid **1**: 238-253.
46. **Simon, E., and I. Tessman.** 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S.A. **50**: 526-532.
47. **Tipton, C. L., J. A. Klun, R. R. Husted, and M. D. Pierson.** 1967. Cyclic hydroxamic acids and related compounds from maize. Isolation and characterization. Biochemistry **6**:2866-2870.
48. **Vervliet, G., M. Holsters, H. Teuchy, M. van Montagu, and J. Schell.** 1975. Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. J. Gen. Virol. **26**:33-48.
49. **Wayne, R., K. Frick, and J. B. Neilands.** 1976. Siderophore protection against colicins M, B, V. J. Bacteriol. **126**:7-12.
50. **Weinberg, E.** 1978. Iron and infection. Microbiol. Rev. **42**:45-66.