Agrobactin, a Siderophore from Agrobacterium tumefaciens*

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A siderophore (microbial iron transport compound) was isolated from low iron cultures of Agrobacterium tumefaciens B6. The substance was characterized as a threonyl peptide of spermidine acylated with 3 residues of 2,3-dihydroxybenzoic acid, the carbonyl group of 1 residue of the latter participating in an oxazoline ring with the β-hydroxyl of the threonine moiety. The compound, \( N-[3-(2,3\text{-dihydroxybenzamido})\text{propyl}]-N-[4-(2,3\text{-dihydroxybenzamido})\text{butyl}]-2-(2,3\text{-dihydroxyphenyl})-\text{trans}-5\text{-methyl-oxazoline-4-carboxamide} \), was given the trivial name agrobactin. Exposure to acid opened the oxazoline ring to afford agrobactin A. Ferric agrobactin A and agrobactin A itself, but not agrobactin or its ferric complex, had some capacity to feed iron to enterobactin-deficient strains of Escherichia coli and Salmonella typhimurium. Agrobactin was produced by A. tumefaciens in response to iron deficiency and was able to reverse the iron starvation in this organism precipitated by the presence of a ferric complexing agent not utilized by the cells.

The successful establishment of bacterial infections in mammals depends in part on the capacity of the microbe to acquire iron from the host (1). In the course of infection, iron is diverted to tissue stores where it is apparently less available for the invading pathogen. Those organisms capable of elaborating the high affinity iron scavenging system consisting of 2,3-dihydroxybenzoic acid, the carbonyl group of 1 residue of the latter participating in an oxazoline ring with the β-hydroxyl of the threonine moiety. The compound, \( N-[3-(2,3\text{-dihydroxybenzamido})\text{propyl}]-N-[4-(2,3\text{-dihydroxybenzamido})\text{butyl}]-2-(2,3\text{-dihydroxyphenyl})-\text{trans}-5\text{-methyl-oxazoline-4-carboxamide} \), was given the trivial name agrobactin. Exposure to acid opened the oxazoline ring to afford agrobactin A. Ferric agrobactin A and agrobactin A itself, but not agrobactin or its ferric complex, had some capacity to feed iron to enterobactin-deficient strains of Escherichia coli and Salmonella typhimurium. Agrobactin was produced by A. tumefaciens in response to iron deficiency and was able to reverse the iron starvation in this organism precipitated by the presence of a ferric complexing agent not utilized by the cells.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Colicin B and ferric enterobactin were prepared as previously described (5). The EDDA* was from Geigy Chemical Co. and octopine was from Sigma Chemical Co. The deuterated solvents were from Aldrich Chemical Co.

Production of Agrobactin—A. tumefaciens B6 was obtained from Dr. N. Panopoulos, Department of Plant Pathology, University of California, Berkeley and was maintained on LB plates (0.5% NaCl) (6). It was shown to be pathogenic for pea seedlings by wounding the stems of the plant with a sterile toothpick which had touched a single colony. Plants were examined after 2 and 4 weeks. The strain was confirmed to be ketolactose-positive (7) and to utilize octopine (8).

Cells were cultured at 30°C on a rotary shaker in a Tris-buffered medium (9) modified to contain 0.1 μM FeSO₄ and 1.0 μM MnSO₄. Glucose (0.4%) was added subsequent to sterilization. Exactly 1 ml of a 24-h Luria broth culture was inoculated into 50 ml of Tris medium. After 24 h of growth, 20 ml was transferred to 10 liters of sterile Tris medium. Following 48 h of growth, the cells were separated by centrifugation and the supernatant was extracted with three 1-liter portions of ethyl acetate. The extracts were combined, reduced to about 100 ml at 30°C on the rotary evaporator and washed three times with 20-ml aliquots of 0.1 M sodium citrate buffer, pH 5.5, three times with water, dried over MgSO₄, filtered and concentrated to a few milliliters. Crude agrobactin was precipitated by the addition of excess n-hexane. The product was collected by centrifugation, washed with n-hexane, dissolved in 0.5 ml of methanol and diluted with 50 ml of ethyl acetate. The latter solution was washed with citrate buffer and water, dried, concentrated to approximately 2 ml and the agrobactin crystallized by slow, slowwise addition of n-hexane. The process of citrate washing and recrystallization from ethyl acetate/n-hexane was repeated once again. The yield of white, crystalline substance, consisting of stubby needles and rosettes, was 50 to 100 mg. For removal of solvent contaminants, samples were dissolved in the least volume of methanol, precipitated with water, and lyophilized overnight. The preparation so obtained was weakly hygroscopic and was stored in vacuo over P₂O₅.

Chromatography and Paper Electrophoresis—The following solvent systems (v/v) were used for thin layer and paper chromatography: (A) chloroform:methanol, 4:1; (B) benzene:acetic acid:water, 2:2:1, organic phase; (C) t-butyl alcohol:methyl ethyl ketone:water:diethylamine, 10:10:5:1; (D) ethyl acetate:cyclohexane, 3:4; (E) benzene:triethylamine, 8:1; and (F) isopropyl alcohol:concentrated HCl:water, 80:30:20. Paper electrophoresis was performed on a flat bed device in the following buffers: (A) 0.1 M sodium phosphate, pH 6.6, and (B) 1.5 M formic acid:2 M acetic acid, 1:1 (v/v), pH 2.

As sprays and detection aids, we used 0.4% ninhydrin in acetone, I₂ vapor, 1.0% ferric chloride in 0.1 N HCl, and ultraviolet illumination.

Thin layer plates of silica gel and cellulose were obtained from EM Laboratories. Thin layer sheets of silica gel on plastic, obtained from Brinkmann Instruments, were used for chromatography of catechols.

Threonine was determined on a Beckman model 120 amino acid analyzer by the method of Spackman et al. (10).

Spectroscopy—Ultraviolet and visible spectra were recorded with a Beckman model 25 instrument. For nuclear magnetic resonance spectroscopy, we employed the following spectrometers located at the University of California, Berkeley: the 220 and 270 MHz instruments previously isolated from Micrococcus denitrificans N.C.I.B. 8944 by Tait (4).

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Agrobactin, a Siderophore from A. tumefaciens

in the Biodynamics Laboratory and the 180 MHz instrument in the Chemistry Department, all of custom design, and the 60 MHz Varian model EM-360 in the Biochemistry Department. One spectrum was recorded on a 220 MHz Varian model HR-220 spectrometer, Chemistry Department, University of California, San Diego.

**Ferric Complexes—** These were prepared in 50% aqueous methanol and brought to neutral pH with a disulfonic recrystallizing ultracare (11). The small amounts required for growth tests were obtained by equilibration with ferric triacetohydroxamate followed by separation on paper electrophoresis, as previously described (12). As a first approximation, the $E_{270} = 5.6 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$ for ferric enterobactin (12) was assumed to hold for ferric agrobactin.

**Gel Permeation Chromatography—** Ferric agrobactin was chromatographed on a column (1 x 29 cm) containing either Sephadex G-10 or Bio-Gel P-4 equilibrated in 10 mM Tris-HCl, pH 7.8.

**Growth Factor Assay—** The ability of the ferric complexes to support the growth of enterobactin-deficient strains of enteric bacteria was examined by placing 6-mm filter paper discs impregnated with 10 μl of test solution on the surface of seeded agar plates. E. coli K-12 strains RW193 and B18 (13) were propagated on nutrient agar containing 100 μM deferriferrihexochromic A (14) while Medium E (15) was seeded with 1000 colony-forming units per ml of A. tumefaciens B6 and approximately 25 ml of agar was dispensed into each Petri dish. Filter paper discs (6 mm) containing 10 μl of test solution on the surface of seeded agar plates. E. coli K-12 was examined by placing 6-mm filter paper discs impregnated with 100 μM deferrified rhodochromic A (14) while Medium E (15) was used as inoculum per 50 ml of agar medium. The diameter of growth was measured after incubation overnight at 37°C.

**Colicin B Protection Assay—** Exactly 5 μl of a colicin B solution (1074 killing units per ml) was added to 2 ml of plain top agar and poured over a plate containing 1.3% tryptone agar. The plate was spotted with 2-μl portions of 100 μM ferric enterobactin (control) and with similar volumes of approximately 100 μM solutions of ferric agrobactin and ferric agrobactin A and then layered with 2 ml of plain top agar seeded with three drops of an overnight culture of E. coli RW193 (6). Incubation was overnight at 37°C.

**Reversal of Iron Starvation of A. tumefaciens—** LB agar (5% NaCl) was prepared containing 1.5% agar and 1 mg per ml of a Millipore-sterilized solution of EDDA. The latter was added to the molten, sterile agar which was then allowed to stand for at least 24 h at 4°C prior to the assay to permit the slow chelation of all adventitious iron. The EDDA stock solution was prepared by dissolving 1 g of deferrated EDDA, the iron having been removed as described by Rogers (17), in 15 ml of 1 N NaOH. The pH was adjusted to 9 with concentrated HCl and the total volume brought to 20 ml per g of EDDA stock solution was prepared by dissolving 100 μM deferriferrichrome A (14) while Medium E (15) was used as inoculum per 50 ml of agar medium. The diameter of growth was measured after incubation overnight at 37°C.

**TABLE I**

| δ | Type | Integration | Assignment |
|---|------|-------------|------------|
| 1.5 | 2 D₀ | 3 | Threonine γ-CH₁ |
| 1.7 | M | 6 | Spermidine C-CH-C |
| 3.5 | M | 8 | Spermidine N-CH-C |
| 4.6 | 2 D₀ | 1 | Threonine α-CH |
| 5.5 | M | 9 | Dihydroxybenzoic acid aromatic CH |
| 6.6-7.2 | M | 10 | Dihydroxybenzoic acid aromatic OH |
| 8.1 | S | ~3' | Dihydroxybenzoic acid meta OH |
| 8.2 | T | 2 | Dihydroxybenzoic acid amide NH |
| 11.8 | S | ~1' | Dihydroxybenzoic acid ortho OH (central) |
| 12.7 | S | ~2' | Dihydroxybenzoic acid ortho OH (terminal) |

*δ = singlet; D = doublet; T = triplet; M = multiplet.

*Integration complicated by broadening of the resonance.

**RESULTS**

**Purity and Properties**

Thin layer chromatography in Solvent A on silica gel revealed a single spot with $R_F$ of 0.64, strong blue fluorescence in the ultraviolet, and positive ferric chloride and I₂ vapor reactions.

Agrobactin was found soluble in the lower alcohols, ether, ethyl acetate, tetrahydrofuran, dioxane, and sparingly soluble in water, hexane, and benzene. It decomposed at 108 to 112°C without melting to a clear liquid.

The electronic absorption spectrum in ethanol showed a broad peak with $E_{230} = 9.6 \times 10^2 \text{M}^{-1} \text{cm}^{-1}$, a minimum at 279 nm and a sharper peak deeper in the ultraviolet with $E_{295} = 28.3 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$.

**Elemental Composition**

$C_{23}H_{23}O_{11}N_9$

Calculated: C, 60.37; H, 5.70; N, 8.80

Found: C, 60.07; H, 5.83; N, 8.63

**Nuclear Magnetic Resonance Spectra**

The chemical shifts for agrobactin relative to tetramethylsilane at 25°C are reported in Table I.

**Effect of Acid on Agrobactin**

The absorption spectra of agrobactin upon exposure to mild acid for varying lengths of time are recorded in Fig. 1.

**Hydrolytic Fragments**

A 6.4-μg (10 μmol) quantity of agrobactin was hydrolyzed in 6 N HCl in a sealed, evacuated tube for 48 h at 110°C. 2,3-Dihydroxybenzoic Acid—The hydrolysate was diluted with a few milliliters of water and extracted exhaustively with ether. The ether was evaporated and the residue was dissolved in a measured volume of ethanol. The absorption spectrum of this solution was virtually identical with that of authentic 2,3-dihydroxybenzoic acid and the yield, based on $E_{270} = 3.2 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$, was 80%. The alcohol was concentrated to a

![Fig. 1. Absorption spectrum of 0.05 mM agrobactin in 10% N HCl, 90% ethanol at room temperature after (a) 0, (b) 85, (c) 355, (d) 1025, and (e) 1440 min, respectively. The $t_{1/2}$ of the first order reaction was 350 min. Isosbestic points were noted at 338, 294, 252, and 241 nm.](http://www.jbc.org/)
small volume and examined by paper electrophoresis and by descending chromatography on Whatman No. 1 paper with ultraviolet fluorescence and ferric chloride spray as detection agents. In Buffer A and in Solvents B and C, the product had the same mobility as 2,3-dihydroxybenzoic acid, namely, an anodic migration and RF values of 0.33 and 0.70, respectively.

Threonine—The aqueous portion of the hydrolysate was evaporated to dryness to remove excess HCl, the residue taken up in a small, measured volume of water, and an aliquot applied to the amino acid analyzer. The single amino acid identified was threonine, which was obtained in a yield of approximately 40%. This low yield may be attributed to a combination of destruction during acid hydrolysis and to incomplete scission of the threonyl-spermidine bond.

The hydrolysate was chromatographed on Whatman No. 1 paper in system C of Dietzler and Strominger (18) and by the method of Hardy and Holland (19). The amino acids were visualized with 0.4% ninhydrin in acetone. The mobilities relative to L-threonine in the first solvent system were DL-threonine, 0.68; unknown, 0.96; and in the second system the RF values were L-threonine, 0.67; DL-threonine, 0.43; and unknown, 0.57.

Spermidine—The hydrolysate, as well as authentic spermidine, putrescine, and cadaverine, were dansylated and then chromatographed on thin layer silica gel first in Solvent D and then after drying at room temperature for 20 min in Solvent E (20). The dansylated polyamine in the hydrolysate co-migrated with dansyl-spermidine and moved more slowly than either dansyl-putrescine or dansyl-cadaverine.

In addition, the undansylated hydrolysate was chromatographed in Solvent F on thin layer cellulose (21). A ninhydrin-positive, nonfluorescing spot was observed with an RF of 0.28, identical with that of authentic spermidine. Threonine and the threonyl-spermidine peptide (see discussion of the position of the threonyl substituent) were detected and shown to have RF values of 0.57 and 0.18, respectively.

Position of the Threonyl Substituent

A 12.7-mg (20 μmol) quantity of agrobactin was hydrolyzed in 6 N HCl in a sealed tube at 110°C for 12 h. The hydrolysate was diluted with a small amount of water and the 2,3-dihydroxybenzoic acid was removed by extraction into ether. The aqueous phase was evaporated to dryness, dissolved in a few tenths of a milliliter of water and applied in a line across a 13-cm-wide sheet of Whatman No. 3 filter paper. After electrophoresis for 1.25 h at 20 mA and 1 kV in Buffer B, a strip cut from the edge of the sheet showed the presence of a number of fluorescent and ninhydrin-positive spots, two of which were identified with the aid of the markers threonine and spermidine. A major, ninhydrin-positive but nonfluorescent zone, judged by its relative mobility to be a trivalent cation, was eluted from the paper, concentrated, and divided into two aliquots, one of which was subjected to total hydrolysis in 6 N HCl for 48 h. Upon electrophoresis of the hydrolysate, new spots appeared corresponding to threonine and spermidine. The remaining aliquot, as well as various standards consisting of primary, secondary, and primary-secondary mixtures of amines, were treated with excess 2,4-dinitrofluorobenzene as described by Dubin (22) and as utilized for this purpose by Tait (4). The spectral ratios 350/390 nm found were: spermidine 1.51, putrescine, 2.45, diethylenamine, 0.70, unknown, 2.23.

Agrobactin A

Exactly 64 mg (100 μmol) of agrobactin was dissolved in 2.7 ml of ethanol, 0.3 ml of 1 N HCl was added, and the solution allowed to stand at room temperature for 24 h. After removal of the solvent, examination of the residue by thin layer chromatography on silica gel in Solvent A showed the presence of only a trace of agrobactin and a major new fluorescent and ferric chloride-positive spot with an RF value of 0.48. The product was dissolved in a trace of methanol, diluted with ethyl acetate, washed with citrate buffer and water, dried over MgSO₄, concentrated, and precipitated with n-hexane. The transition from agrobactin to agrobactin A resulted in approximately a 20% loss in absorbance of the second peak at 252 nm relative to the first one at 316 nm and a blue shift of its maximum of 3 nm. The preparation was contaminated with a small amount of agrobacin.

\[ \text{C}_{10} \text{H}_{16} \text{O}_4 \text{N}_3 \]

Calculated: C, 58.71; H, 5.85; N, 8.56
Found: C, 57.19; H, 5.70; N, 8.11

Ferric Complexes

On paper electrophoresis in Buffer A, ferric agrobactin moved as a discrete spot to the anode with a mobility 0.86 times that of ferrichrome A. In contrast, ferric agrobactin A migrated as an elongated streak with a mobility substantially slower than that of ferric agrobactin. The latter was wine-colored, whereas ferric agrobactin A was blue; the maxima of the broad absorption band in the visible lay at 503 and 513 nm, respectively.

Gel Permeation Chromatography

Ferric agrobactin was not readily chromatographed on either Sephadex G-10 or Bio-Gel P-4. On G-10, the ferric complex streaked down the column and on P-4 it moved as a single, tight band eluting with an apparent molecular weight of less than 200 as judged by its relative mobility compared to the markers vitamin B₇, oxidized glutathione, and the siderophore ferrichrome (M₉ = 740).

Activity with Enteric Bacteria

Agrobactin and ferric agrobactin were found unable to act as siderophores in E. coli RW193 or B18, but agrobactin A and ferric agrobactin A showed approximately 10% of the activity of ferric enterobactin in both of these strains. Similarly, in S. typhimurium TA2442 and TA2443, agrobactin and its ferric derivative did not promote growth while agrobactin A and its ferric complex did so at about 50% of the potency of ferric enterobactin.

Ferric agrobactin had no detectable ability to reverse the killing action of colicin B in E. coli RW193.

Reversal of Inhibition of Growth of A. tumefaciens

As shown in Table II, only agrobactin, agrobactin A, and ferric agrobactin were found unable to act as siderophores in E. coli RW193 or B18, but agrobactin A and ferric agrobactin A showed approximately 10% of the activity of ferric enterobactin in both of these strains. Similarly, in S. typhimurium TA2442 and TA2443, agrobactin and its ferric derivative did not promote growth while agrobactin A and its ferric complex did so at about 50% of the potency of ferric enterobactin.

Reversal of EDDA-inhibited growth of A. tumefaciens B6

| Test solution | Growth (diameter) |
|---------------|------------------|
| 2.5 mM agrobactin | 48 mm |
| 250 μM agrobactin A | 40 mm |
| 25 μM agrobactin | 30 mm |
| 2.5 mM agrobactin A | 46 mm |
| 250 μM agrobactin A | 41 mm |
| 25 μM agrobactin A | 30 mm |
| 2.5 mM 2,3-dihydroxybenzoic acid | nil |
| 250 μM 2,3-dihydroxybenzoic acid | nil |
| 25 μM 2,3-dihydroxybenzoic acid | nil |
| 10 mM FeSO₄ | 25 mm |
| Trace elements mixture | nil |
| Distilled water | nil |

* Methanolic solutions of catechols were employed and the solvent was allowed to evaporate from the discs prior to the assay.

\[ 0.18 \text{ mg of } \text{ZnSO}_4 \cdot 4 \text{H}_2 \text{O}, 0.20 \text{ mg of } \text{CuSO}_4 \cdot 5 \text{H}_2 \text{O}, 0.10 \text{ mg of } (\text{NH}_4)\text{MoO}_4, \text{ and } 0.10 \text{ mg of } \text{H}_2 \text{BO}_3 \text{ in } 200 \text{ ml of distilled water (36).} \]
levels of inorganic iron high enough to saturate the EDDA in the medium promoted the growth of *A. tumefaciens* B6. This stimulatory effect was evidenced by a halo of large, single colonies around discs containing iron or iron-feeding substances while background growth was negligible. Similar results were obtained using minimal medium 925 described by Langley and Kado (23) containing either 0.5 or 1.0 mg/ml of EDDA (data not shown). This system, however, was less satisfactory as incubation times were for 72 h and background growth was more extensive.

**Repression of Biosynthesis by Iron**

Complete repression of agrobactin synthesis was observed at 5 μM added iron and the yield was reduced at 1 μM added iron.

**DISCUSSION**

When cultured on low iron media, *A. tumefaciens* produced a neutral, ethyl acetate-extractable substance. The characteristic blue fluorescence in the ultraviolet (24) and the positive Arnow (25) reaction indicated that it belonged to the catechol rather than to the hydroxamic acid line of siderophores (26). Of the former, the only known neutral members are enterobactin (27) and Compound III of Tait (4). Agrobactin was chromatographically distinct from enterobactin and failed to yield chromatographic or electrophoretic evidence for the presence of salicylic acid following hydrolysis in hot mineral acid. Furthermore, examination of the ultraviolet spectra of model compounds in ethanol showed that 1 mol of a salicyl amide residue in the presence of 2 mol of a 2,3-dihydroxybenzamide was sufficient to shift the 316 nm peak of the latter some 9 nm to the blue. Thus, the major spectral differences observed between Tait’s Compound III and agrobactin can be accounted for by the absence of salicylic acid in the latter substance.

Chromatographic analysis of a vigorous acid hydrolysate of agrobactin afforded evidence for the presence of 2,3-dihydroxybenzoic acid, sperrmine, and threonine, the latter established by special chromatographic techniques to be threonine rather than allo-threonine. Like all 2,3-dihydroxybenzonic acid-containing compounds (24), agrobactin displayed a broad electronic absorption band with a maximum at about 316 nm and a second, sharper and more intense band with a peak at 252 nm. The spectrum in ethanol closely resembled curve (c) in Fig. 1. A careful comparison of the ultraviolet absorption spectrum of agrobactin with those of various model amides of 2,3-dihydroxybenzoic acid revealed that the former displayed substantially more absorption in the range 250 to 290 nm. The absorbance in this wavelength region was enhanced greatly in acidic media; upon standing in acid, the spectrum gradually reverted to one more closely resembling those of simple 2,3-dihydroxybenzamide amides rather than to the hydroxamic acid line of siderophores (26). Of the former, the only known neutral members are 2,3-dihydroxybenzamides. This is further evidence for the absence of a salicyl amide residue in the compounds from *A. tumefaciens*.

The possible presence of an oxazoline ring in the compound produced by *M. denitrificans* cannot be ruled out in view of the routine use of acidic conditions during its isolation from both culture fluids and enzyme reaction mixtures (4).

The method of Dubin (22), which depends on the differential absorbance ratio at 350/390 nm for 2,4-dinitrofluorobenzene derivatives of primary and secondary amines, established the secondary amine nitrogen as the position of the threonyl-spermidine bond.

The NMR spectrum of agrobactin is summarized in Table I. The phenolic protons varied in position and intensity, sometimes being completely absent in solvents containing d<sub>6</sub>-dimethylsulfoxide. Most interesting was the fact that two o-phenolic resonances were observed, integrating in 2:1 ratio. We interpreted this as indicative of different environments for these protons. Amides of 2,3-dihydroxybenzoic acid generally form H-bonds between the o-hydroxyl and the carbonyl oxygen (31), but the central o-hydroxyl of agrobactin cannot do this as the 2,3-dihydroxybenzoyl carbonyl is incorporated into an oxazoline ring. Instead, H-bonding is to the oxazoline nitrogen or oxygen.

The NMR spectrum of agrobactin A was very similar to that of agrobactin, except that the α- and β-threonine protons were shifted slightly and the two o-phenolic resonances were fused into a single resonance. This is consistent with the proposed structure of agrobactin A since all o-hydroxyls are now equivalent. The coupling constants of the threonine residue in agrobactin were measured to determine the conformation of the oxazoline ring. Also examined was the model compound 2-phenyl-4-carbomethoxy-5-methyl-cis-oxazoline,
prepared by the method of Pfister et al. (32). $J_{\alpha \gamma}$ was 6.8 Hz in agrobactin and 10.2 Hz in the model cis-oxazoline. Since $J_{\alpha \gamma} > J_{\alpha \beta}$, we concluded that a trans-oxazoline exists in agrobactin. This corresponds to threonine and not allo-threonine. As stated above, the former was detected in the acid hydrolysate, a procedure which would not be expected to cause inversion.

The $\alpha$ and $\gamma$ resonances of the threonine residue were not simple doublets, but were each two doublets of unequal size, together integrating for one and three protons, respectively. Agrobactin may exist in two conformations in solution. We speculate that the difference between the two forms is that the $\alpha$-hydroxyl of the central 2,3-dihydroxybenzoic acid residue is $H$-bonded alternatively to the oxygen and nitrogen of the oxazoline ring.

The presence of spermine was ruled out by Dubin's (22) assay, by the elemental analysis, by the neutron character and/or lack of an additional acyl substituent, and by the NMR data. The NMR spectra also eliminated bis-(3-aminopropyl)amine as an alternative to spermidine since the ratio of the areas of $C_2 CH_2$ and $N CH_2 C$ resonances was precisely 6:8, as expected for spermidine, whereas the bis-(3-aminopropyl)amine would have given a ratio of 4:8.

Despite numerous attempts at purification and extensive drying in vacuo, the agrobactin crystallized from ethyl acetate/hexane always contained a variable amount (0.15 to 0.80 mol equivalent) of ethyl acetate and lesser amounts of hexane, as determined by NMR. Attempts to crystallize agrobactin from other solvents yielded only gummy, amorphous material, indicating that ethyl acetate was part of the crystal. Ethyl acetate contamination has been noted previously in the case of the siderophore bis-(2,3-dihydroxybenzoyl)lysine (33).

The behavior of agrobactin with ferric ion differed in important respects from that noted with the tricatcchol siderophore of enteric bacteria, enterobactin (34). For example, the complex was more completely formed in acidic media. Acidification of dilute aqueous solutions of ferric agrobactin, which was highly charged and hence very water-soluble, resulted in precipitation of the complex without dissociation of the coordinated ferric ion. The difficulty in removing iron and the lability of the ligand in acidic media frustrated all attempts to isolate the substance via its ferric complex. The characterization of the metal complexes of agrobactin, which appear to be optically active, will be reported in a subsequent communication. It should be recalled that an X-ray diffraction of the metal complexes of agrobactin, which appear to be optically active, will be reported in a subsequent communication.

Because of its aromatic character, ferric agrobactin was severely retarded on permeation gels. Thus the molecular weight of the ferric complex could not be determined by this method.

A number of variations in the composition of the medium were tried in order to enhance the yield of agrobactin extractable into ethyl acetate. While the total amount of catechol was variable, the yield of agrobactin remained constant at about 5 to 10 mg/liter. This is probably close to the solubility limit of agrobactin in water. Indeed, preliminary experiments suggest that extraction of the cell debris with methanol will yield substantial amounts of agrobactin. Obviously, the sensitivity to acid (Fig. 1) precluded the use of the low pH step generally employed for the extraction of catechol type siderophores into organic solvents (27).

Ferric agrobactin did not support the growth of the siderophore auxotrophs E. coli RW193 and B18 or S. typhimurium TA2442 and TA2443, nor compete with colicin B for the outer membrane ferric enterobactin receptor of RW193. Thus the siderophore of A. tumefaciens appears unable to utilize the specific receptor for ferric enterobactin in enteric bacteria. Agrobactin A, however, gave a small growth response. In the disc assay with E. coli RW193, a 10^{-u} volume of 10 to 20 $\mu M$ ferric enterobactin sufficed to give a halo of growth with diameter of 10 to 20 mm. With RWB18, which lacks the outer membrane ferric enterobactin receptor, a concentration of ferric enterobactin in excess of about 100 $\mu M$ was required to achieve this magnitude of response. Since comparable concentrations of agrobactin A or its ferric complex were needed to obtain halos of similar dimension in either RW193 or B18, we speculate that both ferric agrobactin and ferric agrobactin A penetrate the cell by nonspecific transport and that the iron of the latter, which is possibly less firmly bound, is available to the cell. Other reasons for the total absence or low activity of these compounds might be accounted for by the chirality of the coordination center or by a polymeric form of the metal complex. While the first possibility cannot be ruled out, the second probably can as the ligands had comparable activities to their ferric derivatives.

At concentrations of the iron-selective chelating agent EDDA (36) which were inhibitory to growth, agrobactin, agrobactin A, and FeSO$_4$, but not 2,3-dihydroxybenzoic acid or a mixture of trace elements lacking iron, were found capable of stimulating the growth of A. tumefaciens B6. In addition, the synthesis of agrobactin was tightly regulated by the availability of iron in the growth medium, being totally repressed at 5 $\mu M$ added iron. These data suggest that the biological role of agrobactin is the transport of iron in A. tumefaciens and that it is in fact a siderophore. A systematic survey of the cross-reactivity of agrobactin in siderophore-requiring systems in diverse organisms and the ability of exogenous siderophores to transport iron in A. tumefaciens is a study which awaits completion.

Future experiments with agrobactin will be focused on the mechanism of iron transport and on its role in the virulence of A. tumefaciens. Eventually, this work will be extended to fungal as well as other bacterial plant pathogens.

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S A Ong, T Peterson and J B Neilands

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