A catechol-type siderophore, assigned the trivial name chrysobactin, was isolated from the phytopathogenic bacterium Erwinia chrysanthemi and characterized by degradation and spectroscopic techniques as \( N^\bullet(2,3\text{-dihydroxybenzoyl})\text{-L-lysyl}\text{-L-serine} \). Chrysobactin, which was also obtained by chemical synthesis, was shown to be active in supplying iron to a group of mutants of \textit{E. chrysanthemi} defective in biosynthesis of the siderophore.

Most cells have an absolute requirement for iron. Due to the insolubility of ferric hydroxide, the element is often growth limiting under aerobic conditions. Siderophores, highly efficient and virtually Fe\(^{3+}\)-specific chelators, are synthesized by a majority of aerobic and facultative anaerobic microorganisms under conditions of iron deprivation (Neillands, 1981).

Iron can also be growth limiting for microbes in host-microbe interactions. In mammals, where most of the iron is either stored intracellularly or bound to proteins such as transferrin or lactoferrin, bacterial virulence has been correlated to siderophore production (Weinberg, 1984). A correlation between siderophore production and virulence has also been found in fish pathogens (Crosa, 1989). The situation in plant-microbe interactions is less clear (Neillands and Leong, 1986). Siderophores from plant growth-promoting rhizobacteria have been shown to increase crop yields through chelating iron in the rhizosphere (Kloepper et al., 1980). Plant deleterious and plant growth-promoting pseudomonads can be classified according to their ability either to utilize or be inhibited by siderophores (Leong, 1986). In a survey of several bacterial plant pathogens, the majority was found to produce siderophores (Leong and Neillands, 1982). Siderophore production was in the case of \textit{Agrobacterium tumefaciens} A217 shown not to be linked to pathogenicity (Leong and Neillands, 1981).

In agriculturally important pectinolytic \textit{Erwinia}, which causes soft-rot diseases in a variety of plants (Collmer and Keen, 1986), a correlation was found between the lack of pathogenicity and the absence of three outer membrane proteins. These proteins were induced in the wild type under low iron conditions (Expert and Toussaint, 1985). In the same strain, \textit{Erwinia chrysanthemi} 3937 which causes systemic disease in \textit{Sainopaullia} plants, it was found recently that a catechol-type, siderophore-dependent iron assimilation system was required for the expression of bacterial virulence throughout the plant (Enard et al., 1988).

In the present communication we present the isolation, characterization, synthesis, and certain biological properties of the novel siderophore chrysobactin, which may be described as \( N^\bullet(2,3\text{-dihydroxybenzoyl})\text{-L-lysyl}\text{-L-serine} \). Chrysobactin has the unusual property for a catechol siderophore of possessing only three coordination sites. A minimum of two molecules is thus required to chelate hexacoordinated iron. Purified chrysobactin could support the growth of iron-starved mutants defective in the conversion of dihydroxybenzoic acid (DHBA)\(^1\) to chrysobactin. The synthesis of chrysobactin was accomplished through traditional methods. Synthetic chrysobactin was chemically and biologically indistinguishable from the natural product.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—**Chrysobactin was prepared from \textit{E. chrysanthemi} PMV 4098, a transport-defective derivative of the wild type \textit{E. chrysanthemi} 3937 (Enard et al., 1988). The biosynthetic mutants PMV 4096, PMV 4087, and PMV 4088, blocked in the biosynthesis of chrysobactin at three different stages between DHBA and the final product, and the transport mutant PMV 4082 (Enard et al., 1988) were used as indicator strains in a siderophore bioassay.

**Materials—**All glassware was washed in 6 N HCl and rinsed in double-distilled water prior to use. 3-Dimethylaminonaphthalene-1-sulfonic acid (dansyl chloride), N-ethylmorpholine, and polyamide sheets (Cheng Chin) were from Pierce Chemical Co. Benzaldehyde, D, O, XAD-4, EDDA, carboxypeptidase \( \text{Y} \), lysine decarboxylase, pyridoxal phosphate, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), dansylamino acids, N-benzyloxycarbonyl-L-lysine (N-CBZ-L-lysine), N-NCBZ-L-lysine, L-serine benzy ester hydrochloride and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl were from Sigma. Hydrazine, DHBA, and 1,5-diaminopentane were from Aldrich. Sephadex was from Pharmacia LKB Biotechnology Inc., and DEAE-trisacryl M was from LKB. Desferal was a gift from Ciba Geigy.

**Isolation of Chrysobactin—**For chrysobactin production 10 ml of an overnight culture of \textit{E. chrysanthemi} 4098 in LB was inoculated into each of 10 2800-ml Fernbach flasks containing 500 ml of MM-9 broth, 5 g of NaCl, 6 g of Difco agar/liter.

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Chrysobactin, a Siderophore from *E. chrysanthemi*

on a rotary shaker at 30 °C for 36 h, after which the cells were removed by centrifugation.

The cell-free medium was passed slowly through a 5 × 60-cm column of XAD-4, and after washing the column with water adsorbed chrysobactin was eluted with water:methanol (1:1). Fractions positive by the NMR method were collected (187) and analyzed. The fractions were evaporated under reduced pressure at 30 °C. The dark-brown material, approximately 7.5 ml, was filtered through a 2.5 × 90-cm Sephadex G-25 column equilibrated with 5 mM ammonium acetate, pH 5.5. Arrow-positive fractions were collected, evaporated, and rechromatographed on G-25 as above. Fractions containing catechol material were lyophilized and dissolved in a minimal volume of water. This material was chromatographed on a Hewlett-Packard HP1090 liquid chromatograph, equipped with a UV/visible diode-array detector, using a 10 × 250-mm LiChrosorb RP-18 column (Merck). Separation was achieved at a flow rate of 5 ml/min by isocratic elution in 10 mM triethylammonium acetate, pH 5.5, 3% acetonitrile, 2.5% methanol for 5 min and by a linear gradient to 15% methanol for 15 min and to 85% methanol for 10 min in the same buffer. Fractions with significant absorbance at 315 nm were collected. The chrysobactin-containing major peak which eluted after approximately 10 min (data not shown), was concentrated and passed through a 1.5 × 60-cm Sephadex G-10 column equilibrated in 5 mM ammonium acetate, pH 5.5, and Arrow-positive fractions were pooled and lyophilized. As a final purification step, a solution of chrysobactin in water was passed through a short column of DEAE-trisacryl M, chloride form, with the amino acid analyzer operated by the Protein Chemistry Facility. The chrysobactin solution had a pH of approximately 3.5 before deuterium exchange. Chemical shifts were expressed relative to an internal standard of acetone, equal to 2.225 ppm. Peaks were identified by UV illumination or spraying with 0.5% ninhydrin in acetone.

**Amino Acid Analysis**—Chrysobactin equivalent to approximately 90 nmol of DHBA by the Arrow assay was hydrolyzed at 100 °C in vacuo in 6 N HCl for 18 h. Following evaporation of acid at reduced pressure, the hydrolysate was analyzed by paper chromatography or with the amino acid analyzer operated by the Protein Chemistry Laboratory, Scripps Institution of Oceanography, University of California, San Diego, as follows: Following hydrolysis in 6 N HCl for 24 h of 180 μg (500 nmol) of chrysobactin, the sample was derivatized with p-phthalaldehyde/N-acetyl-L-cysteine and analyzed by high performance liquid chromatography.

Lysine decarboxylase was used to determine the chirality of lysine, following the method of Boeker and Fischer (1983). Two mg (5.4 μmol) of chrysobactin was hydrolyzed in vacuo in 0.5 ml of 6 N HCl for 24 h. The hydrolysate was extracted with 3 × 500 μl of ether to remove DHBA, after which excess HCl was removed under reduced pressure. The hydrolysate was dissolved in water and divided into two test tubes, to one of which was added 2.7 μmol of L-lysine. Both samples were lyophilized and dissolved in 400 μl of 0.2 M sodium acetate buffer, pH 5.7, containing 60 μM pyridoxal phosphate, after which 0.45 units of lysine decarboxylase was added. Samples were incubated at 37 °C for 3 h and reaction products identified by paper chromatography at pH 6.5 with 1,5-diaminopentane, lysine, and serine as references, and by amino acid analysis. A sample containing 2.7 μmol each of L-serine and L-lysine was treated identically as a reference.

**Chemical Synthesis**—The two lysine isomers of chrysobactin were synthesized basically as described by Chimiai and Neilands (1984). One mmol (280 mg) of CBZ-L- or D-lysine was dissolved in 20 ml of tetrahydrofuran, 8 ml of H₂O, and 0.6 ml of triethylamine. To this was added 1 mmol (455 mg) of p-nitrophenyl ester of 2,3-dibenzoyloxybenzoic acid (Chimiai and Neilands, 1984) in 2 ml of tetrahydrofuran. The solution was stirred for 1 h and organic solvents removed by evaporation. The reaction mixture was evaporated and dissolved in ethyl acetate. The organic phase was washed with 10% citric acid, saturated ammonium carbonate and brine, after which it was dried with MgSO₄, filtered and evaporated. The residual, yellowish oil was taken up in 20 ml ethanol and 1 ml of 2 N NaOH, which was filtered on a column with Sephadex G-10 and DEAE-trisacryl M as above. The yields of pure products were approximately 85%.

The acid and ethyl ester of the glylicycline analogs of chrysobactin were synthesized according to the procedure of Ito and Neilands (1958), with the following exceptions; Glycicycline ethyl ester HCl was neutralized with N-ethylmorpholine and dissolved in dimethyl formamide. As coupling agent 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl was used. The free acid was purified by gel filtration on a 10-cm column with Sephadex LH-20 using chloroform:methanol (95:5) as eluent. Fractionation was checked by paper electrophoresis. The reaction mixture was evaporated and dissolved in ethyl acetate. The organic phase was washed with 10% citric acid, saturated ammonium carbonate and brine, after which it was dried with MgSO₄, filtered and evaporated. The residual, yellowish oil was taken up in 20 ml ethanol and 1 ml of 2 N NaOH, which was filtered on a column with Sephadex G-10 and DEAE-trisacryl M as above. The yields of pure products were approximately 85%.

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Tris, pH 6.0, was titrated with 5-μL portions of 1.0 mM FeCl₃ and absorbance changes at 550 nm recorded. In addition we used Job's method of continuous variation to assess the formation of a single metal chelate complex (Chaberek and Martell, 1959). The molar chryso·bac·tin fraction in 10 samples of a 0.2 mM chryso·bac·tin/Fe³⁺ mixture in 0.1 M MOPS buffer, pH 6.5, was varied from 0.33 to 0.98 and absorbances were measured.

**Siderophore Bioassay**—Biological activity of chryso·bac·tin for one transport mutant and three mutants of *E. chrysanthemi* 3937 deficient in the synthesis of chryso·bac·tin was determined in a bioassay under low iron conditions. Iron deficient L-broth containing 1.5% agar was prepared by adding 100 μg/ml filter-sterilized EDTA to the liquid medium. The agar was allowed to stand at 4 °C for 48 h to allow slow chelation of iron. The medium was then remelted and seeded with 10⁶ or 10⁷ CFU/ml of indicator cells from exponential cultures in L-broth. Sterile disks with 6-mm diameter were placed on the agar surface and 10-μl portions of the following solutions were added: chryso·bac·tin, 0.01, 0.12, and 1.2 mM; supernatant from *E. chrysanthemi* PM 4098 grown in MM9, 0.01 and 0.1 mM; enterobac·tin, 0.01 and 0.12 mM; DHBA, 0.1 and 1.0 mM; FeCl₃, 1.0 and 10 mM; and H₂O. All concentrations refer to DHBA equivalents, as determined by the Arnow assay. Plates were incubated at 30 °C and diameters of zones of growth of the indicator strains measured after 24, 36, and 48 h.

**RESULTS**

**General Characteristics**—Growth of *E. chrysanthemi* PM 4098 in MM9 medium was accompanied by production of Arnow-positive material, equivalent to at most 0.13 mM DhBA after 24 h of incubation. Purified chryso·bac·tin had spectral properties typical for catechol compounds (Ito and Nei·lands, 1958; Corbin and Bulen, 1969). At pH 6.5 in 0.1 M MOPS buffer, absorption maxima were found at 227 and 318 nm (ε₉₀ = 3.0), with a shoulder at 247. The ferric complex, at the same pH, had absorption maxima at 228, 333 (ε₉₀ = 11.2), and 570 nm (ε₉₀ = 3.8), with a shoulder at 255 nm. The color of the ferric complex was highly dependent on pH and also on the ratio of iron to chelator. At high pH or at a low iron to chelator ratio, the color was burgundy. With decreasing pH or increasing iron ratio at higher pH, the color shifted toward violet blue.

Chryso·bac·tin gave a violet ninhydrin reaction, was neutral upon electrophoresis at pH 4.4 and 6.5, and showed a net charge of +1, pH 1.9. Ferric chryso·bac·tin had a net charge of −1 upon electrophoresis at pH 6.5.

Amino acid analysis revealed lysine and serine with a molar ratio of 1:0.94. This corresponded to roughly 1.4 DHBA equivalents with the Arnow test. A slightly elevated value for DHBA relative to threonine in agrobac·tin was found by Ong et al. (1979), which was attributed to a combination of incomplete scission and destruction during hydrolysis. The reasons may be similar in the case of chryso·bac·tin. In order to determine the number of DHBA molecules per chelate, the CAS assay of Schwy·n and Nei·lands (1987) was used.

One CAS equivalent was equal to one chelated Fe³⁺, whereas one Arnow equivalent was defined as one DHBA. Enterobac·tin which has 3 DHBA residues/molecule (Pollack and Nei·lands, 1970), showed an CAS/Arnow ratio of 0.5, as expected. In the case of chryso·bac·tin 1 DHBA was found to correspond to between only 0.05 and 0.1 CAS equivalents (data not shown).

Since chryso·bac·tin contained serine and DHBA the presence of an oxazoline ring was thought possible, as in agrobac·tin (Ong et al., 1979), pararac·bin (Petersen and Nei·lands, 1979), and vibro·bac·tin (Griffiths et al., 1984). Spectroscopy, however, indicated an absence of an oxazoline ring (data not shown).

**Structure of Chryso·bac·tin**—The ¹H NMR spectrum of chryso·bac·tin in D₂O is shown in Fig. 1a. The three aromatic protons and the lysine and serine ₂C protons had integrals of one, as is shown in Table I. Proton NMR of chryso·bac·tin in 90% H₂O, 10% D₂O revealed two additional peaks, which were assigned to the ₂NH-serine and the ₂NH-lysine protons, respectively. The chemical shifts of most peaks corresponded well to literature values (Llinás et al., 1973; Bundi and Wüth·rich, 1976; Groš and Kalbitzer, 1988). However, it should be noted that the ₂NH-lysine and ₂CH-lysine signals were significantly lower than expected. Nonequivalence of the lysine ₂ protons caused the signal to be split into two multiplets.

Mass spectral analysis of the triethylammonium salt of chryso·bac·tin gave an m/z peak at 370.1614 in the cationic detection mode. This peak was assigned to [MH]+. In the anionic detection mode, a peak was observed at m/z 368 and was assigned to [M]−. Chryso·bac·tin therefore had a M of 369. An m/z peak at 739, assigned to the adduct ion [M₂H]+, was also observed. Ferric chryso·bac·tin failed to produce a molecular ion (data not shown).

Chryso·bac·tin thus consisted of 1 DHBA, 1 Lys, and 1 Ser. Furthermore, the downshifted ₂-lysine signals from proton NMR indicated that DHBA was attached to the ₂-nitrogen of lysine. Evidence for the presence of a free ⁺-amino group was obtained by dansylation, where the labeled product from a chryso·bac·tin hydrolysate comigrated with commercial N’-dansyllysine. A spot comigrating with serine on paper electrophoresis following hydrazinolysis, confirmed the presence of a free seryl carboxyl terminus and the peptide sequence as Ne-DHBA-Lys-Ser.

**Chirality**—Chryso·bac·tin was not a substrate to carboxypeptidase Y. The configuration of the serine residue was therefore determined by chemical modification, which revealed it to be the L-isomer.

The absolute structure of chryso·bac·tin was established by the finding that lysine decarboxylase did not affect lysine in a chryso·bac·tin hydrolysate. Commercial L-lysine in a L-ly·sinel-serine mixture, as well as L-lysine added to the chryso·bac·tin hydrolysate, was degraded and comigrated with 1,5-diaminopentane upon paper electrophoresis. The ratio of Lys/Ser, as determined by amino acid analysis, was 0.83 and 0.82 for the chryso·bac·tin hydrolysate and chryso·bac·tin hydrolysate spiked with an equimolar amount of L-lysine, respectively. The lysine residue therefore had ²- chirality.

The absolute structure of chryso·bac·tin was thus established as N’-[N’-(2,3-dihydroxybenzoyl)-D-lysyl]-L-serine (Fig. 2).

Synthetic chryso·bac·tin was indistinguishable from the natural product by NMR (Fig. 1b), UV/visible absorption spectroscopy, and FAB. The CD spectrum of the D-lysine isomer had a shape very similar to that of natural chryso·bac·tin, thus confirming the chirality (Fig. 3).

**Stoichiometry of Ferric Chryso·bac·tin**—Ferric chryso·bac·tin had a net charge of −1 upon electrophoresis at pH 6.5, as predicted for a 2:1 complex. Titration with Fe³⁺ gave an inflection point at 1.8 DHBA residues/chelate. By varying the mole fraction of chryso·bac·tin and Fe³⁺ in a solution with constant total molarity, absorbance maxima were obtained at the preferred molar ratio. The obtained value of 0.69 corresponds well to a 2:1 stoichiometry (Fig. 4). As mentioned, the ratio of CAS to Arnow was surprisingly low; about 0.1 instead of the expected 0.5. This discrepancy might be explained, as pointed out by Schwy·n and Nei·lands (1987), by a formation constant too low to allow complete exchange of iron from the CAS complex.

The glycy·glycine and glycy·glycine ethyl ester derivatives of DHBA were synthesized as a means to assess the involvement of the carboxyl-terminal carboxyl oxygen in the chelation of Fe³⁺. In these model compounds, the "backbone" is identical to that of chryso·bac·tin. However, the carboxyl oxy-
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Fig. 1. Proton NMR spectrum of chrysobactin (a) and synthetic chrysobactin (b) in D$_2$O (c). Chemical shifts are referred to an internal standard of acetone (r) and are listed in Table I.

### Table I

<table>
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<tr>
<th>Resonance</th>
<th>$\delta$ (ppm)</th>
<th>Coupling constants (Hz)</th>
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*Abbreviations: d, doublet; t, triplet; q, quartet; m, multiplet.

gen of the ester would not likely function as an iron chelating ligand. Chrysobactin and the free acid glycine analog had identical spectral properties, as well as identical Job's plots. However, the ethyl ester was more similar to chrysobactin than to DHBA (data not shown). At high ligand to iron ratios, 3:1 or higher, or at high pH at ratios of ligand to iron at 2:1 or lower, the complex had a red color similar to that of enterobactin. It is not clear if the red color signifies a 3:1 complex and, in the case of a 2:1 complex, if the fifth and sixth ligands are supplied by the carboxyl oxygens or water.

**Biological Activity**—Purified chrysobactin, together with other iron sources, was investigated for its qualitative ability to enhance growth of non-producing mutants. The results of the bioassay, summarized in Table II, showed that both chrysobactin and ferric chrysobactin could reverse iron starvation of biosynthetic mutants, but not of the transport mutant lacking the putative chrysobactin receptor. This indicated that the purified compound is actually a biologically active
siderophore, which is specifically taken up by the cell when chelated to ferric iron. In these preliminary experiments activity was, unexpectedly, associated also with the synthetic L-lysine analog. Growth stimulation, characterized by distinct colonies surrounding the disks, was also observed with Fe(III) at levels high enough to saturate the EDDA. Material from two of the major Arnow-positive peaks separated from chrysobactin by high performance liquid chromatography did not, however, support growth of the mutants (data not shown). These fractions might represent degradation products.

In the same concentration range, the purified compound appeared slightly less active than crude supernatant fluid of a low iron-induced culture of *E. chrysanthemi* PMV 4098. Since this culture supernatant could significantly enhance the growth of the receptor mutant, it may be assumed that iron is taken up through a second, not yet determined pathway.

Finally, the exogenous siderophore enterobactin was highly efficient in promoting growth of all mutants. This suggested that ferric enterobactin probably not was taken up through the chrysobactin outer membrane receptor. It should be mentioned that in the concentration range used, the enterobactin preparation stimulated the growth of an *Escherichia coli* ent A mutant, but not a *fep A* mutant lacking the enterobactin outer membrane receptor (data not shown).

In summary, the proposed structure of chrysobactin, which had a molecular formula of C_{16}H_{24}N_{3}O_{7} and an accompanying calculated mass spectral molecular weight of 370.1614, was consistent with its 1H NMR spectrum and its observed spectral molecular weight of 370.1609. Biological activity of chrysobactin was shown by its ability to support growth of iron-starved *E. chrysanthemi* mutants. Final proof for the proposed structure was obtained by synthesis. Synthetic chrysobactin was identical to the natural product, as determined by UV/visible absorption spectroscopy, FAB, NMR, and CD. Growth of iron-starved mutants was supported by chrysobactin and its L-lysine analog.

**DISCUSSION**

In this study, we have purified, characterized, and synthesized chrysobactin, a novel siderophore from *E. chrysanthemi*. Chrysobactin, with the structure N-[N2-(2,3-dihydroxybenzoyl)-D-lysyl]-L-serine, can supply at most three coordination sites for iron. The presence of L-lysine is notable, although D-amino acids are commonly found with peptide siderophores from, for example, *Pseudomonas* (Teintze et al., 1981). The only siderophores with structures resembling that of chrysobactin of which chirality has been determined is azotochelin (Corbin and Buleu, 1969) and 2,3-dihydroxy-N-benzoylserine (O'Brien et al., 1969). Both amino acids were determined to have the L-configuration.

The ability of the L-Lys-L-Ser isomer to support growth of the iron-starved mutants was unexpected and remains unexplained. O'Brien et al. (1970) found that the isomers of 2,3-
dihydroxy-N-benzylocysine were equally active in supporting growth of *E. coli*. However, in this case DHBA was more active and it therefore can not be ruled out that 2,3-dihydroxy-N-benzylocysine could have served as a source of DHBA for enterobactin synthesis, and not as a siderophore per se.

In hexacoordinated siderophores of both catechol and hydroxamate type, it has been shown that the iron center is specifically recognized by the outer membrane receptor. A change in amino acid chirality may lead to a change in iron center configuration and a biologically inactive siderophore (Neilands et al., 1981; Huschka et al., 1985). It is not known whether this is also the case for non-hexadentate siderophores, such as chrysobactin, or if other parts of the chelate also play an important role in receptor recognition. The question is presently under investigation.

*E. chrysanthemi* 3937 might produce additional siderophore(s), since the supernatant of minimal medium was shown to contain the iron center per se. Specifically, chrysobactin produces enterobactin synthesis. Likewise, biological activity with linear, non-hexacoordinated catechol-containing siderophores has been shown with a number of iron-induced monoamino acid catechols or decarboxylation products, isolated from several microbial sources (Table III). It has been suggested (O'Brien et al., 1970) that 2,3-dihydroxy-N-benzylocysine was present in the supernatant only as a degradation product of biologically active enterobactin. The number of simple, biologically active DHBA derivatives described (Table III) appears, however, to indicate that these compounds are in fact functioning as siderophores. Furthermore, no enterobactin-like trimer of the di- or tripeptide DHBA derivatives has been isolated. With the exception of azotochelin (Page and Huyer, 1984), the structure of the monooamino acid compounds would make a 3:1 siderophore-iron complex likely. No studies on the stoichiometry of siderophore-iron complexes that do not show 1:1 stoichiometry have been described previously. Rhodotorulic acid, a hydroxamate siderophore from *Rhodotorula pilimanae*, was shown by Carrano and Raymond (1978) to form an Fe₂RA₃ complex at physiological pH. In chrysobactin the o-hydroxyl and carboxyl-terminal oxygens are separated by nine atoms, a distance theoretically large enough to form a tridentate ligand and thus to allow the chelation of one hexacoordinated ferric ion by two chrysobactin molecules. This assumption was experimentally supported. However, the synthesis of 2,3-dihydroxybenzylocysine and its tripeptide had not unequivocally shown whether or not the carboxyl oxygens are involved in iron chelation.

Chrysobactin might be the first characterized siderophore from a plant pathogen, where iron has been directly correlated to virulence. Its stoichiometry and structure are unusual, but similar siderophores have been isolated. It thus appears that

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### Table III

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Source</th>
<th>Trivial name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td><em>Bacillus subtilis</em></td>
<td>Dhba</td>
<td>Ito and Neilands (1958)</td>
</tr>
<tr>
<td>Ser</td>
<td><em>E. coli</em></td>
<td>Azotocholin</td>
<td>Modi et al. (1985)</td>
</tr>
<tr>
<td>Thr</td>
<td><em>K. oxyaca</em></td>
<td>Aminochelin</td>
<td>Korth (1970)</td>
</tr>
<tr>
<td>Lys (Bis)</td>
<td><em>A. vinelandii</em></td>
<td>Azotocholin</td>
<td>Corbin and Bulen (1969)</td>
</tr>
<tr>
<td>Orn, Ser</td>
<td><em>Azospirillum brasilense</em></td>
<td>Spiribactin</td>
<td>Bachawat and Ghosh (1987)</td>
</tr>
<tr>
<td>α-Lys-Ser</td>
<td><em>E. chrysanthemi</em></td>
<td>Chrysoactin</td>
<td>This study</td>
</tr>
<tr>
<td>Lys, Leu</td>
<td><em>Azospirillum lipoforum</em></td>
<td></td>
<td>Saxena et al. (1986)</td>
</tr>
<tr>
<td>Lys, Gly, Phe</td>
<td><em>Aeromonas hydrophilica</em></td>
<td>Amonobactin P</td>
<td>Byers (1987)</td>
</tr>
<tr>
<td>Lys, Gly, Thr</td>
<td><em>A. hydrophila</em></td>
<td>Amonobactin T</td>
<td>Byers (1987)</td>
</tr>
</tbody>
</table>

*Pseudomonads* show promiscuity in their ability to utilize structurally related pseudobactin-type siderophores from other *Pseudomonas* strains (Leong, 1986). Griffiths et al. (1984) showed that both vibriobactin and agrobactin supported growth of iron-starved *Vibrio cholerae* Loui15. The similarity in general structural features, as well as around the iron center in the case of both pseudobactins and vibriobactin/agrobactin, makes the use of a common receptor conceivable. However, chrysobactin and enterobactin are structurally, as well as stoichiometrically, quite distinct and would therefore require separate receptors.

Despite its unfavorable stoichiometry, chrysobactin was biologically active, as shown by its ability to support growth of iron-starved *E. chrysanthemi* mutants deficient in chrysobactin synthesis. Likewise, biological activity with linear, non-hexacoordinated catechol-containing siderophores has been shown with a number of iron-induced monoamino acid catechols or decarboxylation products, isolated from several microbial sources (Table III). It has been suggested (O'Brien et al., 1970) that 2,3-dihydroxy-N-benzylocysine was present in the supernatant only as a degradation product of biologically active enterobactin. The number of simple, biologically active DHBA derivatives described (Table III) appears, however, to indicate that these compounds are in fact functioning as siderophores. Furthermore, no enterobactin-like trimer of the di- or tripeptide DHBA derivatives has been isolated. With the exception of azotochelin (Page and Huyer, 1984), the structure of the monoaamino acid compounds would make a 3:1 siderophore-iron complex likely. No studies on the stoichiometry have, however, been undertaken. Siderophore-iron complexes that do not show 1:1 stoichiometry have been described previously. Rhodotorulic acid, a hydroxamate siderophore from *Rhodotorula pilimanae*, was shown by Carrano and Raymond (1978) to form an Fe₂RA₃ complex at physiological pH. In chrysobactin the o-hydroxyl and carboxyl-terminal oxygens are separated by nine atoms, a distance theoretically large enough to form a tridentate ligand and thus to allow the chelation of one hexacoordinated ferric iron by two chrysobactin molecules. This assumption was experimentally supported. However, the synthesis of 2,3-dihydroxybenzylocysine and its tripeptide did not unequivocally show whether or not the carboxyl oxygens are involved in iron chelation.

Chrysobactin might be the first characterized siderophore from a plant pathogen, where iron has been directly correlated to virulence. Its stoichiometry and structure are unusual, but similar siderophores have been isolated. It thus appears that

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*D. Expert, unpublished data.*
Chrysobactin is representative of a class of siderophores which are DHBA derivatives of amino acids or linear peptides.

Acknowledgments—We thank Drs. E. Alvarado, J. L. Bada, P. Hoeprich, and A. Smith for experimental assistance. We also acknowledge the staffs at the University of California, Berkeley, Mass Spectrometry Facility and the University of California, San Francisco, Bio-Organic Mass Spectrometry Resource (A. L. Burlingame, Director) for technical assistance. The latter was supported by National Institutes of Health Division of Research Resources Grant RR01614.

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