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Synthesis of optically pure chrysobactin and immunoassay development

Chuang Lu*, Jeffrey S. Buyer*, John F. Okonya† & Marvin J. Miller†

* *US Department of Agriculture, Agricultural Research Service, Soil Microbial Systems Laboratory, Beltsville, MD and † Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, USA*

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Chrysobactin (α-*N*-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine), a siderophore that is essential for systemic virulence by plant pathogenic *Erwinia chrysanthemi*, was synthesized with high diastereomeric purity. Chrysobactin was prepared by coupling the *N*-hydroxysuccinimide ester of α-*N*-(2,3-dibenzyloxybenzoyl)-ε-*N*-Cbz-D-lysine with L-serine benzyl ester followed by deprotection via hydrogenolysis. Optically pure chrysobactin was obtained with 98% overall yield. A monoclonal antibody to ferric chrysobactin was developed and characterized as IgM. The antibody reacts with chrysobactin, ferric chrysobactin and less strongly with ferric dihydroxybenzoic acid. The antibody reacts weakly with the siderophores ferrichrome, A, ferric pseudobactin and ferric rhodotorulic acid. This antibody was used in a competitive immunoassay to detect ferric chrysobactin at 10^{-8} to 10^{-10} mol. This immunoassay may provide a useful method for the detection of chrysobactin in plant samples.

Keywords: chrysobactin, *Erwinia chrysanthemi*, immunoassay, siderophore, synthesis

Introduction

Iron is an essential element for virtually all living organisms. Because of the poor solubility of ferric hydroxide in aerobic environments ($K_{sp} = 10^{-38}$) (Latimer 1952), most microorganisms depend on the production of highly specific iron chelators, termed siderophores, to make sufficient iron available for growth. The role of siderophores in plant–microbe interactions was reviewed previously (Loper & Buyer 1991).

Chrysobactin, α-*N*-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine, is a catecholate siderophore first isolated from the bacterium *Erwinia chrysanthemi* (Persmark *et al.* 1989). *E. chrysanthemi* is a plant pathogen capable of systematic virulence following initial infection. Mutants deficient in either chrysobactin synthesis or transport were shown to produce a localized necrosis on *Saintpaulia* plants but were unable to induce systemic necrosis (Enard *et al.* 1988). This result suggested that chrysobactin production and transport *in planta* was required for systemic virulence by this pathogenic bacterium. An operon responsible for production of an outer membrane receptor protein to chrysobactin and for several enzymes required for chrysobactin synthesis was induced when *E. chrysanthemi* was inoculated into *Saintpaulia* plants (Masclaux & Expert 1995). Direct detection and quantitation of chrysobactin in infected plants would provide confirmation of the role of the siderophore in systemic infection and would be useful in studying the physiology of the plant pathogen *in vivo*.

The conventional method for detection of siderophores in environmental samples is a bioassay with indicator microorganisms able to use specific siderophores (Powell *et al.* 1980, 1983, Bossier & Verstraete 1986, Nelson *et al.* 1988). This method is

Address for correspondence: J. S. Buyer, USDA, ARS, SMSL, Building 318 BARC-East, Beltsville, MD 20705-2350, USA. Tel: +1(301) 504-8436; Fax: +1(301) 504-8370. E-mail: jbuyer@asrr.arsusda.gov.

qualitative rather than quantitative, and specificity depends on finding an indicator organism only capable of transporting the siderophore in question. Immunoassays using monoclonal antibodies have become a powerful method to detect components in environmental samples directly due to high specificity and low detection limits. Monoclonal antibodies have been developed to the siderophores ferric pseudobactin (Buyer *et al.* 1990, 1993) and ferric aerobactin (Le Roy *et al.* 1992, 1993). Monoclonal antibodies to nicotianamine were found to react with phytosiderophores (Mihashi *et al.* 1992). Polyclonal antibodies have been developed to nicotianamine (Shojima *et al.* 1991) and to iron-binding compounds produced by the fungus *Gloeophyllum trabeum* (Jellison *et al.* 1991).

The present study describes the chemical synthesis of optically pure chrysobactin, development of a monoclonal antibody to ferric chrysobactin and a quantitative immunoassay for chrysobactin.

Materials and methods

Synthesis of chrysobactin

Instruments and general methods used have been described previously (Teng & Miller 1993). ^{1}H and ^{13}C-NMR spectra were obtained on a General Electric GN-300 spectrometer. 1,4-Dioxane was used as a reference in ^{13}C-NMR spectra recorded in D_2O. Solvents used were dried and purified by standard methods (Perrin *et al.* 1986).

The stereogenic integrity of protected chrysobactin (**4**) was determined using a Beckman HPLC system consisting of a model 110 A pump, a model 420 controller and a model 332 injector. Chromatography was accomplished using an Alltech Econosil column (25 cm × 4.6 mm, 5 μm silica) run at a flow rate of 1.5 ml min^{-1}.

The diastereomeric purity of chrysobactin (**1**) was confirmed by analysis of its *o*-phthalaldehyde (OPA) derivatives using reversed-phase (C-18) HPLC with fluorescence detection (Lotz *et al.* 1990). A 2% sodium dodecyl sulfate (SDS) solution was prepared by dissolving 0.20 g of sodium dodecyl sulfate in 10 ml of 0.4 M sodium borate buffer (pH 9.5). The OPA reagent was prepared by dissolving 50 mg of OPA in 1.25 ml of MeOH. Then, 50 μl of 2-mercaptoethanol and 11.2 ml of 0.4 M sodium borate buffer (pH 9.5) were added. The solution was stored refrigerated when not in use and could be used for about 1 month. Derivatization was done by placing 10 μl of the sample aliquot into a 500 μl polypropylene microcentrifuge vial and adding 10 μl of the SDS solution. Next, 10 μl of the OPA solution were added and after exactly 1 min the mixture was quenched with 20 μl of 0.1 M potassium phosphate buffer (pH 4.0). This mixture was injected without delay onto the HPLC column for analysis.

HPLC analysis of the OPA derivatives was done using an ISCO model 2360 gradient programmer, model 2350 HPLC pump, model FL-2 fluorescence detector, Spectra-Physics model SP-420 intergrator, and an Alltech Econosil C_{18} 25 cm × 4.6 mm 5 μm reversed-phase column. The solvent flow rate was 1.4 ml min^{-1} using a gradient elution program. Solvent A consisted of 1:19:80 THF:CH_3OH:NaOAc buffer (50 mM; pH 5.8). Solvent B consisted of 80:20 CH_3OH:NaOAc buffer (50 mM; pH 5.8). The gradient program was typically as follows: isocratic at 0% B for 3 min, linear step to 14% B over 5 min, isocratic at 14% B for 5 min, linear step to 50% B over 5 min, linear step to 100% B over 5 min and linear step to 0% B over 3 min.

*α-N-(2,3-Dibenzyloxybenzoyl)-ε-N-Cbz-D-lysine (**3**)*

2,3-Dibenzyloxybenzoic acid (Sharma *et al.* 1989) (**2**, 1.34 g, 4.00 mmol) was converted to 2,3-dibenzyloxybenzoic acid-*N*-hydroxysuccinimide ester using *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide by the method described previously (Chimiak & Neilands 1984). The *N*-hydroxysuccinimide ester in 8 ml THF was added to a solution of ε-*N*-Cbz-D-lysine (Aldrich, 1.12 g, 4.00 mmol) in 80 ml THF, 32 ml H_2O and 2.4 ml (17 mmol) triethylamine. After brief heating, the solution was stirred at room temperature overnight. The organic solvent was removed by evaporation under reduced pressure and the residue was acidified with 8 ml 12 N HCl then dissolved in EtOAc. The EtOAc layer was washed with saturated sodium chloride solution, dried over $MgSO_4$, filtered and evaporated to provide **3** as a yellow oil (2.39 g, 100%): HPLC R_t 10 min (25% 2-propanol in hexanes); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.20–1.75 (m, 6H, lys CH_2), 3.05 (dd, J = 12.8 and 6.4 Hz, 2H, lys δ-CH_2), 4.61 (dd, J = 12.4 and 7.0 Hz, 1H, lys α-H), 5.00–5.20 (m, 6H, benzylic H), 7.10–7.50 (m, 18H, aromatic H), 7.75 (dd, J = 7.2 and 2.4 Hz, 1H, NH), 8.60 (d, J = 7.2 Hz, 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 22.5, 29.1, 31.1, 40.5, 52.6, 66.5, 71.2, 76.2, 117.4, 123.2, 124.4, 126.0, 127.7, 128.0, 128.2, 128.4, 128.5, 128.5, 128.6, 128.8, 136.1, 136.2, 136.5, 147.0, 151.7, 156.4, 165.7, 175.5; HRMS (EI) calcd for $C_{35}H_{36}N_2O_7$ (M^+) 596.2523, found 596.2525.

*α-N-(2,3-Dibenzyloxybenzoyl)-ε-N-Cbz-D-lysyl-L-serine benzyl ester (**4**)*

To a mixture of α-*N*-(2,3-dibenzyloxybenzoyl)-ε-*N*-Cbz-D-lysine (**3**, 2.39 g, 4.00 mmol) and *N*-hydroxysuccinimide (0.46 g, 4.0 mmol) in 12 ml anhydrous THF at 0°C under N_2 was added dropwise a solution of *N,N'*-dicyclohexylcarbodiimide (0.84 g, 4.0 mmol) in 3 ml anhydrous THF. The solution was stirred at room temperature overnight. The solvent was evaporated under reduced pressure and the residue was dissolved in 40 ml EtOAc and filtered to remove dicyclohexylurea. The filtrate was added to a solution of D-serine benzyl ester hydrochloride (0.93 g, 4.0 mmol) and $KHCO_3$ (0.40 g, 4.0 mmol) in 20 ml H_2O and stirred at room temperature for 20 h. The mixture was

diluted with EtOAc and washed with water, saturated sodium chloride, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was chromatographed on silica gel eluting with EtOAc:hexanes (2:1) and recrystallized from a mixture of EtOAc and hexanes to provide **4** as a white crystalline solid (3.04 g, 98%): m.p. 98–100°C; HPLC R_t 11 min (10% 2-propanol in hexanes); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.15–1.60 (m, 6H, Lys CH_2), 3.00 (m, 2H, Lys δ-CH_2), 3.99 (dd, J = 27.6 and 11.0 Hz, 2H, Ser β-CH_2), 4.38 (dd, J = 12.9 and 6.4 Hz, 1H, Ser β-OH), 4.65 (m, 1H, Lys α-H), 4.80 (m, 1H, Ser α-H), 5.10–5.25 (m, 8H, benzylic H), 7.00–7.50 (m, 23H, aromatic H), 7.51 (dd, J = 7.3 and 2.3 Hz, 1H, NH), 8.53 (d, J = 6.2 Hz, 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 22.7, 29.4, 30.7, 40.4, 54.4, 55.0, 62.6, 66.6, 67.4, 71.3, 76.37, 117.6, 123.3, 124.5, 125.9, 127.7, 128.1, 128.1, 128.3, 128.4, 128.5, 128.6, 128.7, 129.0, 135.1, 136.17, 136.19, 136.5, 147.1, 151.7, 156.5, 166.0, 170.1, 171.4; IR (KBr) 3320 and 3260, 1740, 1680, 1655, 1630 cm^{-1}; HRMS (FAB) calcd for $C_{15}H_{48}N_3O_9$ (MH^+) 774.3391, found 774.3383.

α-N-(2,3-Dihydroxybenzoyl)-D-lysyl-L-serine (chrysobactin, 1)

α-*N*-(2,3-dibenzyloxybenzoyl)-ε-*N*-Cbz-D-lysyl-L-serine benzyl ester (**4**) (0.89 g, 1.1 mmol) in 50 ml MeOH was hydrogenolyzed over 10% Pd-C (0.18 g) at atmospheric pressure for 7 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was concentrated and lyophilized to yield 0.38 g (100%) of chrysobactin (**1**) as an off-white powdery solid. Reversed-phase HPLC of the OPA derivative (Lotz *et al.* 1990) R_t 3 min; ^{1}H-NMR (300 MHz, D_2O) δ 1.47 (m, 2H, Lys CH_2), 1.68 (m, 2H, Lys CH_2), 1.90 (m, 2H, Lys CH_2), 2.96 (t, J = 7.4 Hz, 2H, Lys δ-CH_2), 3.80 (d, J = 1.6 Hz, 1H, Ser β-H), 3.82 (s, 1H, Ser β-H), 4.29 (dd, J = 9.6 and 4.4 Hz, 1H, Ser α-H), 4.60 (dd, J=8.5 and 5.5 Hz, 1H, Lys α-H), 6.80 (td, J = 8.0 and 1.7 Hz, 1H, aromatic H), 7.02 (d, J = 7.9 Hz, 1H, aromatic H), 7.24 (d, J = 8.0 Hz, 1H, aromatic H); ^{13}C-NMR (75 MHz, D_2O) δ 22.9, 27.1, 31.7, 40.0, 54.6, 58.0, 62.8, 118.0, 120.1, 120.4, 145.7, 148.2, 171.0, 174.3, 176.8; IR (KBr) 3680–2300 (br), 1700–1480 (br) cm^{-1}; HRMS (FAB) calcd for $C_{16}H_{24}N_3O_7$ (MH^+) 370.1614, found 370.1619.

Synthesis of chrysobactin–bovine serum albumin conjugate

Ten milligrams (1.5×10^{-6} mol) of bovine serum albumin (BSA, Sigma, St. Louis, MO) were dissolved in 400 µl of 0.1 M borate buffer, pH 7.5, followed by 5.5 mg (1.5×10^{-5} mol) of chrysobactin. Then, 50 µl of 0.11 g ml^{-1} disuccinimidyl suberate (Pierce, Rockford, IL, 1.5×10^{-5} mol) in DMSO were added and the mixture was stirred at room temperature for 2 h. Excess disuccinimidyl suberate and chrysobactin were removed by ultrafiltration using a 30 000 molecular weight cut-off filter. Phosphate buffered saline (PBS) was added and ultrafiltered five times. Chrysobactin–keyhole limpet hemocyanin (KLH) conjugate was synthesized by the same procedure except that 62 mg (1.1×10^{-7} mol) KLH (Pierce), 4.0 mg (1.1×10^{-5} mol) chrysobactin and 4.0 mg (1.1×10^{-5} mol) disuccinimidyl suberate were used.

Immunization and hybridoma production

The chrysobactin–BSA conjugate (0.1 mg/ml) was emulsified with an equal volume of monophosphoryl lipid A-trehalose dicorynomycolate adjuvant (MPL–TDM; Sigma, St. Louis, MO) and 0.2 ml of the mixture was injected intraperitoneally into BALB/c mice three times at 1 month intervals. One week after the final injection, the mouse spleen cells were fused with mouse myeloma cell line P3X63 AG8.653 at a ratio of 4:1 in the presence of 44% polyethylene glycol. The hybridoma cells were then transferred to 96-well plates and grown in RPMI 1640 medium with hypoxanthine–aminopterin–thymidine selection and nystatin at 37°C in a 5% CO_2 atmosphere. Cells were re-fed twice weekly by replacing half of the growth medium.

Screening and monoclonal antibody secreting cell lines

Enzyme-linked immunosorbent assay (ELISA) was applied to screen the cell lines secreting antibodies to chrysobactin. A 96-well microtitration plate was coated with 50 µl of 20 µg ml^{-1} chrysobactin–KLH conjugate for 2 h at 37°C, washed five times with PBS containing Tween-20 (PBS–Tween), blocked with 100 µl of 1% casein in PBS for 2 h at 37°C and then washed five more times with PBS–Tween. Then, 50 µl of hybridoma cell supernatant were added to the plates in duplicate and incubated for 2 h for binding, followed by washing five times with PBS–Tween. Then, 50 µl of anti-immunoglobulin A, G, M (Zymed, San Francisco, CA) alkaline phosphatase conjugate (1:1000 diluted in Tris-buffered saline) were added and incubated for another hour at 37°C. Finally the plate was washed five times with PBS–Tween and once with 1 M diethanolamine buffer (pH 9.8) before receiving 50 µl of 1 mg ml^{-1} disodium *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer. The plate was then incubated at 37°C for 1 h and the absorbances at 405 nm were read with a microplate reader (Thermomax; Molecular Devices). PBS buffer and mouse serum were used as negative and positive controls, respectively.

Cell lines which responded positively to chrysobactin–KLH conjugate were cloned by limiting dilution in RPMI 1640 medium with hypoxanthine thymidine.

Antibody characterization and purification

One antibody secreting cell line was grown to its maximum density and the supernatant was collected and isotyped by ELISA with an anti-mouse isotyping kit (Sigma). The collected supernatant was concentrated by ultrafiltration using a 30 000 molecular weight cut-off filter followed by purification on an ImmunoPure MBP IgM affinity column (Pierce).

Competitive ELISA

ELISA plates used in a competitive assay were prepared by cross-linking chrysobactin to the surface of a 96-well surface-modified plate (Covalink, Nunc). To accomplish this, 50 μl of 0.5 mM chrysobactin in 0.1 M borate buffer, pH 7.5, and 50 μl of 1 mM disuccinimidyl suberate in 1:1 DMSO:0.1 M borate buffer, pH 7.5, were added to each well and the plate was then incubated at room temperature overnight. After washing five times with distilled water, the plate was blocked with 1% casein and 1 mM ferric EDTA in PBS for 2 h. The plate was then washed five times with PBS-Tween and stored at 4°C.

Then, 1 ml of 1 mM chrysobactin in PBS was mixed with 0.05 ml of 10 mM $FeCl_3$, followed by adding 1 ml of 1 M sodium phosphate buffer (pH 7.5). PBS was used to bring the final volume to 5 ml. The resulting ferric chrysobactin was diluted in PBS by eight 2-fold serial dilutions and 125 μl of each ferric chrysobactin dilution (from 2.0×10^{-4} to 7.8×10^{-7} M) were mixed with 125 μl of 1:4000 diluted antibody (in PBS). The chrysobactin and antibody mixtures were incubated at 37°C for 1.5 h. Then, 100 μl of the incubated samples were transferred to ferric chrysobactin cross-linked plates in duplicate and the plates were incubated at 4°C overnight. After washing five times with PBS–Tween, 100 μl of alkaline phosphatase conjugate were added to each of the wells, followed by performing the remaining steps of the ELISA procedure as described above.

Antibody specificity

The specificity of the antibody was tested by performing a competitive ELISA, as described above, with ferric chrysobactin cross-linked plates but with a different ferric complex in solution. Ferric complexes included ferric dihydroxybenzoic acid, ferric pseudobactin, ferrichrome, ferrichrome A and ferric rhodotorulic acid.

Immunoassays were conducted to determine if the antibody was reactive with chrysobactin, ferric chrysobactin or both. Chrysobactin was covalently linked as described above. During the blocking stage half of the plate received casein and 1 mM EDTA while the other half of the plate receive casein and 1 mM ferric EDTA. The antibody was then added and titrated with 2-fold serial dilutions. In order to keep the free chrysobactin from scavenging iron, 1 mM EDTA, 1 mM pseudobactin or 10 mM pseudobactin were added, while the same concentration of ferric EDTA or ferric pseudobactin was added to the side of the plate with ferric chrysobactin. After incubation for 1 h the ELISA was continued as described above.

Results and discussion

Synthesis of chrysobactin

Initially we chose to prepare chrysobactin (**1**) by the reported 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ)-mediated coupling of α-*N*-(2,3-dibenzyloxybenzoyl)-ε-*N*-CBz-D-lysine (**3**) to L-serine benzyl ester (Persmark *et al.* 1989). Surprisingly, careful examination of the products and intermediates for diastereomeric purity by HPLC and ^{13}C-NMR revealed that the EEDQ coupling resulted in greater than 30% epimerization of the α-H of lysine. We suppose then that the electron withdrawing dihydroxybenzoic functionality on the α-amine of lysine enhances the acidity of the α-H making it more prone to racemization following formation of azalactone under the reaction conditions (Bodanszky 1993). Interestingly, two diastereoisomers of chrysobactin containing D- or L-lysine synthesized by the EEDQ method were both reported to be effective growth promoters for *E. chrysanthemi* (Persmark *et al.* 1989, 1992). A close examination of the reported circular dichroism (CD) spectra of the synthetic diastereomers and the natural chrysobactin suggested that the synthetic compounds were not diastereomerically pure, and cross-contamination of the two diastereomers might account for the observed biological activity.

Careful modification of the synthetic methodology led to the route shown in Figure 1. α-*N*-(2,3-dibenzyloxybenzoyl)-ε-*N*-Cbz-D-lysine (**3**) was prepared as described previously (Chimiak & Neilands 1984, Persmark *et al.* 1989, Sharma *et al.* 1989). In the key coupling process, *N*-hydroxysuccinimide ester of lysine derivative **3** was preformed using *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in THF, and then coupled to L-serine benzyl ester in a mixture of organic and aqueous solvents buffered with bicarbonate (Figure 1). Protected chrysobactin **4** was obtained diastereomarically pure in 98% yield. Hydrogenolysis of **4** on 10% Pd-C afforded chrysobactin (**1**) quantitatively. The optical purity of the peptide derivatives **4** and **1** was assessed by HPLC and ^{13}C-NMR. This modified synthetic route provides optically pure chrysobactin in 98% overall yield.

Monoclonal antibody

The initial screen of hybridomas involved ELISA plates coated with chrysobactin–KLH conjugates. Some of the antibodies were observed to cross-react with KLH, so we directly linked chrysobactin to surface-modified plates and eliminated the interference from KLH. The covalently linked plates also produced a higher response to the antibody. Covalently linked plates were also useful in an immunoassay for ferric pseudobactin (Buyer *et al.* 1993).

The antibody produced in this study was isotyped as an IgM. This may make the antibody less useful

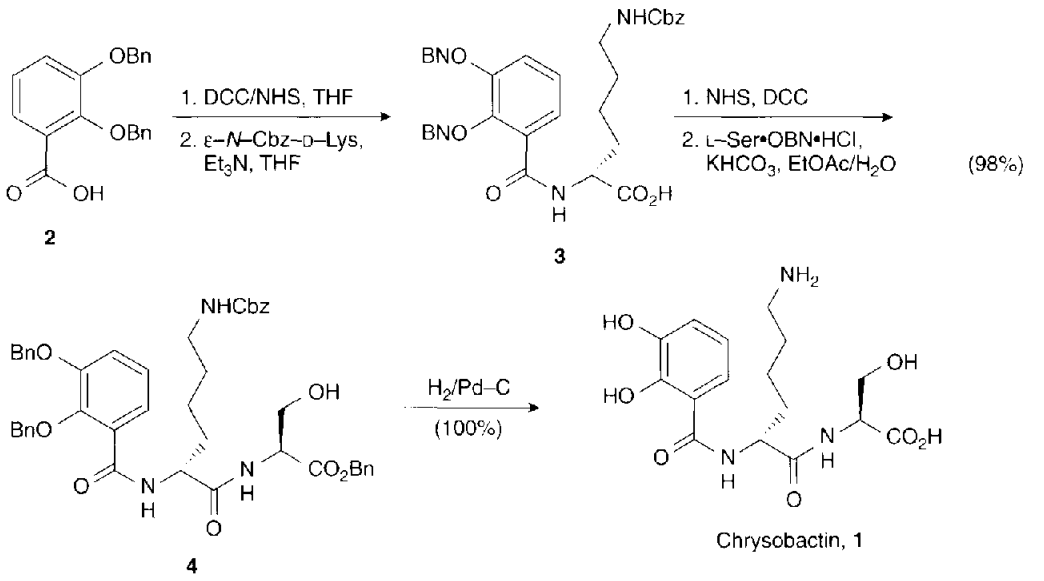

Figure 1.

for certain studies than if it was an IgG (Matthew & Reichardt 1982). We were unable to identify any IgG antibodies reactive to chrysobactin or ferric chrysobactin. While antibodies to ferric pseudobactin (Buyer *et al.* 1990) and ferric aerobactin (Le Roy *et al.* 1992) were identified as IgG1 and IgG2 isotypes, respectively, only IgM antibodies were developed to the nicotianamine (Mihashi *et al.* 1992).

In the competitive ELISA, the antibody was first incubated with ferric chrysobactin solution, forming an antibody–siderophore complex. Further incubation in the ferric chrysobactin-coated ELISA plate allowed free antibody to bind to siderophore immobilized on the plate surface. The final absorbance was proportional to the amount of antibody bound to immobilized ferric chrysobactin, so a greater concentration of ferric chrysobactin in solution lowered the absorbance. Figure 2 presents a competitive immunoassay for the antibody to the antigen ferric chrysobactin. The assay could be used to quantitate ferric chrysobactin with a range of 10^{-8} to 10^{-10} mol. The immunoassays for ferric pseudobactin (Buyer *et al.* 1990, 1993), phytosiderophores (Mihashi *et al.* 1992) and ferric aerobactin (Le Roy *et al.* 1992) are considerably more sensitive. However, this immunoassay may still be useful for quantifying chrysobactin production in infected plants, depending on the concentration of chrysobactin in the plant samples.

The chelating groups in chrysobactin are believed to be the catechol oxygens (Persmark *et al.* 1989,

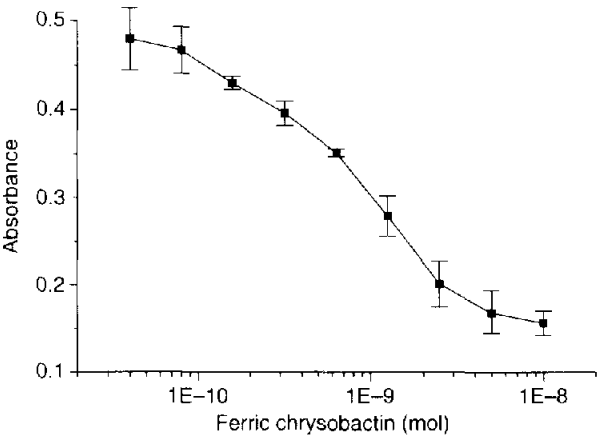

Figure 2. Competitive ELISA for ferric chrysobactin. Each point is the mean of three replicates. Error bars represent standard deviation.

Persmark & Neilands 1992) so chrysobactin is a bidentate ligand. Under our assay conditions ferric chrysobactin would exist as a mixture of bis and tris complexes (Persmark & Neilands 1992). We do not know if one or both complexes are reactive in our immunoassay. Chrysobactin was covalently bound to BSA before injection, so it seems unlikely that it formed bis and tris complexes *in vivo*.

In a competitive immunoassay with other ferric complexes the antibody reacts most strongly with ferric dihydroxybenzoic acid followed by ferrichrome A, ferrichrome, ferric pseudobactin and ferric rhodotorulic acid (Figure 3). Interpretation

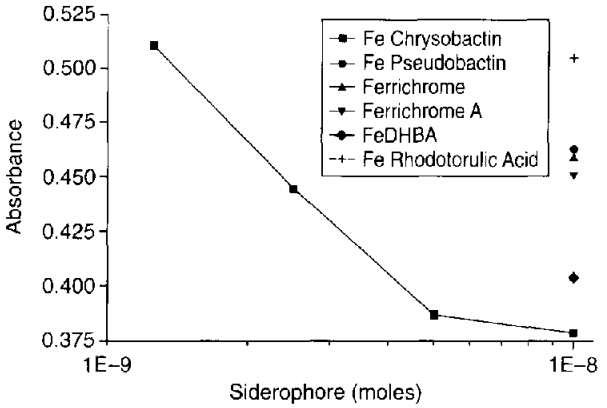

Figure 3. Competitive ELISA for ferric chrysobactin and five other ferric complexes. Each point is the mean of two replicates.

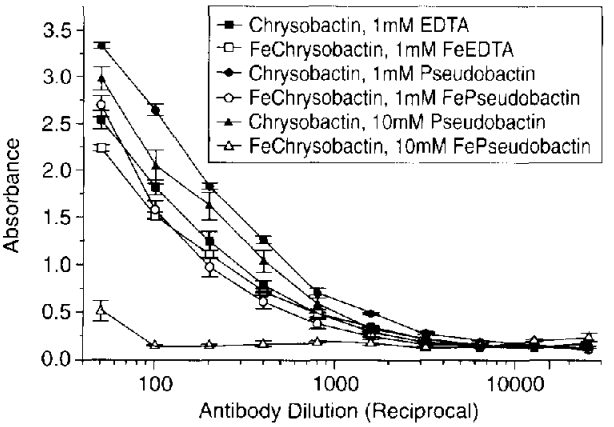

Figure 4. Titration of the antibody against chrysobactin or ferric chrysobactin. Each point is the mean of three replicates. Error bars represent standard deviation. The horizontal axis represents the reciprocal of the antibody dilution (i.e. a dilution of 1:100 is plotted as 100).

of these results is complicated by the varying stoichiometry of the metal complexes. Ferric dihydroxybenzoic acid should occur as a mixture of mono, bis and tris complexes, while ferric rhodotorulic acid is found as the complex Fe_2 (rhodotorulic acid)$_3$ (Carrano *et al.* 1979). The other three siderophores form 1:1 complexes with iron. The data in Figure 3 are plotted by the concentration of the ligand, not the concentration of the metal complex, since we do not know if the antibody is reacting with the intact complex or with each ligand molecule in the complex. If the data were instead graphed by the concentration of the metal complex the apparent concentration of the ferric chrysobactin, ferric rhodotorulic acid and ferric DHBA would all be decreased, making the three 1:1 complexes appear less reactive. However, it is clear that the antibody reacts most strongly to ferric chrysobactin, followed by ferric DHBA; and reacts weakly with the other complexes.

Both chrysobactin and ferric chrysobactin appear to react with the antibody (Figure 4). Interpretation of these results is complicated by the addition of the chelating agents, which were necessary to prevent scavenging of iron by chrysobactin. The 10 mM ferric pseudobactin clearly interfered with the assay. Furthermore, it is impossible to prove that the chrysobactin was in fact free and not ferrated. However, it seems reasonable to suppose that the chrysobactin, at least in the presence of 10 mM pseudobactin, was in fact not ferrated and that it did react with the antibody. In every case the curve for chrysobactin has higher absorbance than the corresponding curve for the ferric complex. This may indicate that chrysobactin is more reactive than ferric chrysobactin.

While iron acquisition by certain plant pathogens is known to be required for virulence, chrysobactin is the only siderophore shown to be essential to infection (Loper & Buyer 1991). If the monoclonal antibody could bind sufficient chrysobactin *in planta*, and if *E. chrysanthemi* does not have an alternative iron transport system, it might suppress growth of *E. chrysanthemi*. This would provide a novel method for protecting plants from a pathogen.

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