Confirmation of Occurrence of Hydroxamate Siderophores in Soil by a Novel *Escherichia coli* Bioassay

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The occurrence of ferrichrome-type hydroxamate siderophores in soil was confirmed. In the presence of the iron-scavenging chelator ethylenediamine[di(*o*-hydroxyphenylacetic)acid], soil extract stimulated the growth of an *Escherichia coli* strain possessing the ferrichrome transport protein (TonA) but did not stimulate growth of a strain lacking this protein (TonA$^-$). The siderophore concentration in a 1:1 (soil-water) extract was estimated to be approximately 78 nM. Specificity of the assay was supported by the absence of significant differential strain responses to ferric citrate, ferric 2,3-dihydroxybenzoate, enterochelin, ferrioxamine B, coprogen, and triacetylfusigen.

Most fungi and bacteria respond to low iron availability by the secretion of high-affinity, ferric-specific chelators termed siderophores (13, 14). Hydroxamate siderophores of the ferrichrome type are of special ecological interest because of their production by many soil fungi (23), including symbiotic ectomycorrhizal fungi (20), and their ability to mobilize iron in neutral and alkaline soils in which other naturally occurring compounds are ineffective as iron chelators because of competition from other metal ions (2). Experimental evidence indicates that hydroxamate siderophores can supply iron to some plant species (16, 18, 19).

The occurrence of hydroxamate siderophores in soils has been demonstrated previously in bioassays with *Arthrobacter terregens* (10) and *Arthrobacter flavescens* (17) and has been corroborated by a less specific *Salmonella typhimurium* bioassay (1). Attempts to isolate sufficient quantities of these compounds for chemical characterization have, however, failed, probably because adsorption to soil organic matter upon drying substantially decreases their extractability (17).

We report here the results of a soil extract bioassay specific for the ferrichrome-type hydroxamate siderophores. The assay is based on the differential growth response of EDDHA (ethylenediamine[di(*o*-hydroxyphenylacetic) acid]-inhibited *Escherichia coli* K-12 strains which either are able to utilize ferrichrome (TonA strain) or are unable to do so because

they lack the ferrichrome membrane transport protein (TonA$^-$ strain) (6, 22). The specificity of the assay is supported by the absence of differential strain response to several siderophore and nonsiderophore iron chelates.

MATERIALS AND METHODS

The *E. coli* K-12 enterochelin-defective TonA strain RW193 and its TonA$^-$ parental strain AN193 (9) were obtained from S. M. Payne (Department of Microbiology, The University of Texas at Austin). The medium used for all experimental, inoculum, and maintenance cultures contained the following components, expressed in grams per liter: K$_2$HPO$_4$, 2.0; (NH$_4$)$_2$HPO$_4$, 0.5; MgSO$_4$ · 7H$_2$O, 0.1; yeast extract (Difco Laboratories), 1.0; vitamin-free Casamino Acids (Difco), 1.0; and sucrose, 10.0. This formula gives a final pH of about 7.3 and an iron concentration of approximately 8 μM, as determined by flame atomic absorption spectroscopy.

The inoculum consisted of late-logarithmic-phase cells which were harvested after 6 h and then washed by centrifugation three times with deionized distilled water. After the inoculum was adjusted to an optical density of 50 Klett units, 0.1 ml was transferred to each 10 ml of experimental medium in culture tubes (20 by 175 mm). All cultures were incubated for 12 h at 37°C on a gyratory shaker. Since there appears to be a slow utilization of EDDHA iron, the age of the cultures was important in these studies. The growth of TonA and TonA$^-$ strains in 10-fold or 2-fold dilution series of soil extract, test chelates, or inhibitor (EDDHA) was determined as optical density in a Klett-Summerson colorimeter with a blue-400 filter.

The test soil, TC1 (17), was taken from the upper 10 cm of a Typic Ustochrept sandy clay loam which had a pH of 7.9 and an organic matter content of 7%. Soil was air dried and stored at room temperature until used. Aqueous soil-water extracts (1:1, wt/wt) were obtained by shaking for 1 h at 4°C. After centrifuga-

tion, the supernatant was reduced to dryness under a stream of dry air in a boiling-water bath. Extracts were rehydrated to 10 ml, filter sterilized, and then refrigerated at 4°C until assayed. Sufficient soil extract was added to the initial dilution tubes to give a concentration of 10%.

Deferrated EDDHA was provided by S. M. Payne and was used as an iron-scavenging inhibitor (11) at a concentration of 5 μM unless otherwise stated. Ferrichrome was produced and purified by the method of Emery (4). Citric acid (Baker Chemical Co.), 2,3-dihydroxybenzoic acid (DBA) (Aldrich Chemical Co.), and desferrioxamine B (Ciba-Geigy Ltd.) were of the highest available purity and were ferrated by mixing with equimolar amounts of $FeCl_3$. After filter sterilization, the solutions were allowed to come to equilibrium at room temperature for several days. Coprogen, prepared by H. Zähner, was supplied by G. C. Frederick (Department of Chemistry, The University of Texas at Austin), and triacetylfusigen was supplied by T. Emery (Department of Chemistry and Biochemistry, Utah State University). Both were shown to be chromatographically pure on silica gel thin-layer chromatography plates (Eastman Chromagram 13179) in a 1-butanol–acetic acid–water (60:15:25) solvent system. Enterochelin was provided by C. F. Earhart (Department of Microbiology, The University of Texas at Austin) and ferrated as described above. The concentrations of citric acid, DBA, and desferrioxamine B were determined gravimetrically, and the concentrations of ferrichrome and coprogen were determined spectrophotometrically by using published extinction coefficients. The concentration of triacetylfusigen was estimated by the absorbance of mevalonic acid at 210 nm (12).

The data presented are the means of three or four replications of each treatment. Experiments were performed at least twice, yielding essentially similar results.

RESULTS AND DISCUSSION

EDDHA-inhibited cultures of both the TonA and TonA⁻ strains responded similarly to increasing concentrations of enterochelin, the native siderophore of E. coli, indicating that when iron was supplied in a usable form, no significant physiological differences between the strains existed under these experimental conditions (Fig. 1). However, increasing concentrations of ferrichrome reversed the inhibition of the TonA strain but not the TonA⁻ strain (Fig. 1). Although the general shape of the ferrichrome dose-response curve for the TonA strain was sigmoidal, optical density was an approximately linear function of the natural logarithm of the ferrichrome concentration between 0.250 an 0.015 μM ($y = 1,200 + 64.0 \ln x$; $r = 0.994$, $n = 5$). Precision in this range was acceptable for most purposes, as indicated by an average standard error of 2.3%.

It is interesting to note that the fungal siderophore ferrichrome was five to seven times more efficient in stimulating the growth of E. coli than
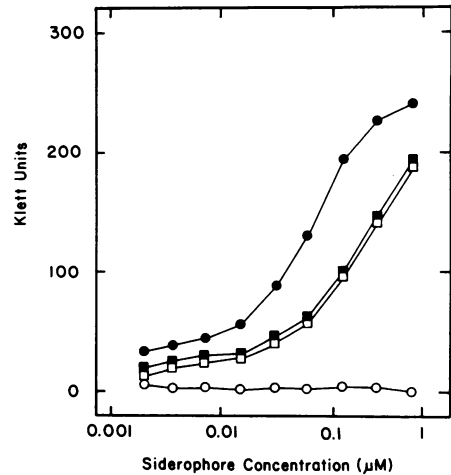

FIG. 1. Reversal of EDDHA inhibition of E. coli RW193 (TonA) and AN193 (TonA⁻) by ferrichrome (TonA, ●; TonA⁻, ○) and enterochelin (TonA, ■; TonA⁻, □) at various concentrations as indicated by optical density (Klett units) of the cultures after 12 h of growth.

the native siderophore enterochelin of the bacterium was.

A differential response of the E. coli strains to soil extracts was expected if ferrichrome-type siderophores occur in soil. The dilution response curve of soil extract was quite similar to that of ferrichrome, with a significant reversal of EDDHA inhibition only in the TonA strain (Fig. 2). From these data, we estimated the concentration of ferrichrome-type siderophores in the original extract to be approximately 78 nM. This compared favorably with the value of 34 nM estimated for the same soil as determined previously by the somewhat less specific hydroxamate siderophore bioassay with A. flavescens JG-9 (17). These data also suggest that most of the hydroxamate siderophores present in this soil were of the ferrichrome type.

The remote possibility that the differential growth of the two strains in the presence of soil extract resulted from the presence of some factor other than an iron chelate was tested by using single concentrations of soil extract and ferrichrome while varying the concentration of the inhibitor, EDDHA. Differential growth response was a linear function of the logarithm of the concentration of inhibitor in the presence of soil extract or ferrichrome, confirming that natural iron chelates recognized by the TonA receptor protein occur in soil (Fig. 3). When comparisons were made between the two strains within both ferrichrome and soil extract treatments, TonA values were significantly greater than TonA⁻ values at EDDHA concentrations of 6,
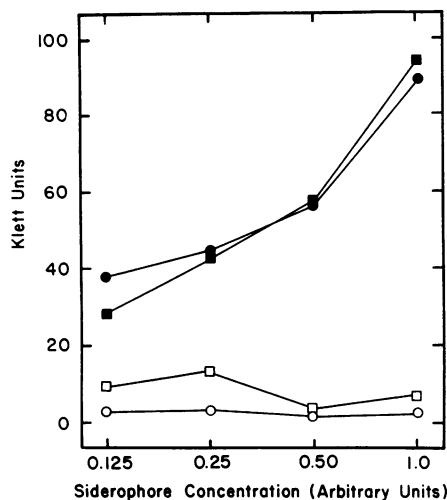
FIG. 2. Reversal of EDDHA inhibition of *E. coli* RW193 (TonA) and AN193 (TonA⁻) by various dilutions of ferrichrome (1.0 = 31 nM; TonA, ●; TonA⁻, ○) and natural siderophores in soil extracts (1.0 = 0.4 [1:1, soil-water]; TonA, ■; TonA⁻, □) as indicated by optical density (Klett units) of the cultures after 12 h of growth.

FIG. 3. Effect of EDDHA concentration on the growth response of *E. coli* RW193 (TonA) and AN193 (TonA⁻) to 11.3 nM ferrichrome (– – –) and to soil extract (——). Relative differential growth response = (TonA growth − TonA⁻ growth)/(difference in growth at 6.25 μM EDDHA). Growth was indicated by optical density (Klett units) of cultures after 12 h.

12, and 25 μM (Student *t* test; α values of 0.001, 0.01, and 0.05, respectively).

The specificity of the *E. coli* TonA-TonA⁻ assay system was examined by adding ferrated siderophore and nonsiderophore chelators in 10-fold dilution series to cultures inhibited by 5 μM EDDHA (Table 1). There were no significant differences in the growth of TonA or TonA⁻ at either the high concentration (10 μM), at which growth inhibition was completely reversed, or at the low concentration (0.1 μM), at which growth stimulation by ferric citrate and ferric DBA was almost completely inhibited and growth stimulated by ferrioxamine B, coprogen, and triacetylfusigen was somewhat less strongly inhibited. At the intermediate concentration (1 μM), there were no significant differences in growth between the strains in the presence of ferric citrate, ferrioxamine B, or coprogen. The small differential stimulation of TonA by ferric DBA was reproducible, but DBA was approximately 1,000 times less efficient than ferrichrome (cf. Fig. 1). The differential stimulation of growth by 1 μM ferric DBA was equivalent to that found in the 0.125 soil dilution (Fig. 2). However, for the ferric DBA to give a differential stimulation equal to the most concentrated soil extract treatment, it would have had to be at a concentration of 10 μM, a level at which both strains were stimulated equally.

The two hydroxamate siderophores, ferrioxamine B and coprogen, affected a partial reversal of EDDHA inhibition in both the TonA and
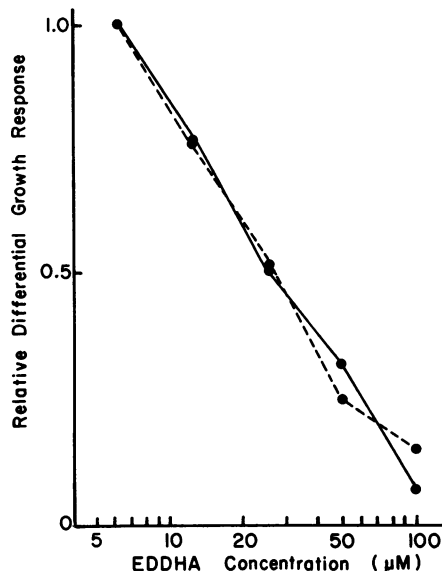
TonA⁻ strains at concentrations of 1 μM. This confirmed previous reports of iron transport by these siderophores in *E. coli* (5, 9). Ferrioxamine B was only 2% as effective as ferrichrome in relieving iron stress, whereas coprogen was twice as effective as ferrichrome (Table 1 and Fig. 1). Triacetylfusigen also partially reversed EDDHA inhibition in both strains, although the TonA strain was much more efficient in utilizing this non-ferrichrome-type hydroxamate siderophore (Table 1). These data suggest that the TonA receptor has a weak affinity for triacetylfusigen, approximately 2% as strong as that for ferrichrome, and that this siderophore is also weakly associated with another independent transport system. The absence of growth simulation of the TonA⁻ strain by soil extract (Fig. 2) argues against a significant contribution of triacetylfusigen to the differential growth stimulation observed in the soil extract bioassays.

Since the EDDHA-inhibited TonA⁻ strain did not respond to soil extracts, it may be concluded that if other siderophores known to be utilized by *E. coli* via independent transport systems were present in soil, they occurred at concentrations too low to be detected by this assay. These include enterochelin (6, 22), rhodoturulic acid (8), aerobactin (21), dimerium acid (5), coprogen B (5), and ferrioxamine B (9).

The strong growth response of the *E. coli* K-

TABLE 1. Effect of iron chelate concentration on growth of *E. coli* RW193 (TonA) and AN193 (TonA⁻) cultures inhibited by 5 μM EDDHA

Iron chelate	Growth[a] at chelate concn:					
	10 μM		1 μM		0.1 μM	
	TonA	TonA⁻	TonA	TonA⁻	TonA	TonA⁻
Citrate	274	274	13	8	8	5
2,3-Dihydroxybenzoate	280	276	27	9	6	4
Ferrioxamine B	299	287	138	134	38	37
Coprogen	280	272	278	273	214	207
Triacetylfusigen			152	86	32	27

[a] Growth is measured in Klett units; 1 Klett unit = optical density times 500.

12 TonA strain RW193 to soil extract in the presence of EDDHA, together with the lack of response of the TonA⁻ strain AN193 under similar conditions, confirms the occurrence of biologically significant concentrations of ferrichrome-type siderophores in soil. Transport of ferrichrome by the *tonA* gene product is well established (13), and other siderophores of the ferrichrome type, e.g., ferricrocin, may also be strictly *tonA* dependent (3). Ferrichrome C and ferrichrysin in this group have been shown to stimulate the growth of *E. coli* under iron-limiting conditions, but their transport mechanisms have not been determined (5). The value of 78 nM for the concentration of ferrichrome-type siderophores in our test soil may reflect an underestimation of the total amount of these compounds present since their activity can be reduced by acetylation of the *N*-hydroxy functional group by some bacteria (7). The concentration of the biologically active component is, however, high enough to be significant in plant nutrition according to the calculations of O'Conner et al. (15), which predict that chelate concentrations of 10 to 100 nM are sufficient to supply iron to corn plants growing in soil.

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