Microbial Degradation of the Ferrichrome Compounds

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SUMMARY
An unidentified Pseudomonas capable of growth on ferrichromes as sole sources of carbon and nitrogen was isolated by enrichment culture on ferrichrome A. The spectral shifts observed for the culture supernatant fluids and the degradation products which accumulated in the culture media during growth on these compounds indicated that initially the cyclic peptide rings are broken to yield simpler hydroxamates. These simpler hydroxamates were then deacylated, perhaps after reduction to the corresponding N-substituted amides.

INTRODUCTION
The ferrichromes are typical representatives of a class of ferric polyhydroxamates (Emery & Neilands, 1959), known as siderochromes (Zähner, Bachmann, Hütter & Nuesch, 1962), which are widely distributed among microbial genera (Zähner et al. 1962, 1963). The siderochromes frequently display growth factor and/or antibiotic activity; they may be divided into two groups: peptide and non-peptide. Ferrichrome and ferrichrome A (Fig. 1) belong to the former group (Emery & Neilands, 1961; Rogers, Warren & Neilands, 1963), as also do the albomyccins (Turkova, Mikeš & Šorm, 1962); coprogen (Hesseltine et al. 1953); ferricrocin, ferrichrysin, ferrirhodin, ferrirubin (Zähner et al. 1963). The growth-promoting siderochromes, termed sideramines, may function as iron transfer cofactors in microbial iron metabolism (Neilands, 1957; Burnham & Neilands, 1961; Burnham, 1962, 1963; Zähner et al. 1962). Very little is known about the metabolism of the ferrichromes. Work is in progress on the biosynthesis of ferrichrome (Emery, personal communication). In the present report, we describe the isolation, growth characteristics and some of the metabolic products of an organism which is capable of growth on the ferrichromes as sole sources of carbon and nitrogen.

METHODS

Chemicals. Ferrichrome and ferrichrome A were isolated from the culture medium of Ustilago sphaerogena grown under conditions of iron deficiency (Garibaldi & Neilands, 1955). They were converted to the iron-free, or apo-forms, by the methods of Emery & Neilands (1960). Ferrioxamine E was prepared from nocardamin by the addition of iron sufficient to form a 3:1 complex at pH 7.0 (Keller-Schierlein & Prelog, 1961).

Syntheses. Acethydroxamic acid was synthesized by the method of Wise & Brandt (1955). N-methyl- and N-propyl-acethydroxamic acids and N-hydroxy-N-
acetyl-L-leucine were synthesized from the appropriately substituted hydroxylamines by the method of Ulrich & Sayigh (1963). N-hydroxy- and N-hydroxy-N-propylsuccinamic acids were prepared by the reaction of succinic anhydride with one equivalent of hydroxylamine and N-propyl hydroxylamine, respectively. N-methyl- and N-propyl-acetamides were synthesized according to Muller (1958). Succinamic and N-propyl-succinamic acids were made by reacting succinic anhydride with excess ammonium hydroxide and propylamine, respectively.

![Figure 1. Structures of the ferrichromes.](image)

Ferrihrome: \( R = CH_3 \)
\( R' = R'' = H \)

Ferrihrome A: \( R = HOOC.CH_2C=CH \)
\( R' = H \)
\( R'' = HOCH_2 \)

Ferrihrome was reduced to the ornithine analogue by the method of Gipson, Pettit, Skinner & Shive (1963). Attempts to reduce ferrihrome A by this method were not completely successful.

**Culture media.** Supplements were added to the salts solution of Barnett & Ingram (1955) to give the following concentrations: carbon and nitrogen source, 0.2%; carbon source 0.2% + \((NH_4)_2SO_4\) 0.2%. All media were adjusted to pH 7.4.
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before sterilization by autoclaving, or to pH 7.2 for media to be sterilized by filtration. For solid media, Difco agar was added to a concentration of 2.0%.

**Incubation.** Unless stated otherwise, all cultures were incubated at 30° on a New Brunswick rotary shaker.

**Determinations.** Growth was measured turbidimetrically at either 660 m\(\mu\) (Process & Instruments Recording Spectrophotometer; 1 cm. light path) or 650 m\(\mu\) (Bausch & Lomb Spectronic 20 Colorimeter), depending on the experiment. Extinction against dry-weight curves were prepared for each set of conditions; in no case was there a significant deviation for extinction up to 0.8.

Oxygen uptake was measured by the standard manometric techniques (Umbreit, Burris & Stauffer, 1957).

Absorption spectra were recorded with a Process and Instruments recording spectrophotometer. (Process & Instruments Co., 15 Stone Avenue, Brooklyn, N.Y., U.S.A.).

**Chromatography and electrophoresis.** Paper chromatography and paper electrophoresis were performed with Whatman No. 1 paper. The solvent systems and buffers used are designated in the text. Ninhydrin-positive compounds were detected by spraying with 1% ninhydrin in acetone followed by gentle heating. Non-volatile acids were detected by spraying with sugar + aniline followed by gentle heating (Michl, 1959), while the procedure of Kennedy & Barker (1951) was used for the chromatography and detection of volatile acids.

**RESULTS**

**Isolation of the organism**

Ten ml. of ferrichrome A medium contained in a 50 ml. Erlenmeyer flask were inoculated with about 1 g. garden soil (Ferrichrome A, which is available in larger quantities than ferrichrome, was used for all preliminary experiments). After incubation for several days without shaking, the medium was markedly decolorized. Four successive subcultures were made from this enrichment culture, the flasks being incubated with shaking. Plating of the final subculture on ferrichrome A agar yielded well-defined colonies after incubation for 5 to 6 days. A colony was transferred to the liquid medium and this culture replated. Finally, a colony from the second plate was transferred to the liquid medium. Plating out on ferrichrome A and nutrient agars showed the culture to be pure. The isolate was maintained by monthly transfers on nutrient agar slopes. The organism isolated was a small Gram-negative rod. It was motile by means of a single polar flagellum. On all media tested, it produced a non-diffusible lemon-yellow pigment. Further examination of the organism placed it in the genus *Pseudomonas*. It has been given the temporary designation *Pseudomonas FC 1*.

**The role of inducible enzymes in the degradation of the ferrichromes**

When cultures of *Pseudomonas FC 1* grown on ferrichrome A and glucose + salts media were inoculated into fresh ferrichrome A solution, a similar lag period before the onset of growth was observed in each case. A much longer lag was observed when such cultures were inoculated into acetate salts medium. This suggested the non-involvement of inducible enzymes in the growth of the organism on the ferrichromes. Attempts to confirm this observation by means of respirometer experi-
ments were unsuccessful because of the slow rate of oxygen consumption by washed suspensions of the organism in the presence of ferrichrome A, and the very high rates of endogenous respiration of organisms grown in glucose salts medium. Further experiments are in progress in order to determine the effect of chloramphenicol on the metabolism of ferrichrome A by washed cell suspensions of organisms.

Non-involvement of extracellular enzymes in the degradation of the ferrichromes

After prolonged periods of incubation, the medium became decolorized around areas of heavy growth of the organism on ferrichrome A agar. As pointed out by Pollock (1963) this was not necessarily an indication of extracellular destruction of substrate. By use of the cup-technique used for penicillin assay, it was not possible to demonstrate, in sterile culture filtrates, the appearance of enzymes capable of decolorizing ferrichrome A agar. Incubation of culture filtrates with solutions of ferrichrome A gave the same result. Ferrichrome A cultures were tested in this way during the first 40 hr of growth, by which time the organism was well into the stationary phase. Extracellular enzymes thus appeared not to be involved in the degradation of the ferrichromes by Pseudomonas FC1.

Growth of Pseudomonas FC1 on components of the ferrichromes

The compounds released from ferrichrome by acid hydrolysis are glycine, L-2-amino-5-hydroxaminopentanoic acid (AHAPA) and acetic acid. In ferrichrome A, L-serine is present, and trans-β-methylglutaconic acid (TMGA) is obtained instead of acetic acid. The hydroxamino group of AHAPA is unstable at neutral pH values; if the free amino acid were released from the ferrichromes by the action of cellular enzymes, reduced (ornithine) and oxidized (perhaps glutamic acid) compounds might be formed by disproportionation. Alternatively, the hydroxamate moieties might be reduced to the corresponding N-substituted amides; subsequent hydrolysis would yield ornithine. All these compounds were tested as growth substrates for Pseudomonas FC1. None of the amino acids which are directly obtainable from the ferrichromes served as substrates for growth (Table 1); a mixture of them was also ineffective. The acyl moieties of the hydroxamate functions of the ferrichromes served as carbon sources, although the rate of growth on TMGA was much slower than that on acetate. Glutamic acid was an effective substrate. The failure of the organism to grow on the amino acids from the ferrichromes was surprising. It is possible that permeability barriers prevented utilization of these compounds.

Growth on the ferrichromes and the detection of degradation products

The rate of growth of Pseudomonas FC1 on the ferrichromes was proportional to the rate of disappearance of substrate as measured by the decrease in absorption of the ferric hydroxamates (Fig. 2). During growth, there were also significant changes in the spectra at pH 2.0 of the remaining ferric hydroxamates. Ferric acethydroxamic acid shows a change in its visible absorption spectrum between pH 7.0 and pH 2.0 (Fig. 3). This shift of the λ_max on acidification, and the decrease in the extinction, are characteristic of simple hydroxamic acids (Dutta, 1959). The ferrichromes are much more stable; at pH 2.0 the metal-binding centre of ferri-
Microbial degradation of the ferrichrome compounds

Table 1. Growth of Pseudomonas FC 1 on degradation products of the ferrichromes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Increment of extinction at 650 μm after 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0</td>
</tr>
<tr>
<td>L-2-Amino-5-hydroxaminopentanoic acid</td>
<td>0</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>0</td>
</tr>
<tr>
<td>Acetate; salts</td>
<td>0.75</td>
</tr>
<tr>
<td>Trans-β-methylglutaconic acid; salts</td>
<td>0.82</td>
</tr>
<tr>
<td>L-δ-N-Acetylornithine</td>
<td>0</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.82</td>
</tr>
<tr>
<td>Glycine + L-serine + L-ornithine + trans-β-</td>
<td>0</td>
</tr>
<tr>
<td>methylglutaconic acid</td>
<td></td>
</tr>
<tr>
<td>Ferrichrome A</td>
<td>0.87</td>
</tr>
<tr>
<td>Ferrichrome</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Media were dispensed in 7.0 ml. amounts in 15.0 cm. x 2.2 cm. test tubes. The inoculum was an aseptically prepared washed suspension of organisms in distilled water, from an overnight culture of Pseudomonas FC 1 grown on ferrichrome A. The tubes were incubated at an angle of 30° on a reciprocal shaker.

Fig. 2. Growth of Pseudomonas FC1 on the ferrichromes. An aseptically prepared washed cell suspension from an overnight culture was used to inoculate 20 ml. quantities of the media shown. At intervals during incubation 3.0 ml. samples were withdrawn, the cells centrifuged down and resuspended in a suitable volume of distilled water for determination of the optical density at 660 μm. The supernatant was diluted 10 x for determination of the optical density at 440 μm (ferrichrome A) or 425 μm (ferrichrome). O—O 440 (425) μm. ●—● 660 μm.

Ferrichrome A is probably not changed (Fig. 3), while that of ferrichrome is changed only slightly. After incubation for 4 days, the remaining hydroxamate in a ferrichrome A culture had a spectrum at pH 2.0 which was very similar to that of a simple hydroxamate (Fig. 3). This spectral change can be expressed as the change at pH 2.0 in the value of the ratio of the extinction at 480 μm to that at 440 μm. In ferrichrome A the value is 0.76, and in ferric acethydroxamic acid 1.18. The value of the ratio changed during growth of Pseudomonas FC1 on ferrichrome A (Fig. 4)
and after four days (Fig. 3) reached 1·18. Similar results were obtained with ferrichrome (Fig. 4). Since \( \lambda_{\text{max}} \) for ferrichrome is 425 m\( \mu \), the ratio is for 480 m\( \mu \) to 425 m\( \mu \). For ferrichrome this ratio has a value of 0·72, and for ferric acethydroxamic acid 1·86. After growth for 2 days on ferrichrome, the value was 1·18. The rates of change of the ratios appeared to be most marked in the later stages of incubation when the cultures were in or entering the stationary phase.

![Fig. 3. Spectra of ferric hydroxamates. Absorption spectra of ferric acethydroxamic acid, and ferrichrome A medium before and after growth of Pseudomonas FC 1; ○○ at pH 7·0; △△ at pH 2·0.](image)

![Fig. 4. Changes in the spectra of the culture supernatant fluids during growth on the ferrichromes. Experimental conditions as for Fig. 3. The pH value of each sample was adjusted with 6 N-HCl by the use of a pH meter.](image)

Ninhydrin-positive compounds appeared in the culture media during growth of Pseudomonas FC1 on the ferrichromes. Paper electrophoresis in 4% formic acid will separate glycine, serine, ornithine and AHAPA; examination of the medium during growth on ferrichrome A showed the release of only traces of these amino acids. Free glycine was readily detected during growth on ferrichrome. Several of the ninhydrin-positive compounds observed were peptides. When the iron was removed from samples of the culture supernatant fluid after growth for 40 hr on the ferrichromes, and the resulting solutions hydrolysed, the only amino acids detectable were those contained within the ferrichromes, plus ornithine. Free TMGA appeared in the medium during growth on ferrichrome A, but no acetic acid was detected during growth on ferrichrome. Quantitative determination of the release of TMGA agreed with the result of the chromatographic examination of the culture medium (Fig. 5). No ninhydrin-positive compounds, TMGA or acetic acid were detected in the culture medium when Pseudomonas FC1 was grown on a glucose + salts medium.
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The finding of ornithine in hydrolysates of the culture fluid but not in the culture fluid itself, suggested that the hydroxamate functions might be reduced to N-substituted amides. Examination of the iron-free supernatant fluids from ferrichrome A cultures showed that after treatment with excess periodate to remove hydroxamate-bound TMGA, further TMGA was released by acid hydrolysis. The acid was identified by chromatography and reaction with permanganate. Unfortunately, its quantitative determination was prevented by materials which obscured its spectrum. The same difficulty was encountered when the solution was treated in two parts, one of which was reacted with periodate, the other hydrolysed; the TMGA from the latter was again contaminated with interfering substances which could not be removed chromatographically. Ferrichrome culture supernatant fluid, when freed from iron, appeared to contain \(\delta\)-N-acetylornithine. Preparative paper chromatography gave a compound having the same \(R_F\) value as authentic \(\delta\)-N-acetylornithine in several solvent systems. However, the compound gave a ferric chloride test and on acid hydrolysis yielded only acetic acid and AHAPA. It appeared to be \(5\)(N-acetyl)-hydroxamino-2-aminopentanoic acid. In \(n\)-propanol + water + conc. \(\text{NH}_2\text{OH}\) (sp. gr. 0.88) (80 + 18 + 2, by vol.), it moved more slowly than \(\delta\)-N-acetylornithine and showed the same \(R_F\) value as an authentic sample of the hydroxamate compound (Neilands, unpublished). From these results it seems likely that Pseudomonas FC1 cleaves the peptide rings of the ferrichromes to form simpler hydroxamates which are then deacylated, perhaps after reduction.
**Rates of growth on ferrichromes and apoferrichromes**

The iron can be removed from the ferrichromes under mild conditions (Emery & Neilands, 1960). The rates of growth of Pseudomonas FC1 on the apoferrichromes were lower than those on the ferric complexes (Fig. 6), the difference being more striking for the ferrichromes. After incubation, the values of the ratios discussed previously were determined; they were 0.88 for ferrichrome A, 0.78 for apoferrichrome A, 0.87 for ferrichrome, and 0.76 for apoferrichrome. After the addition of iron to the apoferrichrome samples, older (4-day) culture supernatant fluids from the apo- and ferric forms contained the same ninhydrin-positive compounds. Apart from the slower rates of growth on these compounds, the iron-free forms appear to be degraded in the same way as the ferric complexes.

**Table 2. Growth of Pseudomonas FC1 on other ferric hydroxamates and the corresponding amides**

Hydroxamate media were sterilized by filtration, after sufficient iron as FeCl₃ had been added to give a 3:1 complex at pH 7-0. Amides were autoclaved. Other experimental conditions as for Table 1.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Increment in extinction at 650 mµ in 5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acethydroxamic acid</td>
<td>0-10</td>
</tr>
<tr>
<td>Acetamide</td>
<td>0-06</td>
</tr>
<tr>
<td>N-methyl-acethydroxamic acid</td>
<td>0-03</td>
</tr>
<tr>
<td>N-methyl-acetamide</td>
<td>0-05</td>
</tr>
<tr>
<td>N-propyl-acethydroxamic acid</td>
<td>0</td>
</tr>
<tr>
<td>N-propyl-acetamide</td>
<td>0-05</td>
</tr>
<tr>
<td>DL-N-acetyl-N-hydroxyleucine</td>
<td>0</td>
</tr>
<tr>
<td>L-N-acetylleucine</td>
<td>1-40</td>
</tr>
<tr>
<td>N-hydroxysuccinamic acid</td>
<td>0-15</td>
</tr>
<tr>
<td>Succinamic acid</td>
<td>0-30</td>
</tr>
<tr>
<td>N-propyl-N-hydroxysuccinamic acid</td>
<td>0-06</td>
</tr>
<tr>
<td>N-propylsuccinamic acid</td>
<td>0-06</td>
</tr>
</tbody>
</table>

**Growth on other ferric hydroxamates**

Ferrichrome and ferrichrome A may be regarded as complex derivatives of acethydroxamic acid and of the half-hydroxamate of TMGA, respectively. The ability of the organism to utilize a series of acethydroxamic acids and the corresponding amides was investigated. Since the synthesis of the half-hydroxamates of TMGA proved too difficult, succinic acid was used as a dicarboxylic acid to make compounds corresponding to ferrichrome A. These substances were tested as carbon sources only. Since Pseudomonas FC1 showed a long inductive response before growth on succinate and acetate, the inocula were grown on acetate + salts for the acetyl compounds and on succinate + salts for the succinyl compounds. The results (Table 2) indicate that Pseudomonas FC1 does not have the ability to utilize hydroxamates in general as growth substrates. It appears also to be devoid of general amidase activity. It had previously been observed to grow readily on asparagine as a carbon + nitrogen source.
Microbial degradation of the ferrichrome compounds

Growth on other naturally-occurring ferric hydroxamates

Several other naturally-occurring ferric hydroxamates were tested as growth substrates for Pseudomonas FC1 (Table 3). Of the four compounds tested, only coprogen, which is probably quite closely related to the ferrichromes (Garibaldi & Neilands, 1955), supported growth of the organism. The shift in the spectrum at pH 2·0 also occurred. The albomycins, which are fairly closely related to the ferrichromes (Turkova et al. 1962) did not support growth. Ferrioxamine E is a cyclic ferric trihydroxamate, but it does not contain a peptide ring (Prelog, 1963).

Table 3. Growth of Pseudomonas FC1 on naturally-occurring ferric hydroxamates

<table>
<thead>
<tr>
<th>Substance</th>
<th>Increment in extinction at 650 μm</th>
<th>O.D. 480</th>
<th>O.D. 440 (at pH 2·0)</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coprogen</td>
<td>0·53 (16 hr)</td>
<td>0·86</td>
<td>1·20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albomycin δ</td>
<td>0·00 (72 hr)</td>
<td>0·88</td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albomycin ε</td>
<td>0·11 (72 hr)</td>
<td>0·89</td>
<td>0·91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferrioxamine E</td>
<td>0·14 (96 hr)</td>
<td>0·86</td>
<td>0·74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Growth on cyclic peptides

Pseudomonas FC1 grew on only one of five other cyclic peptides tested as growth substrates (Table 4).

Table 4. Growth of Pseudomonas FC1 on other cyclic peptides

<table>
<thead>
<tr>
<th>Substance</th>
<th>Extinction at 650 μm in 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced ferrichrome</td>
<td>0·04</td>
</tr>
<tr>
<td>his-gly-gly-tyr-gly-gly</td>
<td>0·45</td>
</tr>
<tr>
<td>Circulin</td>
<td>0</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>0·04</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>0</td>
</tr>
</tbody>
</table>

Experiments with washed suspensions of Pseudomonas FC1

Washed suspensions prepared from overnight cultures of Pseudomonas FC1 grown on glucose + salts medium rapidly metabolized the ferrichromes. The spectral shifts seen during growth of the organism on these compounds were again observed, and the same patterns of ninhydrin positive compounds were also seen on chromatograms. Such suspensions did not cause decreases in the absorbancies of solutions of any of the ferric hydroxamates listed in Table 2. Circulin, polymyxin and tyrocidin were broken down to their constituent amino acids by the washed organisms, but neither of the albomycins nor ferrioxamine E were degraded under these conditions.
DISCUSSION

The spectral shifts observed in the culture supernatant fluids during growth of Pseudomonas FC 1 on the ferrichromes indicate that initially the hexapeptide rings of these trihydroxamates are cleaved to yield simpler hydroxamates. Ferrioxamine B, an open chain ferric trihydroxamate, does not show a significant spectral shift between pH 4·0 and pH 2·0 (Schwarzenbach & Schwarzenbach, 1963). Hence, it seems unlikely that the shift observed in the present case is the result merely of opening the peptide ring. This conclusion is supported by the finding of 5(N-acetyl)-hydroxamino-2-aminopentanoic acid in ferrichrome cultures. Also several of the peptides which accumulate during growth on ferrichrome A are capable still of binding ferric iron (Rogers et al. 1963; Warren & Neilands, unpublished). The detection of free trans-β-methylglutaconic acid (TMGA) in ferrichrome culture fluids further indicates that these simpler hydroxamates are deacetylated. Although free ornithine was not detected in the culture fluids, it was released by acid hydrolysis of preparations from which the iron had been removed. Before removal of the iron, none of the peptides appeared to be very basic, as judged by paper electrophoresis in 4% formic acid. These observations, together with the qualitative demonstration of TMGA bound in non-hydroxamate groupings, strongly suggest that the simpler hydroxamates are reduced to substituted amides before deacetylation. This point is being further investigated. Pseudomonas FC 1 thus produces an endopeptidase capable of cleaving small cyclic peptides and possibly produces a hydroxamate reductase. These enzymes appear to be intracellular.

Since the rates of growth on the apoferrichromes were slower than on the iron complexes, it seems that the presence of the iron influences the metabolism of these compounds. This might occur in one of two ways: the permeability of the apo-form could be lower than that of the iron complex; or the absence of the iron produces steric hindrance at some stage of the breakdown. Charged groups seem to be important, whichever the case, since the organism grew more slowly on ferrichrome than on ferrichrome A, a difference even more noticeable in the apo-forms; it did not grow at all on fully reduced ferrichrome, whereas it did grow on the synthetic peptide \texttt{his-gly-gly-tyr-gly-gly}.

Several cyclic peptide antibiotics were degraded by washed suspensions of Pseudomonas FC 1, even though it was unable to grow on these compounds. This was not true of the sideromycin, albomycin \(\delta\), or the related biologically inactive albomycin \(\epsilon\); in this case the presence of 3-methyluraecil attached to one of the serine residues (Mikes & Turková, personal communication) might interfere with the initial ring cleavage or permeation of the molecules.

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