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Identification of enterobactin and linear dihydroxybenzoylserine compounds by HPLC and ion spray mass spectrometry (LC/MS and MS/MS)

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Summary. The production of catecholate siderophores was studied in some selected species of *Enterobacter* (Enterobacteriaceae). The extracted catecholates were separated as iron-free compounds by HPLC on a C_{18} reversed-phase column using methanol/0.1% phosphoric acid or methanol/0.1% formic acid as a solvent system and identified by ion spray mass spectrometry (LC/MS, MS/MS). Five catecholate compounds were identified which include 2,3-dihydroxybenzoylserine, its linear dimer and trimer, the cyclic enterobactin and an unidentified isomer of enterobactin. In addition, a new large-scale method for the isolation of catecholate siderphores is described which is based on adsorption on XAD-2 and subsequent purification on Sephadex LH20.

Key words: Enterobactin - Dihydroxybenzoylserine - Isolation - HPLC-separation - Ion spray mass spectrometry

Introduction

Bacteria of the family Enterobacteriaceae are known to produce enterobactin, also named enterochelin, which is a low-molecular-mass iron-chelating agent (siderophore) in which three 2,3-dihydroxybenzoylserine molecules are linked to form a cyclic triester. Ferric-enterobactin transport in *Escherichia coli* has been described in detail by Earhart (1987). Enterobactin was first identified in *E. coli* (O'Brien et al. 1970) and in *Salmonella typhimurium* (Pollack and Neilands 1970). Since then, enterobactin has been detected in most other enterobacterial genera, such as *Shigella, Klebsiella, Enterobacter, Serratia.* However, some genera such as *Proteus, Providencia, Morganella* and *Yersinia* seem not to produce any catecholate-type siderophore (Payne 1988).

The biosynthesis of enterobactin is regulated by the prevailing iron concentration of the cell. Therefore, maximal production of enterobactin is observed when the iron concentration of the cultivation medium is low (0.1-1 μM). After complexation with iron, ferric-enterobactin is transported into the enterobacterial cells via an outer membrane receptor protein (FepA). Inside the cells iron is released from enterobactin, possibly by reduction, and the ligand is assumed to be degraded by an esterase. It was found that the ferric complex has the highest formation constant ($K_f = 10^{52}$) known for any Fe(III) species and a highly negative reduction potential (−790 V vs the normal hydrogen electrode at pH 7.4) which precludes a direct intracellular reduction. The mechanism of iron removal has been the subject of some controversy which has recently been discussed in detail (Cass et al. 1989). Earlier reports have focussed on enterobactin and its degradation products, showing that an esterase attack is required for iron removal inside the cells and that esterase-negative mutants were unable to acquire iron from ferric enterobactin (Langman et al. 1972; Porra et al. 1972). However, an esterase attack prior to reduction of ferric-enterobactin has been questioned because the iron-free ligand, enterobactin, was more rapidly hydrolyzed than the predicted substrate ferric-enterobactin (Greenwood and Luke 1978).

Isolation of enterobactin from a receptor-deficient mutant has been described earlier by Young and Gibson (1979); it requires careful handling of the mutant and a special amino-acid-containing media. Moreover, purification was suggested for ferric-enterobactin using DEAE-cellulose column chromatography, followed by acid dissociation and precipitation with hexane. However, since the criteria for judging the purity were not highly developed in the previous studies, we reinvestigated the production of catecholate siderophores using modern identification methods. The exact determination of enterobactin and its hydrolysis products is essential to an investigation of its physiological role. Great interest exists in the production of larger amounts of enterobactin and its corresponding linear dihydroxybenzoylserine compounds since it is known that the linear trimer and dimer are able to transport iron into *E. coli* (Scarrow et al. 1990); also the monom-

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er, dihydroxybenzoylserine, functions as a siderophore (Hantke 1990). We have therefore isolated the catecholate fraction from low-iron cultures of different enteric bacteria and separated the various components by HPLC followed by mass spectrometric identification. We have further developed a large-scale method for the isolation of enterobactin which is based on adsorption of the catecholate fraction on XAD-2 and a subsequent purification step on Sephadex LH20.

Materials and methods

Bacterial strains and growth conditions. The following strains were used: *Escherichia coli* AN311, *Enterobacter intermedium* and strains of *Enterobacter agglomerans* which we have identified earlier as *Erwinia herbicola*. *Erwinia herbicola* K4 (wild type) was obtained as a clinical isolate from U. Ullmann (Berner et al. 1988). The mutant *E. herbicola* E8 is a derivative of *E. herbicola* K4 (wild type), obtained by a mutant screening and selection for ferrioxamine biosynthesis mutants using Chrome-azurol-S plates (Schwyn and Neilands 1987). *E. herbicola* EH23, an isolate that produces high amounts of polysaccharides, was provided by K. Poralla (Tübingen). *Enterobacter intermedium* was from the Robert Koch-Institut (Wernigerode). All strains were maintained on agar slants containing peptone/yeast extract/glucose or nutrient broth. Chemically defined iron-deficient medium contained M9 salts and glucose. In order to enhance iron-deficiency, M9 medium was further deferrated by passing it through a Chelex-100 column (Bio-Rad).

Isolation of catecholate siderophores. Bacterial suspension cultures (100 ml) were grown under aeration and iron-limiting conditions over a time period of 24 h or longer and then centrifuged at $6000 \times g$ in a Sorvall centrifuge (SS24). The supernatant (10 ml) was acidified to pH 2 and extracted twice with ethyl acetate (10 ml). The extract was dried over anhydrous Na_2SO_4 and evaporated on a rotary evaporator. The catecholates were dissolved in methanol and used directly for HPLC measurements.

Isolation of enterobactin. Enterobactin from *E. coli* AN311 (*fep*$^-$) was isolated according to the method described by Young and Gibson (1979). In addition, a new large-scale isolation method is described. From 10 l culture medium, cells were removed by a tangential filtration device (Pellicon, Millipore). The culture filtrate was then acidified to pH 2 and passed through a XAD-2 column (Serva, Heidelberg). The column was washed with one volume of distilled water and the enterobactin fraction was then desorbed by one volume of methanol, evaporated to dryness, dissolved in methanol and further purified by chromatography on Sephadex LH20 using methanol as an eluting solvent.

HPLC analysis. Ethyl acetate extracts and purified iron-free enterobactin were analyzed by HPLC (Knauer) on a C_{18} reversed-phase column (Nucleosil 5 µm, 125 × 4.6 mm) using methanol/0.1% phosphoric acid (1:1, by vol.) as an isocratic solvent system. The flow rate was 1 ml/min and the compounds were detected at a wavelength of 220 nm.

Mass spectrometry. Samples were analyzed by ion spray mass spectrometry and ion spray tandem mass spectrometry (MS/MS) either directly or on-line (LC/MS) after separation on a C_{18} reversed-phase column (Synchropak C_{18}, 7 µm, 100 × 2 mm; Fa. Grom, Herrenberg, FRG) at a flow rate of 200 µl/min and methanol/0.1% formic acid (1:1) as an eluent.

Ion spray mass spectra and ion spray tandem mass spectra were recorded on a Sciex API III triple-quadrupole mass spectrometer with 2400-Da mass range equipped with an ion spray ion source (Sciex, Toronto, Canada). The mass spectrometer was operated under unit mass resolution conditions for all determinations; profile spectra were obtained by acquiring data points every 0.1 Da. Ion spray voltage was 5 kV.

The sample was dissolved in 0.1% formic acid/methanol (1:1). Lithium chloride was added in form of a 10 mM aqueous solution in order to improve identification of molecular peaks and to suppress fragmentation. Both final analyte and salt concentrations were about 100 µM. For direct injection, the solution was introduced into the ion spray source at a constant flow rate of 5 µl/min with a microliter syringe using a medicinal infusion pump (Harward apparatus, USA). Total sample consumption was in the picomolar range. Argon at a target gas thickness of about 1.3×10^{14} atoms cm^{-2} was used as collision gas for tandem mass spectrometry. The collision energies used ranged over 30–60 eV, laboratory frame.

Results

HPLC analysis of ethyl acetate extracts from low-iron cultures of enterobacterial strains generally revealed a characteristic five-peak pattern as shown for an extract obtained from a culture of *Erwinia herbicola* EH23 (Fig. 1). Good separation of all five peaks was obtained on a C_{18} reversed-phase column using methanol/0.1% phosphoric acid (1:1) as a solvent system. The relative proportions of these peaks varied depending on the time of cultivation and the strains used; additional peaks have been observed in some strains and under certain conditions. After separating the catecholate fraction from *E. herbicola* EH23 on a Sephadex LH20 column, we were able to isolate peak 4 in a highly purified form. Ion spray mass spectrometry confirmed the identity of this fraction with enterobactin, which we have isolated from *E. coli* AN311 as a reference compound (Fig. 2). Ion spray tandem mass spectra (MS/MS) of purified enterobactin showed, besides the parent $[M+H]^+$ peak (m/z 670), a typical fragmenation into two daugther ions ($m/z = 447.4$ and 224.0) which represent the $[M+H]^+$

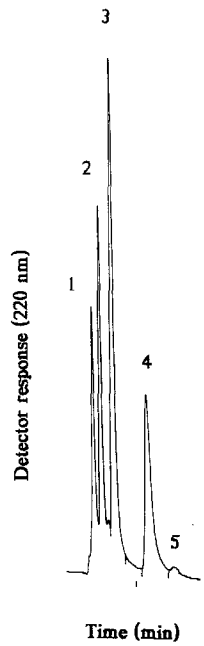

Fig. 1. HPLC separation of catecholate siderophores from *Erwinia herbicola* EH23 (*Enterobacter agglomerans*) on a C_{18} reversed-phase column (Nucleosil 5 µm, 125 × 4.6 mm) using methanol/0.1% phosphoric acid (1:1) as an eluting solvent. The flow rate was set at 1 ml/min and the peaks were monitored at a wavelength of 220 nm. The siderophores were extracted with ethyl acetate from cultures grown aerobically for 12 h in M9 medium at 27° C. Retention times: (1) 1.83 min, (2) 2.15 min, (3) 2.66 min, (4) 4.05 min, (5) 5.37 min

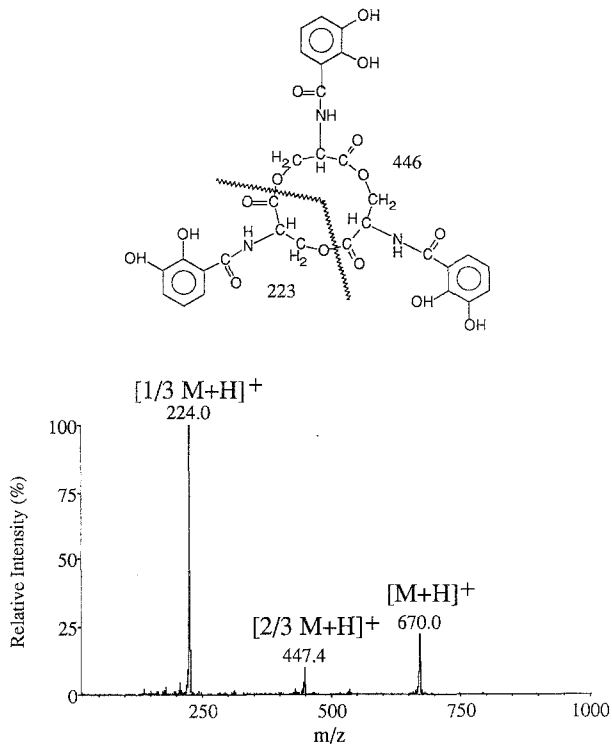

Fig. 2. Ion spray tandem mass spectrum of enterobactin showing the parent ion $[M+H]^+$ *m/z* 670.0 and the fragments 1/3 $[M+H]^+$ *m/z* 224.0 and 2/3 $[M+H]^+$ *m/z* 447.4, which originate from cleavage of the lactone bonds giving one and two dihydroxybenzoylserine residues, respectively. The sample was measured with a triple quadrupole mass spectrometer (API III, Sciex, Toronto) using argon as collision gas. Samples were injected using the solvent system MeOH/0.1% formic acid at a flow rate of 5 µl/min

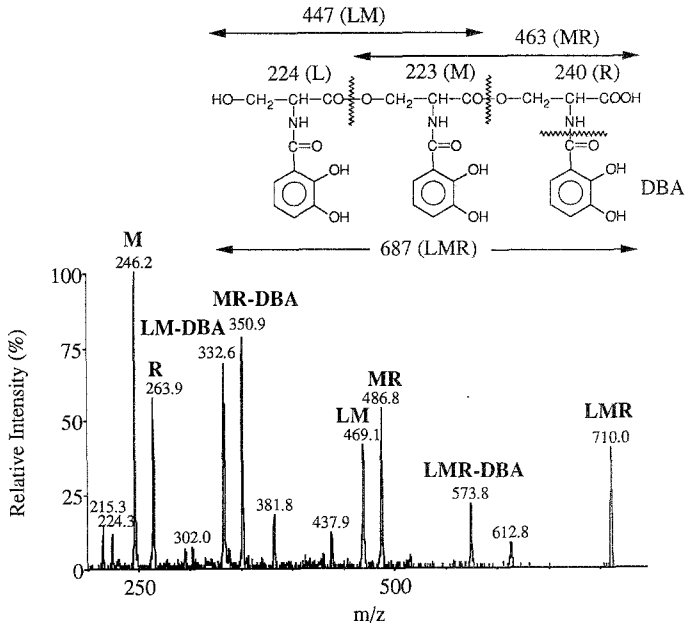

Fig. 3. Fragmentation scheme and ion spray tandem mass spectrum (Na^+-adducts) of the linear trimer from a purified main fraction of *E. herbicola* EH23. Fragments are assigned as follows: left side of the molecule (L), middle (M), right (R), double fragments LM, RM and LMR, the parent ion $[M+Na]^+$. In addition, fragments which have lost the dihydroxybenzoyl (DBA) residues could be assigned (LMR-DBA, MR-DBA, LM-DBA). Conditions are as described in Fig. 2

ions of two-third and one-third masses of the parent $[M+H]^+$ quasi-molecular ion. Obviously, the lactone bonds are the preferred splitting sites after collision of this ion with argon atoms.

For the assignment of peak 3, we used a Sephadex LH20 fraction which contained that peak as the principal compound. This main fraction was subjected to ion spray tandem mass spectrometry (MS/MS) giving a fragmentation pattern (Na^+ adducts) of the corresponding linear trimer of 2,3-dihydroxybenzoylserine (DHBS; Fig. 3). Because of the additional free OH and COOH groups, the linear trimer exhibited a higher ion mass of $m/z=710$ for the $[M+Na]^+$ quasi-molecular ion. The fragmentation pattern revealed Na^+-adduct fragments of the left (L), right (R) and middle (M) part of the molecule as well as two-thirds (LM, MR) and the parent ion (LMR) ions. Furthermore, dihydroxybenzoic acid (DBA) residues are split off resulting in LMR−DBA, LM−DBA and RM−DBA ion fragments. Thus, mass spectrometry confirmed that peak 3 in Fig. 1 is identical with the linear trimer of 2,3-dihydroxybenzoylserine, $(DHBS)_3$.

Since it was more difficult to isolate highly purified samples of the linear dimer and monomer of 2,3-dihydroxybenzoylserine from ethyl acetate extracts, we investigated the ethylacetate extract additionally by HPLC/MS by linking the mass spectrometer on-line to a microbore HPLC system (Applied Biosystems 140A). An LC/MS chromatogram (total ion current versus time) was obtained, in which the peaks for the linear dimer and trimer could be identified. Fig. 4 shows the reconstructed chromatogram after extraction of the expected values for $[M+H]^+$ and $[M+Na]^+$ by a computer program and also the ion spray mass spectra of the two compounds, with *m/z* values (222.0, 465.6, 487.2) and (224.2, 447.2, 688.0, 710.0) for the linear dimer and trimer (peak 2 and peak 3 in Fig. 4) respectively. Thus, peak 2 in Fig. 1 corresponds to the linear dimer of 2,3-dihydroxybenzoylserine $(DHBS)_2$.

The first peak in Fig. 1 was suspected to be the monomer of 2,3-dihydroxybenzoylserine. However, when subjected to ion spray mass spectrometry the $[M+H]^+$ quasi-molecular ion ($m/z=242.1$) was not as prominent as those for the dimer and trimer, suggesting that the proportion of the monomer in peak 1 is smaller than assumed from the ultraviolet detector signal. As shown in a mass spectrum of the whole extract with added lithium chloride (Fig. 5), the monomer species, $[M+H]^+$ $m/z=242.1$ and $[M+Li]^+$ $m/z=248.0$ are clearly detectable. The mass spectrum shows four molecular ion clusters, representing the H^+, Li^+, Na^+ and K^+ adducts of the quasi-molecular ions of DHBS,

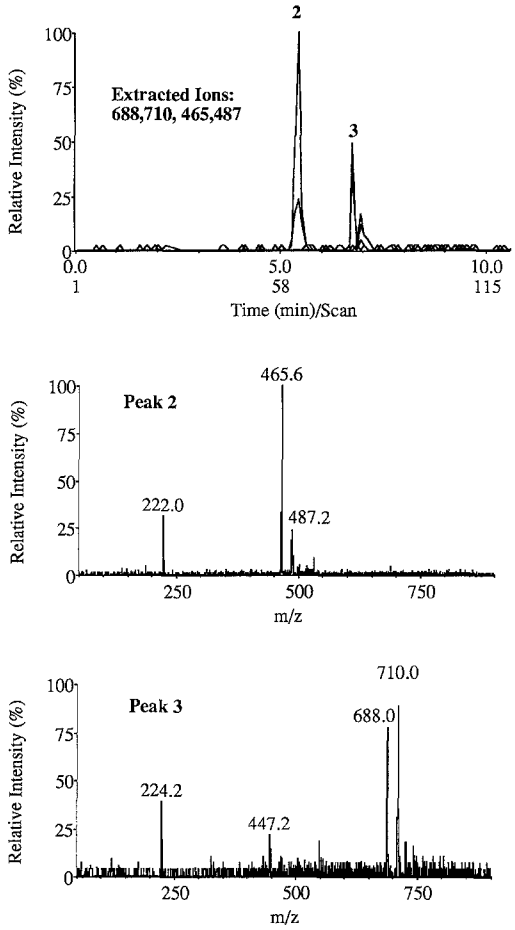

Fig. 4. Extracted ions from an LC/MS chromatogram. A sample of an ethyl acetate extract, isolated from a low-iron culture of *E. herbicola* EH23, was separated on a microbore C_{18} reversed-phase column using a gradient (20–100% methanol in 0.1% formic acid within 10 min). The $[M+H]^+$ and $[M+Na]^+$ ions of the dimer (2) and trimer (3) were extracted from the whole spectrum.

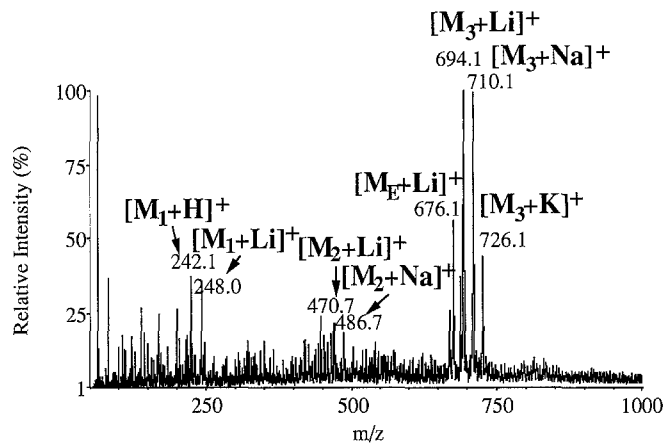

Fig. 5. Mass spectrometric identification of total catecholate siderophores from an extract. Quasi-molecule ion peaks of enterobactin $[M_E+Li]^+$, the linear trimer ($[M_3+Li]^+$, $[M_3+Na]^+$, $[M_3+K]^+$), the linear dimer ($[M_2+Li]^+$, $[M_2+Na]^+$) and the monomer (*N*-2,3-dihydroxybenzoylserine) ($[M_1+H]^+$, $[M_1+Li]^+$) are assigned. Conditions are as described in Fig. 2

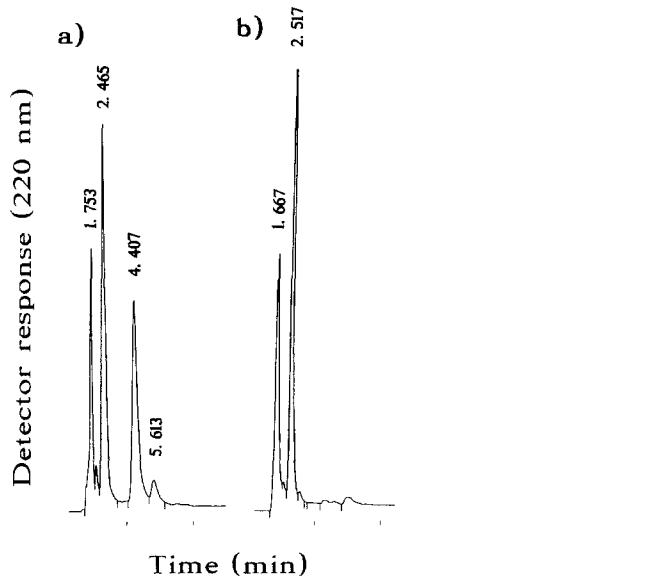

Fig. 6. HPLC separation of catecholates from *Enterobacter intermedium*. (**a**) Iron-free catecholates; (**b**) extract after addition of iron. Retention times and assignments are as follows: 1.667 min, iron-containing catecholate fraction; 1.753 min, monomer fraction; 2.465 min, 2,3-dihydroxybenzoic acid and linear trimer (unresolved); 4.407 min, enterobactin; 5.613 min, isoenterobactin. Conditions are as described in Fig. 1

$(DHBS)_2$ and $(DHBS)_3$. In addition the Li^+-adduct ($m/z=676.1$) of the cyclic enterobactin is clearly visible.

Peak 5 in Fig. 1 is very small. We therefore used an extract from another strain, *Enterobacter intermedium* (Fig. 6a) which contained predominantly the monomer fraction ($t_R=1.753$ min), 2,3-dihydroxybenzoic acid ($t_R=2.465$ min) together with the linear trimer (unresolved peak) as well as enterobactin ($t_R=4.407$ min) and the additional peak 5 ($t_R=5.613$ min). This latter compound, although scarcely detectable in other enterobacterial extracts, appeared in *E. intermedium* after two days of cultivation in equal amounts with enterobactin. Surprisingly, both peaks (4 and 5) gave identical mass spectra ($m/z=670$) when analyzed by LC/MS. This suggested that compound 5 represents an isomer of enterobactin. As the fragmentation into two-third and one-third masses is equivalent to enterobactin, the cyclic nature and the connection of DHBS units via lactone bonds corresponds to enterobactin. Proton-NMR studies with purified material will be performed to substantiate this suggestion. Evidence for the iron-binding capacity of compound 5 has been obtained when iron was added to this extract and the HPLC chromatogram was compared with that of the original iron-free extract. After addition of iron (Fig. 6b), all iron-free compounds, the linear compounds, enterobactin and peak 5 disappeared from the chromatogram and a new peak at a retention time (t_R) of 1.667 min appeared which obviously contained the unresolved ferric-catecholate complexes. The 2,3-dihydroxybenzoic acid peak ($t_R=2.517$ min), however, was not significantly changed. Thus, this procedure allowed all iron-complexing catecholates to be identified from non-com-

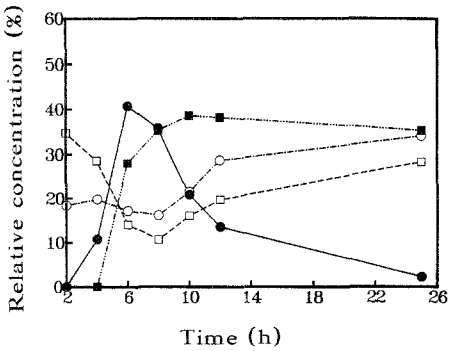

Fig. 7. Time-dependent production of catecholates in low-iron medium (M9) by *E. herbicola* EH23. Samples (10 ml) of low-iron culture filtrates were taken at intervals, extracted and separated by HPLC. The areas of the individual peaks, measured as a percentage of the total area, were taken as an approximate quantitative estimation of catecholate production and plotted against the time of cultivation. (□) Monomer fraction; (○) dimer; (■) trimer; (●) enterobactin

plexing compounds prevailing in an ethyl acetate extract or in otherwise purified material.

Although Fig. 1 represents a general HPLC pattern of catecholate compounds in enteric bacteria, certain deviations from this pattern could be observed. For example, HPLC separations of extracts from *Erwinia herbicola* E8 and *E. herbicola* EH23, two non-ferrioxamine-producing strains, showed high amounts of the monomer fraction and relatively small amounts of the dimer and trimer when grown in Chelex-100-treated M9 medium (not shown), whereas in M9 medium which still contained residual iron, high amounts of the linear compounds are observed as seen in Fig. 1. Thus, the composition of catecholate siderophores may vary with different strains but is more strongly dependent on the degree of iron deficiency. As expected, the HPLC pattern of catecholates also significantly changed with the duration of cultivation. As shown in Fig. 7 for *E. herbicola* EH23, enterobactin production increased with time and had its maximum at about 6 h under the prevailing growth conditions. On the other hand, the linear trimer, and to some extent the linear dimer, showed a relatively late onset followed by a more constant increase with time. As the first peak did not represent pure monomer (DHBS), a kinetic analysis of its production was not possible. However, as judged from the intensities of the molecular signals in the mass spectra, its concentration was similar to that of the dimer, this is to be expected if equimolar degradation products (dimer and monomer) are formed during trimer degradation. Most interestingly, the dimer and trimer seem to be present in higher amounts in M9 medium containing some residual iron, which supports the commonly accepted view that enterobactin has to be transported into the cell in the ferric form in order to be degraded by a ferric-enterobactin esterase.

Discussion

Enterobactin production in bacterial culture fluids is often followed by simply measuring the phenolate concentration by the Arnow reaction (Arnow 1937). However, as shown in the present investigation, only a small part of the catecholate fraction produced represents cyclic enterobactin. A significant fraction of the extracted catecholates represent linear 2,3-dihydroxybenzoylserine compounds which may even predominate in low-iron medium.

Several isolation methods for enterobactin have been described so far. The first method published was the extraction of iron-free enterobactin with ether from acidified culture filtrates (Pollack and Neilands 1970). A subsequent precipitation in excess hexane and removal of oxidation products by a short silica gel column has been recently suggested to improve purification (Neilands and Nakamura 1991). The method used in the present investigation is the extraction with ethyl acetate as first described by Cox et al. (1970). Briefly, the culture filtrate is acidified with H_2SO_4 to pH 2 and extracted twice with an equal volume of ethyl acetate. The combined extracts are dried with Na_2SO_4 or $MgSO_4$ and concentrated by rotary evaporation. A further method which we describe in the present investigation is particularly suited for the preparation of enterobactin on a larger scale. After percolation of the acidified culture filtrate through an XAD-2 column and washing with two volumes of distilled water, the catecholate siderophores are desorbed with methanol. A subsequent purification on Sephadex LH20 with methanol as an eluting solvent yields highly purified enterobactin.

The results of the present investigation show that enterobactin and the linear trimer, dimer, monomer and the supposed isoenterobactin can now be separated by HPLC on C_{18} columns. The five main peaks have been assigned by ion spray mass spectrometry which can be run on-line with liquid chromatography when connected by an ion spray LC/MS interface (Bruins et al. 1987). Enterobactin and its linear DHBS compounds are split at the lactone bonds during MS/MS which allow an easy mass spectrometric identification even from unseparated catecholate mixtures. While the two enterobactins and the corresponding linear compounds have been unequivocally identified by HPLC and mass spectrometry, several compounds which have been detected in the HPLC chromatograms of other strains have not yet been identified and are still under investigation. Moreover, a comparison of iron-free and iron-containing catecholates during HPLC separation could be demonstrated which allows siderophores and non-siderophore compounds to be distinguished. However, the HPLC conditions used did not allow the various iron-containing catecholates to be separated as had been shown earlier for the hydroxamate siderophores (Konetschny-Rapp et al. 1988; Berner et al. 1988; Reissbrodt et al. 1990).

Although there is a common appearance of the HPLC patterns from various enteric bacteria, character-

istic changes within different strains and genera may be detected. In all strains studied, enterobactin was accompanied by the linear DHBS compounds. However, the proportion of these compounds changed in a characteristic manner when iron deficiency was increased. For example, in Chelex-100-treated media the amount of trimer and dimer is greatly reduced and the monomer fraction predominates. The reason for this is unknown but, since the dimer and trimer seem to increase when residual iron is present in the M9 cultivation medium, the involvement of an esterase activity seems likely.

In the present investigation, it was intended to isolate and characterize enterobactin and the linear compounds (trimer, dimer and monomer) from strains of the genus *Enterobacter* (*E. agglomerans, E. intermedium*). The *Erwinia herbicola* (*Enterobacter agglomerans*) group has recently been transferred to a new genus, *Pantoea* (Gavini et al. 1989; Beji et al. 1988). We have shown earlier that species of the *Erwinia herbicola* (*Enterobacter agglomerans*) group and *Hafnia alvei* produce ferrioxamines (Berner et al. 1988; Berner and Winkelmann 1990; Reissbrodt et al. 1990). However, *Erwinia herbicola* EH23 isolate and a mutant strain *E. herbicola* E8, derived from a ferrioxamine-producing *E. herbicola* K4 (wild type) have been found to produce only enterobactin and its linear analogs and no ferrioxamines. Thus, while enterobactin production seems to be more widespread within enteric bacteria, ferrioxamine production is confined to the genera *Enterobacter, Hafnia* and *Pantoea.*

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