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HPLC separation of enterobactin and linear 2,3-dihydroxybenzoylserine derivatives: a study on mutants of *Escherichia coli* defective in regulation (*fur*), esterase (*fes*) and transport (*fepA*)

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Reversed-phase HPLC separation of enterobactin and its 2,3-dihydroxybenzoylserine derivatives was used for a comparative analysis of mutants of *Escherichia coli*, defective in the regulation of enterobactin biosynthesis (*fur*), enterobactin transport (*fep*A) and enterobactin esterase (*fes*). A complete separation of all 2,3-dihydroxybenzoylserine compounds was achieved: the monomer (DHBS), the linear dimer $(DHBS)_2$ and trimer $(DHBS)_3$, the cyclic trimer, enterobactin, as well as 2,3-dihydroxybenzoic acid. The production of all these compounds was followed after ethylacetate extraction from acidified culture fluids. Enterobactin was found to be the predominant product in all mutant strains. The mutant strains behaved differently with regard to the breakdown products. All degradation products, such as DHBS, $(DHBS)_2$ and $(DHBS)_3$, were detected in the overproducing *fur* mutant where both transport and esterase are still functioning, while only the monomer, DHBS, was detected in the *fep*A mutant and no degradation was found in the esterase-deficient *fes* mutant. From the pattern of breakdown products it may be inferred that the esterase acts in two different ways, depending on whether transport is functioning or not. Thus, esterolytic cleavage of ferric enterobactin after entering the cells results in a mixture of all three hydrolysis products, i.e. DHBS, $(DHBS)_2$ and $(DHBS)_3$, while cleavage of iron-free enterobactin subsequent to its biosynthesis yields only the monomer. Thus, the results of quantitative HPLC analysis of enterobactin and its breakdown products show that different enterobactin esterase products arise, depending on whether iron is bound to enterobactin or not.

Keywords: 2,3-dihydroxybenzoylserine, enterobactin, *Escherichia coli*, HPLC

Introduction

Enterobactin, the cyclic triester of 2,3-dihydroxybenzoylserine, is the prototype siderophore of the family of Enterobacteriaceae (Earhart 1987, Payne 1988, Winkelmann 1991). However, the biosynthetic capacity of siderophore production in the family of Enterobacteriaceae is greater than assumed earlier. Thus, in some genera such as *Enterobacter*, *Escherichia*, *Shigella* and *Salmonella* an additional hydroxamate siderophore, named aerobactin, has been found which has been shown to enhance virulence (Payne 1988). Moreover, strains of *Enterobacter*, *Hafnia*, *Erwinia*, *Pantoea* and *Ewingella* produce ferrioxamines (Berner *et al.* 1988, Berner & Winkelmann 1990, Reissbrodt *et al.* 1990), and members of the tribe Proteeae like *Proteus*, *Providencia* and *Morganella* use simple α-keto acids as siderophores which they produce by deamination of amino acids (Drechsel *et al.* 1993, Thieken & Winkelmann 1993). Despite these variations in siderophore production, all enterobacteria and some related Gram-negative bacteria express an outer membrane receptor for the transport of enterobactin. This could be confirmed by the use of monoclonal antibodies against the FepA receptor of *E. coli*, showing a wide-spread occurrence of similar epitopes in the outer membrane of all enterobacteria studied and also in related Gram-negative bacteria

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such as *Aeromonas*, *Vibrio*, *Neisseria* and *Haemophilus* (Rutz *et al.* 1991).

The ferric enterobactin transport system of *E. coli* has been described in detail by Earhart (1987) and the outer membrane receptor protein, FepA, has been cloned and sequenced (Lundrigan and Kadner 1986). However, rapid identification of outer membrane receptors for enterobactin in the various Gram-negative strains by SDS gel electrophoretic methods remains difficult as the molecular and electrophoretic mobility may vary. Therefore, analytical HPLC is the method of choice to follow enterobactin production. Enterobactin and derivatives from culture fluids have previously been identified spectroscopically (Scarrow *et al.* 1991, Berner *et al.* 1991) or by bioassays (Seiffert *et al.* 1993). However, the relative quantities of hydrolysis products of enterobactin esterase (Brickman and McIntosh 1992) have never been determined in detail. The present investigation demonstrates the use of HPLC for the study of enterobactin esterase activity, by identifying the linear trimer $(DHBS)_3$, the linear dimer $(DHBS)_2$ and the monomer DHBS (Figure 1) in different mutant strains of *E. coli*.

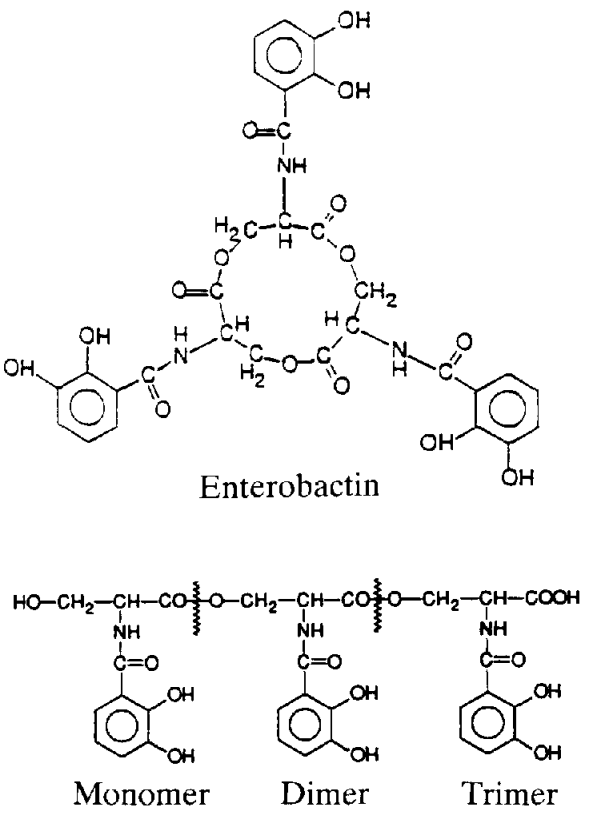

Figure 1. Structural formula of enterobactin and its linear 2,3-dihydroxybenzoylserine derivatives: monomer = DHBS; dimer = $(DHBS)_2$; trimer = $(DHBS)_3$.

Materials and methods

Strains and growth conditions

The following strains were used: *E. coli* AN311 (F, *pro*, *leu*, *trp*, *thi*, *fep*), an enterobactin transport defective mutant of *E. coli* K-12, required the addition of proline, tryptophan, leucine and thiamin for growth as described by Young & Gibson (1979). The presence of 0.2 mM $FeSO_4$ does not lead to a repression of enterobactin biosynthesis because of the *fep* mutation. Thus the accumulation of ferric enterobactin results in a deep red color of the culture broth.

The esterase deficient strain *E. coli* AN 272 (*fes*$^-$) is a mutant of *E. coli* AN92 and was obtained after treatment with nitrosoguanidine (Langman *et al.* 1972). The *fur* mutant, *E. coli* H1941, is a deletion mutant from the parent MC 4100, kindly provided by K. Hantke, Tübingen.

HPLC separation

Enterobactin and its degradation products were identified on an analytical HPLC column (Nucleosil C_{18}, 5 μm, 4.6 × 250 mm; Grom, Herrenberg, Germany) using a gradient of acetonitrile (10–50%) in water, a detector wavelength of 220 nm and a flow rate of 1 ml min^{-1}. Both solvents contained additionally 0.1% trifluoroactic acid (TFA) to maintain a constant pH of 3. Separation was performed on a Shimadzu HPLC system consisting of two LC-9A pumps, an SCL-6B controller, an SIL-6B autoinjector, a C-R4 AX-Chromatopac computer and an SPD-6AV UV/vis spectrometric detector. Samples of 10–50 μl were injected by an autoinjector and separated using a gradient of 10–50% acetonitrile in water (plus 0.1% TFA). Chromatograms were plotted after baseline substraction.

Sample preparation

Samples (3 ml) were taken from shake cultures after 24 h of growth or as in the case of *E. coli* AN 311 at intervals (6, 12, 24, 28, 36 and 48 h) and centrifuged at 13000 r.p.m. in Epppendorf cups. The clear supernatant was acidified with 10 N HCl (15 μl) resulting in an approximate pH of 2 and extracted twice with ethylacetate (3 ml). The solvent was evaporated and the residue dissolved in 1 ml 10% acetonitrile/water (0.1% TFA).

Mass spectra

Electrospray mass spectra were recorded by direct injection of siderophore solutions on a Sciex API III triple-quadrupole mass spectrometer with 2400 Da mass range equipped with an ion spray ion source (Sciex, Toronto, Canada), as described earlier by Berner *et al.* (1991).

Results

HPLC separation of ethylacetate extracts of the receptor-deficient *E. coli* mutant AN311 (*fepA*) revealed a characteristic pattern of peaks consisting of mainly enterobactin, very small amounts of the linear trimer $(DHBS)_3$ and dimer $(DHBS)_2$, and relatively large amounts of the monomer DHBS (Figure 2). Identification of all HPLC peaks was based on electrospray mass spectroscopy. While the spectra of enterobactin and the linear trimer and dimer have been published in a previous paper (Berner *et al.* 1991), the spectrum of the monomer, DHBS, taken from a sample after semipreparative HPLC, is shown in Figure 3. The spectrum of DHBS contains four cluster representing multiple molecular ions (M, M_2, M_3 and M_4) and their Na^+ and K^+ adducts. Additional peaks can be identified which contain mixed adducts (M_x + Na + K, M_x + Na + 2K, M_x). The peak at m/z = 241.8 was assigned to the MH^+ ion of the monomer DHBS species, followed by peaks corresponding to MNa^+ (m/z = 264) and MK^+ (m/z = 280). The second cluster at m/z = 505.2 and 521.2 represents the M_2Na^+ and M_2K^+ molecular ion adducts. Additional clusters of M_3 and M_4 molecular Na^+ and K^+ adducts can be assigned in the range of m/z = 746 and m/z = 1003.2. Thus, the monomer DHBS showed a characteristic ion spray mass spectrum of four molecular clusters and their Na^+ and K^+ ion adducts. A mass peak at m/z = 137 (Figure 2) was assigned to the 2,3-dihydroxybenzoyl ion fragment (DHB), resulting from the fragmentation at the amide bond. A peak occuring at 11.46 min in between the peaks of the dimer and monomer was not a derivative of DHBS but turned out to be tryptophan (MH^+, m/z = 205) as determined by electrospray mass spectrometry. As the AN311 mutant is a *trp*$^-$ mutant the incubation medium was supplemented with tryptophan which is co-extracted by ethylacetate. The HPLC chromatogram of AN311 (Figure 2) contained also a small peak at 12.7 min which was assigned to 2,3-dihydroxybenzoic acid (DHBA) by co-chromatography with an authentic sample of DHBA.

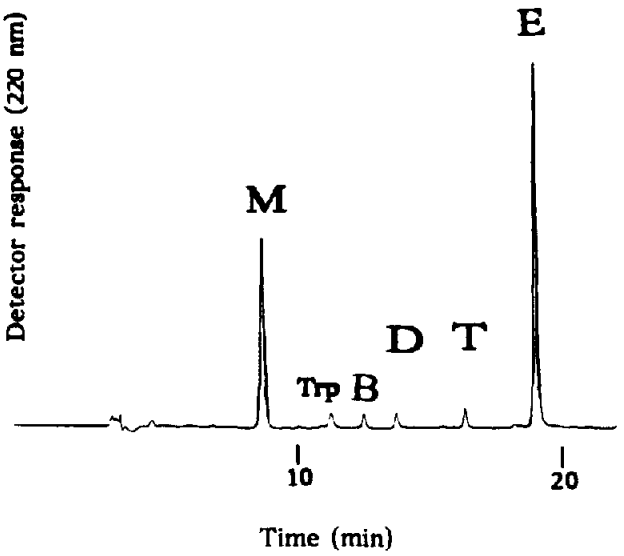

Figure 2. HPLC analysis of an ethylacetate extract from a culture of the enterobactin transport-deficient *fep*A mutant, *E. coli* AN311. The culture was grown in M9 medium, previously deferrated with Chelex 100. HPLC conditions are as described in Materials and methods. Symbols are as follows: enterobactin (E); monomer (M); dimer (D); trimer (T); 2,3-dihydroxybenzoic acid (B); tryptophan (Trp).

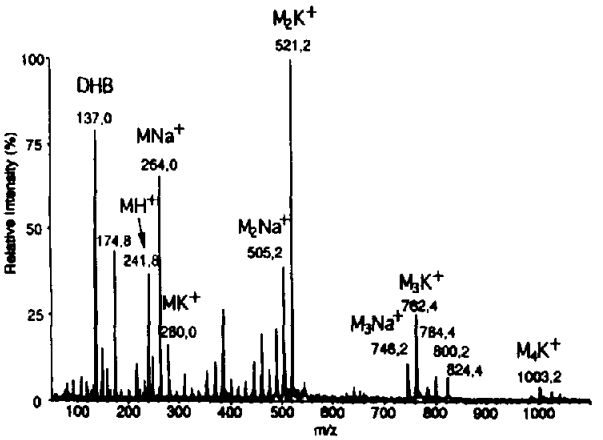

Figure 3. Electrospray mass spectrum of the monomer (DHBS) isolated by semipreparative HPLC from culture extracts of *E. coli* AN311. For details see text.

After having identified all important peaks resulting from HPLC separation of ethylacetate extracts of culture fluids of *E. coli* AN311, we started a time dependent analysis of enterobactin production. Enterobactin (E) was the prominent product during the whole incubation time of 6–48 h (Figure 4). However, the monomer (M) seems to be produced very early, reaching a maximum of production at 28 h and then suddenly disappears from the culture medium. In contrast, the amount of the linear trimer and dimer never exceeded 1–2% of the total enterobactin fraction, suggesting that esterolytic degradation into dimer and trimer is prevented in the transport-defective *fep*A mutant.

The proportion of linear dimer and trimer increased when extracts from a *fur* deletion mutant of *E. coli* (H1941) was studied (Figure 5). A similar pattern was obtained with the *fur* point mutant H1673 (data not shown). The *fur* mutants generally

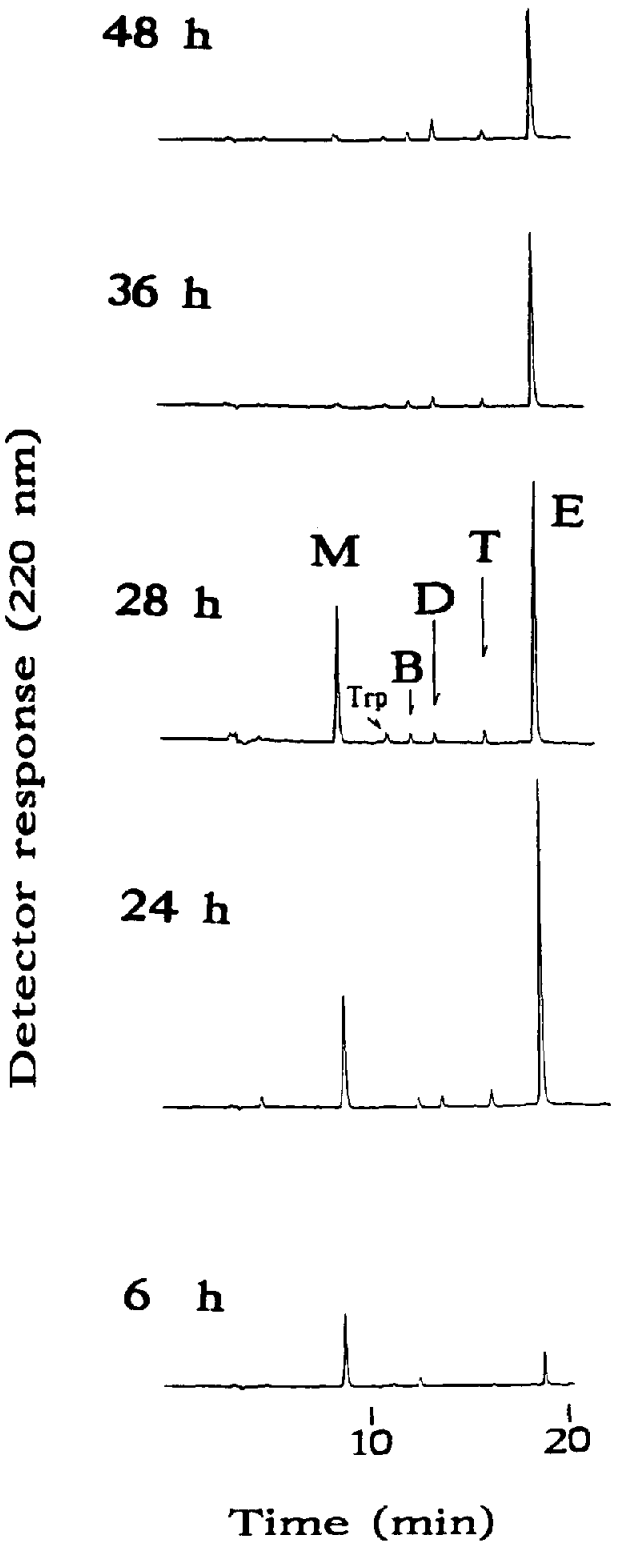

Figure 4. Time dependent production of enterobactin and DHBS compounds by *E. coli* AN311 followed by HPLC after ethylacetate extraction of acidified culture filtrates. Samples of 2 ml were taken at intervals (6–48 h) from a 100 ml culture of AN311. Extraction procedure and growth conditions of cultures were as described in Materials and methods.

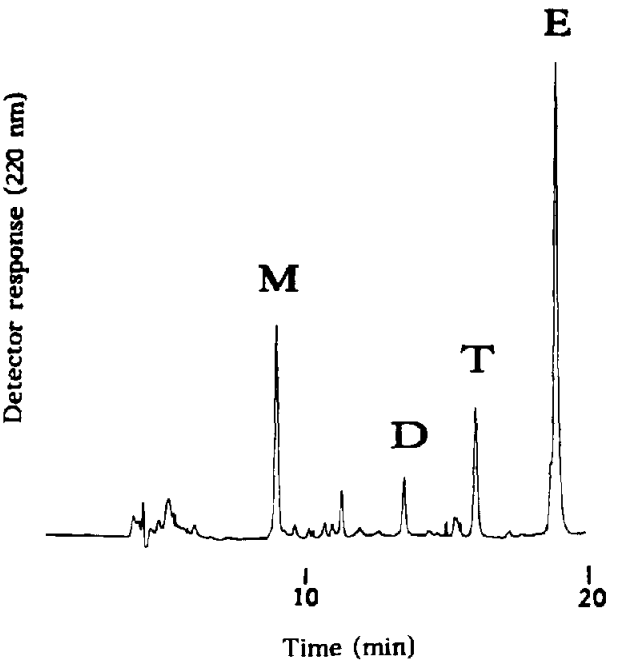

Figure 5. HPLC analysis of ethylacetate extracts from culture filtrates of the *fur* mutant *E. Coli* H1941. The culture was grown in Chelex-100 treated M9 medium for 12 h and the medium was extracted with ethylacetate. Symbols: monomer DHBS (M); dimer $(DHBS)_2$ (D); trimer $(DHBS)_3$ (T); enterobactin (E); tryptophan (Trp).

show overproduction of enterobactin but are otherwise normal with respect to transport and biosynthesis.

A completely different pattern is seen with the esterase-defective *fes* mutant *E. coli* AN273 (Figure 6). In this mutant enterobaction is the only DHBS

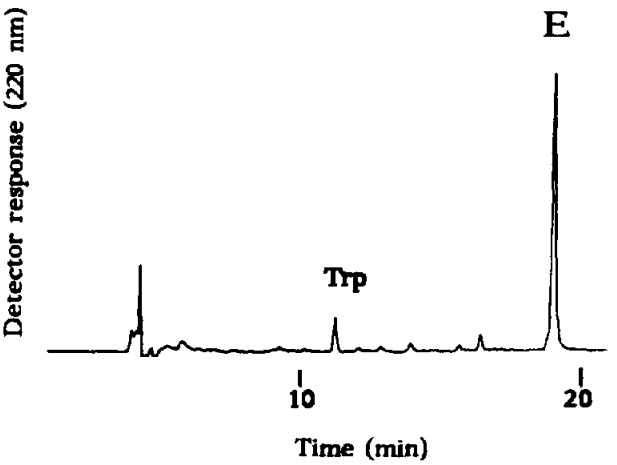

Figure 6. HPLC separation of ethylacetate extracts from culture filtrates of the *fes* mutant *E. coli* AN273. Conditions are as in Figure 5. Symbols: enterobactin (E); tryptophan (Trp).

compound produced. Small amounts of tryptophan were also detected. The results confirm that esterase-deficient strains are unable to produce linear DHBS compounds as a result of biosynthetic precursors. Also the monomer is not biosynthesized as an independent siderophore as might be expected from earlier reports on its siderophore activity.

Discussion

Fermentation and isolation procedures of enterobactin have been described earlier by Young & Gibson (1979). Recently the use of XAD-2 adsorbents for large-scale preparation and analytical reversed-phase HPLC systems were reported. Two HPLC systems have been recommended, an isocratic system consisting of methanol/0.1% phosphoric acid (Berner *et al.* 1991) and a gradient system using acetonitrile (0–100%) in 0.1% phosphoric acid (Seiffert *et al.* 1992). Here we use a gradient system of acetonitrile (10–50%) in water plus 0.1% TFA added to both solvents. Trifluoroactic acid guarantees a continuous acidity (pH 3) over the whole gradient and it can easily be removed by evaporation or lyphilization.

While in earlier studies additional bioassays were used for the identification of HPLC peaks (Seiffert *et al.* 1993), the present investigation makes use of electrospray mass spectrometry which proved to be especially well suited for the analysis of DHBS compounds because the molecules are preferentially split at the ester bonds. While in our previous investigation mass spectra of the linear dimer and trimer were shown (Berner *et al.* 1991), the present paper focuses on the spectroscopic identification of the monomer.

HPLC analysis of ethylacetate extracts from different *E. coli* mutant strains revealed a characteristic pattern of DHBS compounds as summarized in Table 1. Thus we found that the relative amounts of enterobactin and the hydrolysis products were different, depending on whether enterobactin was allowed to enter the cells via the *fep*A receptor or not. While in wild-type strains (data not shown) and *fur* mutant strains (H1941, H1632) all three esterolytic products could be observed, the *fep*A mutant only showed the monomer as an accompanying product of enterobactin. As the *fep*A mutation prevents entrance of the ferric form of enterobactin, it may be inferred that excretion of the monomer results from cleavage of enterobactin during or subsequent to its biosynthesis. On the other hand, in *fep*A$^+$ strains where ferric enterobactin can enter the cells, additional cleavage products, like $(DHBS)_2$ and $(DHBS)_3$ are produced, suggesting that the presence of iron directs the esterolytic cleavage into different products. The esterase-deficient *fes* mutant revealed no hydrolysis products at all, excluding the possibility that linear DHBS compounds might originate biosynthetically. This explanation obviates the possibility of the existence of two different esterases, one acting on iron-free and one on iron-containing enterobactin.

Table 1. Production of enterobactin and linear 2,3-dihydroxybenzoylserine derivatives

Mutants	Entero-bactin	DHBA	DHBS	$(DHBS)_2$	$(DHBS)_3$
fur (H1941)	60	–	20	8	12
*fep*A (AN311)	57	1	37	2	3
fes (AN272)	94	–	1	2	3

Values given as area % of total dihydroxybenzoyl-containing compounds after growth of 12 h in shake cultures.

It has been previously shown that the monomer, DHBS, functions as a siderophore in *E. coli* via several outer membrane receptor proteins, such as FepA, Fiu and to a minor extent via Cir (Hantke 1990). Uptake of ferric DHBS via Fiu and Cir receptors is possibly the reason for the final disappearance of the monomer during cultivation of the *fep*A strain (*E. coli* AN311) as shown in Figure 4.

Acknowledgments

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