Purification of yersiniabactin: a siderophore and possible virulence factor of *Yersinia enterocolitica*

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(Received 14 January 1993; revised 6 April 1993; accepted 13 April 1993)

HPLC analysis revealed that *Yersinia enterocolitica* WA-C produced two substances under iron-limiting conditions one of which was identified as 2,3-dihydroxybenzoyl-L-serine. The other compound had iron-complexing activity and was called yersiniabactin. The *fur* mutant H1852 was shown to produce yersiniabactin constitutively in an iron-independent manner. Yersiniabactin was isolated by ethyl acetate extraction from the spent medium of H1852, size-fractionation chromatography and preparative HPLC. A catechol function was demonstrated with different chemical assays and by UV-visible spectroscopy. The molecular mass of yersiniabactin was determined to be 482 Da. Purified yersiniabactin stimulated growth of *Y. enterocolitica* and *Escherichia coli* φ under iron-limiting conditions and apparently served as an iron carrier. Transport of 59Fe-yersiniabactin was TonB-dependent, indicating a receptor-mediated uptake across the outer membrane. A pesticin-resistant mutant missing the receptor protein FyuA was unable to transport and use yersiniabactin as a siderophore.

Introduction

Many micro-organisms excrete specific iron chelators under iron-limiting conditions. These metabolites are called siderophores, they complex iron and transport it into the cell via specific receptor proteins located in the outer membrane of Gram-negative bacteria (Braun & Hantke, 1991). In mammalian body fluids, iron is firmly bound to carrier proteins such as lactoferrin or transferrin. This limitation of free iron in mammals is part of the unspecific immune system, the so-called nutritional immunity (Weinberg, 1984), because the bound iron is not available for most micro-organisms attempting to colonize these environments. Pathogenic micro-organisms have developed various systems to overcome this shortage of iron. In some cases the production of a siderophore is associated with increased virulence, as in the case of anguibactin produced by *Vibrio anguillarum* (Crosa, 1989). Similarly, siderophore production in *Yersinia enterocolitica* has been postulated to be a virulence factor (Heesemann, 1987).

Several non-pathogenic *Yersinia* such as *Y. frederiksenii*, *Y. intermedia* and *Y. kristensenii* produce the hydroxamate-type siderophore aerobactin (Stuart et al., 1986) in contrast to the pathogenic strains of *Yersinia*, which do not produce aerobactin. Iron supply in these strains is still a matter of debate. In the 1970s conflicting results on siderophore production by the pathogenic *Yersinia* were published (Perry & Brubaker, 1979; Wake et al., 1975). Heesemann (1987) was able to demonstrate the secretion of a siderophore by virulent mouse-lethal strains of *Y. enterocolitica* (serotypes O:8, O:13 and O:40) with the colorimetric chromazurole S (CAS) assay (Schwyn & Neilands, 1987). In this assay the blue CAS-iron complex is destroyed since the chelating activity of the siderophore removes the iron from the complex and the orange colour of chromazurole S appears. Strains of *Y. pseudotuberculosis*, which are mouse-lethal, were also found to be CAS-positive. However, the less virulent *Y. enterocolitica* strains of serotypes O:3, O:9, O:5:27 (Heesemann, 1987) were found to be CAS-negative. From these results it was concluded that virulent, mouse-lethal *Yersinia* may produce novel siderophores that have not yet been

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Abbreviations: CAS, chromazurole S; EDDA, ethylenediamine-di-(O-hydroxyphenyl acetic acid).
detected using common siderophore assays. In addition, in common with many bacteria, Y. enterocolitica is able to use siderophores produced by other micro-organisms, e.g. ferrichrome, ferrioxamine B and E, enterochelin (enterobactin) and 2,3-dihydroxybenzoyl-l-serine (Bäumler et al., 1993). The utilisation of haem via a siderophore-like uptake mechanism was demonstrated by Stojilkovic & Hanke (1992).

The characterization of the siderophore yersiniabactin from the culture broth of Y. enterocolitica O:8 is described. HPLC in combination with the CAS assay was used for the detection and isolation of yersiniabactin. Furthermore, the yersiniabactin-mediated uptake of iron via the pesticin-receptor is demonstrated.

Methods

Media, growth conditions and bacteria. Yersinia enterocolitica WA-C is cured of the virulence plasmid lcr (Heesemann, 1987); WA-1 is a mutant of WA-C unable to produce yersiniabactin (Heesemann, 1987); WA-8 fyuA and WA-9 fnuA were derived from WA-1 (Bäumler et al., 1993) by selection for pesticin resistance; H2000 is a tonB mutant derived from WA-C (Koeblrik et al., 1993). For siderophore production Y. enterocolitica H1852 fur was used (Heesemann et al., 1993; Stagg & Perry, 1992) which showed constitutive siderophore production. Y. enterocolitica 5030, a mutant unable to produce yersiniabactin, was selected after NTG mutagenesis and used for the bioassays. Escherichia coli 01293 was obtained from B. A. D. Stocker, Stanford, CA, USA.

Strains were grown on TY medium (8 g tryptone, 5 g yeast extract and 5 g NaCl 1-) or on nutrient broth medium (8 g nutrient broth, 5 g NaCl 1-). To create iron-limited conditions in these media ethylenediamine-di(0-hydroxyphenol acetic acid) (EDDA) was added at the concentrations given. The strains were stored at -70°C in TY medium with 30% (v/v) glycerol.

A modification of the medium of Cove et al. (1980) was used for siderophore production. The medium contained: A (mg 1-, final concn), l-alanine, 200; l-leucine, 200; l-arginine, 300; l-lysine, 200; l-aspartic acid, 300; l-methionine, 200; l-cystine, 50; p-phenylalanine, 200; l-cystine, 50; l-proline, 300; l-glutamic acid, 400; l-serine, 400; l-glutamine, 200; l-threonine, 300; l-histidine, 300; l-tryptophan, 200; hydroxyproline, 200; l-tyrosine, 200; l-isoleucine, 300; l-valine, 200; l-arginine, 20; 7-glucose, 20 B (mg 1-, final concn), KH2PO4, 100; (NH4)2SO4, 100; NaCl, 1000; MOPS, 20900, pH 7.0 adjusted with NaOH. C, glucose 20 g 1-

Preparation of yersiniabactin. After separating the biomass by passing the culture broth through a cross-flow module (Filtron), yersiniabactin was extracted twice with an equal volume of ethyl acetate at pH 7. The organic phase containing yersiniabactin was evaporated to dryness. The residue was dissolved in methanol (3 ml) and chromatographed on a column (2.8 x 40 cm) containing Fractogel TSK HW 40 (8) with methanol as mobile phase and a flow rate of approximately 15 ml h-

Preparation of outer-membrane proteins by SDS-PAGE. Antiserum and blotting has been described by Heesemann et al. (1993).

Bioassay for siderophore detection. The bioassay was carried out by modification of the method described by Miles & Khimji (1975). A modified production medium as described above was used. MOPS was replaced by TES (50 mm) and the pH was adjusted to 7.5. Deferrated EDDA (30 mg) and 15 g agar (Difco) were added to solution B prior to sterilization. Molten agar (10 ml, 45°C) was inoculated with 30 µl strain 5030 (OD754 0.8 in nutrient broth) and poured into a Petri-dish. A sterile paper disc, loaded with test solution (20 µl), was placed on the solidified agar and the plate was incubated at 30°C for 18 h. A halo of bacterial growth surrounded these discs bearing siderophore activity or iron.
Iron transport. Cells were grown overnight at 30 °C in nutrient broth [8 g nutrient broth (Difco), 5 g NaCl L⁻¹] and diluted 1:100 into fresh nutrient broth with EDDA as indicated. After growth to a density of about 3 x 10⁶ cells ml⁻¹, the cells were collected by centrifugation at 4 °C, and washed once in cold M9 medium (Miller, 1972) with 0.1 % (w/v) glucose and kept on ice. A suspension containing 1 x 10⁶ cells (ml M9 medium⁻¹) was used for the transport assay at 27 °C in the presence of 100 μM-nitrilotriacetate (NTA). Desferrioxamine B was used at a concentration of 0.5 μM with 0.2 μM-Fe²⁺yersiniabactin was used at a concentration of 1 μM with 0.2 μM-Fe²⁺. The 100-fold concentrated stock solution of yersiniabactin contained 20 % (v/v) methanol, 10 mM-NTA, 0.2 mM-NaOH, 100 μM-yersiniabactin dissolved in methanol, and 20 μM-FeCl₃, 0.2 mM-HCl. Cells were collected on filters at the times indicated. Radioactivity was determined by liquid scintillation counting.

Results

Growth under iron-restricted conditions

*Y. enterocolitica* WA-C was grown in a defined medium treated with Chelex 100 and in an iron-enriched (100 μM-FeCl₃) medium. Growth was decreased approximately threefold by iron-limiting conditions. The latter cultures contained CAS and biological siderophore activity. The appearance of iron-regulated metabolites was further investigated by HPLC monitoring at 320 nm to detect any catechol-containing siderophores and at 430 nm to detect any hydroxamate-iron complexes. Two substances were found to be synthesized specifically under iron-limiting conditions, with retention times of 3.5 and 7.4 min. The iron-regulated compound with a retention time of 7.4 min was designated yersiniabactin. By comparison with a standard reference compound, the other metabolite was found to be 2,3-dihydroxybenzoyl-L-serine. This compound was produced only in low amounts although after enrichment from the medium it showed growth-stimulating activity for *E. coli* and was positive in the CAS test (data not shown).

Fermentation of yersiniabactin

The influence of Fe³⁺ concentration on the production of yersiniabactin by *Y. enterocolitica* strains WA-C and H1852fur are shown in Table 1. For large-scale production of yersiniabactin, the constitutive siderophore producer *Y. enterocolitica* H1852fur (Heesemann et al., 1993) was used to avoid repression of siderophore synthesis by iron leaching from the vessel. Fig. 1 shows that the synthesis of yersiniabactin by H1852fur occurred during the exponential growth phase and reached a maximum after 16 h with a maximum yield of 135 mg l⁻¹ as anticipated for a siderophore and therefore represented type A kinetics, as described by Gaden (1959). The carbon source glucose was consumed after 14 h. No ammonium-suppression of yersiniabactin synthesis was observed, as has been described for antibiotic synthesis (Aharonowitz, 1980).

<table>
<thead>
<tr>
<th>FeCl₃ added to the medium (μM)</th>
<th>Yersiniabactin (mg l⁻¹) produced by:</th>
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<td>Strain WA-C</td>
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<tr>
<td>0</td>
<td>101</td>
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<tr>
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Fig. 1. (a) Production of yersiniabactin [■] and growth (measured as OD₅₇₈: □) of strain H1852fur during batch culture in modified Cove medium. Yersiniabactin was determined by analytical HPLC. (b) Utilization of substrates and the change of pH during fermentation. (●) Glucose, (▽) pO₂, (○) pH, and (▲) NH₄⁺.
Purification of yersiniabactin

The iron-chelating metabolite was purified in the desferri form. Preliminary studies with non-ionic polymeric adsorbents indicated adsorption to XAD 2 and XAD 4. However, elution with methanol containing 1% (w/v) formate led to several degradation products. For this reason yersiniabactin was extracted from the neutralized culture supernatant with ethyl acetate. The organic phase was evaporated to dryness and redissolved in methanol for size-fractionation on Fraktogel. Siderophore-containing fractions were detected by HPLC with monitoring at 210 nm and the CAS assay. Pure material was obtained using preparative HPLC with a water/acetonitrile gradient: yersiniabactin eluted at about 60% acetonitrile. The siderophore-containing fractions were concentrated by evaporation and lyophilized to dryness. A colourless powder (approx. 40% yield) was obtained.

Physico-chemical characterization of yersiniabactin

Atmospheric pressure ion-spray mass spectra showed that yersiniabactin has a molecular mass of 482 Da and could easily be divided into two main fragments of 295 Da and 190 Da. The purified compound showed CAS- and growth-stimulating activity for Y. enterocolitica 5030 on EDDA plates. Using Csaky’s (1948) method, no hydroxamate residues were detected in the siderophore. According to the tests of Arnow (1937) and Rioux et al. (1983), the presence of catechol groups could be demonstrated without ambiguity. However, the yersiniabactin–iron complex was colourless, in contrast to the wine-red iron complex of enterochelin (enterobactin), indicating the presence of additional non-catechol-containing iron-chelating groups. For UV spectroscopy the isolated compound was dissolved at a concentration of 1 mM (molecular mass 482 Da) in double-distilled water. The spectra, without iron and as a 1:1 complex with iron, were recorded at different pH values. The desferri ligand showed absorption maxima at 210, 260 and 320 nm. The addition of iron resulted in a charge-transfer band at 400 nm which had already been observed in the diode-array spectrum (Fig. 2). This supported the assumption that it is not a pure catecholate-type complex which would absorb at about 500 nm. Variation of the pH between 3.7 and 8.9 did not significantly alter the spectrum.

Iron transport with yersiniabactin

Y. enterocolitica WA-C was grown to about $3 \times 10^8$ cells ml$^{-1}$ in nutrient broth to which 20 μM-EDDA had been added to derepress the iron transport systems. Iron(III) uptake was observed with yersiniabactin and ferrioxamine B (Fig. 3). Without addition of EDDA, very low uptake rates were observed indicating that an excess of iron in the medium led to repression of both transport systems. As a control, iron transport was measured in the TonB mutant Y. enterocolitica H2000 (Koebnik et al., 1993) which was grown in the same medium with only 10 μM-EDDA added to allow sufficient growth of the cells. No uptake was observed with ferrioxamine B as would be expected for a TonB mutant. Also no uptake was observed with yersiniabactin, indicating TonB and receptor-dependent uptake.

The pesticin-resistant strains WA-8 and WA-9 lacked the outer-membrane protein FyuA (Fig. 4a). These strains were derived from WA-1 which showed a very low expression of FyuA (Fig. 4a). This was confirmed by
Purification of yersiniabactin

**Fig. 3.** Iron uptake with yersiniabactin (a) and ferrioxamine B (b) as a siderophore. (●) Strain WA-C, (▲) strain WA-C grown with 20 μM-EDDA, (▲) WA-8 furA grown with 10 μM-EDDA, and (■) H2000 tonB grown with 10 μM-EDDA. A representative example of three experiments is shown.

**Discussion**

It is now more than 15 years since Wake *et al.* (1975) published the first observation that *Y. pestis* may produce a siderophore. Under iron-limiting conditions 'iron responsive' material was isolated from *Y. pestis* by Perry & Brubaker (1979). This compound had no biological activity which led the authors to conclude that there is no siderophore produced by *Y. pestis*. It was only recently that the siderophore activity was rediscovered in a group of highly pathogenic *Yersinia* (Heesemann, 1987).

A combination of three different assays, bioassay, colorimetric CAS assay and the screening for iron-regulated peaks by HPLC proved to be a powerful tool in the detection of iron complexing metabolites. Two compounds were found in the supernatant of *Y. enterocolitica*, 2,3-dihydroxybenzoyl-L-serine as a minor component and yersiniabactin as the main product. In fermenters where it was difficult to establish iron-limiting growth conditions, the fur mutant of *Y. enterocolitica* proved to be a good choice for the isolation of yersiniabactin from large-scale cultures.

Isolation of yersiniabactin by adsorption to the non-ionic adsorbents of the XAD type was inappropriate since the siderophore was destroyed during desorption from this material. Similar problems may have occurred during the work of Perry & Brubaker (1979) when they
tried to elute the substance from charcoal. Extraction with ethyl acetate followed by HPLC in a water/acetonitrile gradient proved to be a simple and efficient isolation procedure.

Yersiniabactin caused a halo of growth in the biological assays with Y. enterocolitica HS30 and E. coli (as indicator strains). The preliminary chemical characterization of yersiniabactin showed that it is a relatively small siderophore with a molecular mass of 482 Da which contains at least one catechol moiety. Further evidence for a catechol-containing siderophore was the observation that the aroA mutant Y. enterocolitica YAM-1 was unable to produce yersiniabactin (Heesemann et al., 1993). The other coordination sites for iron(III) are still to be elucidated. Yersiniabactin mediated the uptake of $^{55}$Fe into Y. enterocolitica O:8 strain WA-C in about the same amount as ferrioxamine B. Relatively high binding of $^{55}$Fe to the cells was observed in the transport assay in contrast to the uptake experiments with ferrioxamine B. One possible reason is the hydrophobic nature of yersiniabactin which may enhance non-specific binding to the cell surface.

The transport experiments showed that the uptake of yersiniabactin–iron was regulated by the iron supply of the cell. In addition, transport was TonB-dependent as is usually the case for siderophore-mediated iron uptake in enteric bacteria (Braun & Hantke, 1991).

It has been claimed that the pesticin receptor FyuA found in Y. pestis, Y. pseudotuberculosis and in certain Y. enterocolitica strains is the receptor for yersiniabactin (Heesemann et al., 1993). In the present work we have shown that a mutant lacking the pesticin receptor FyuA was unable to use yersiniabactin as a siderophore. Additional evidence was obtained from experiments with E. coli cells, one of the rare E. coli strains sensitive to pesticin. This strain was able to use and transport yersiniabactin as a siderophore in contrast to E. coli K12 which was unable to grow with yersiniabactin as an iron source. The pesticin receptor FyuA therefore appears to be responsible for transport of the yersiniabactin–iron complex.

Members of Y. enterocolitica can be divided into two groups of strains with respect to their pathogenicity: the so-called mouse-lethal serotypes (O:8, O:13, O:40) and the less pathogenic ones (O:9, O:5). Robins-Browne & Prpic (1985) showed that mouse lethality can be conferred on the less pathogenic strains by supplementation with ferrioxamine B. This suggests that the efficiency of iron acquisition determines the degree of pathogenicity. A major difference between these two groups of strains is their sensitivity to pesticin and ability to produce yersiniabactin (Heesemann et al., 1993). The less pathogenic strains are pesticin-resistant and do not produce yersiniabactin. It is therefore probable that yersiniabactin is an important virulence factor for Y. enterocolitica.

Pesticin sensitivity is also found in Y. pestis and in Y. pseudotuberculosis (Ferber et al., 1981; Heesemann et al., 1993). For these strains there is evidence that they also produce a siderophore (Heesemann, 1987; Heesemann et al., 1993; Wake et al., 1975; Perry & Brubaker, 1979). Since the pesticin receptor is the receptor for yersiniabactin, it is highly likely that they all produce the siderophore yersiniabactin described in the present paper.

This work was supported by the SFB 323. We thank Dr Taraz and Professor Dr Budzikiewicz, University of Cologne, Germany, for measuring high-resolution FAB-MS. We also thank J. Heesemann (Institut für Hygiene, Würzburg) for antisera and V. Braun and A. Griffin for critical reading of the manuscript.

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