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

HUBERT HAAG,¹† KLAUS HANTKE,²* HARTMUT DRECHSEL,³ IGOR STOJILJKOVIC,² GÜNTHER JUNG³ and HANS ZÄHNER¹

¹Lehrstuhl für Mikrobiologie I, ²Lehrstuhl für Mikrobiologie II, ³Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany

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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 ⁵⁵Fe-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 angiubactin 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 *Yersiniae* 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 *Yersiniae*, 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 *Yersiniae* 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 *Yersiniae* may produce novel siderophores that have not yet been

* Author for correspondence. Tel. 07071 294645; fax 07071 294634.

† Present address: Sandoz Pharma AG, Building 506 Room 413, CH 4002 Basel, Switzerland.

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 Stojiljkovic & Hantke (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 *fyuA* were derived from WA-1 (Bäumler *et al.*, 1993) by selection for pesticin resistance; H2000 is a *tonB* mutant derived from WA-C (Koebnik *et al.*, 1993). For siderophore production *Y. enterocolitica* H1852 *fur* was used (Heesemann *et al.*, 1993; Staggs & 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* ϕ 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 l⁻¹) or on nutrient broth medium (8 g nutrient broth, 5 g NaCl l⁻¹). To create iron-limiting conditions in these media ethylenediamine-di(*O*-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 l⁻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; L-phenylalanine, 200; L-cysteine, 50; L-proline, 300; L-glutamic acid, 400; L-serine, 400; L-glycine, 200; L-threonine, 300; L-histidine, 300; L-tryptophan, 200; hydroxyproline, 200; L-tyrosine, 200; L-isoleucine, 300; L-valine, 200; adenine, 20; guanine, 20; uracil, 20. B (mg l⁻¹, final concn), KH₂PO₄, 1000; (NH₄)₂SO₄, 2000; NaCl, 1000; MOPS, 20900, pH 7.0 adjusted with NaOH. C, glucose 20 g l⁻¹. The solutions were combined after sterilization and 1 ml of a sterile stock solution of vitamins (mg ml⁻¹, stock solution: folic acid, 10; biotin, 6; *p*-amino-benzoic acid, 200; thiamin.HCl, 1000; panthothenic acid, 1200; riboflavin, 1000; nicotinic acid, 2300; pyridoxine.HCl, 12000; cyanocobalamin, 100) and 5 ml of a sterile solution of trace elements (mg ml⁻¹, stock solution: CaCl₂, 1000; MgSO₄·7H₂O, 1000) were added. If necessary, traces of contaminating metals were removed by passing the medium through a Chelex 100 column prior to use. The glassware was rinsed several times with 0.1 M-HCl, 0.5 M-EDTA and double-distilled water. For large-scale siderophore production a 4 litre stirring flask fermenter (Braun) was used.

Preparation and analysis 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. Deferred EDDA (30 mg) Rogers, 1973) 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 (OD₅₇₈ 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 those discs bearing siderophore activity or iron.

Analytical HPLC. An HP-1050 liquid chromatograph equipped with a C-18 reverse phase column was used (Nucleosil-ODS, particle size 5 µm, 125 × 4.6 mm i.d., Grom, Herrenberg, FRG). FeCl₃ (1 mM) solution was added to the siderophore-containing solutions and the samples were cleared by centrifugation at 12000 g. Samples (20 µl) of these solutions were injected and separated by gradient elution. Solvent A consisted of double-distilled water with 14.8 mM-H₃PO₄, solvent B was acetonitrile. The gradient was from 0–60% acetonitrile in 10 min. The flow rate was 2 ml min⁻¹. The effluent was monitored by a diode-array detection system which allowed the recording of UV-visible spectra of single peaks.

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 × 40 cm) containing Fractogel TSK HW 40 (s) with methanol as mobile phase and a flow rate of approximately 15 ml h⁻¹. The siderophore-containing fractions were detected by CAS activity and HPLC. For further purification the Beckman System Gold 126 with the Waters 712 WISP (auto sampler) and a Shandon ODS Nucleosil column (5 µm, 300 × 20 mm) were used. The samples were separated by gradient elution. Solvent A was double-distilled water, solvent B was acetonitrile. The gradient was 55–75% solvent B in 24 min with a flow rate of 10 ml min⁻¹. The compound was monitored on-line by diode-array detection and checked off-line by the analytical HPLC system and the bioassay described above. The substance eluted at approx. 60% acetonitrile. Finally the yersiniabactin-containing fractions were concentrated *in vacuo* and lyophilized.

Spectroscopic and mass spectrometric methods. UV-visible spectra were recorded on an Ultrospec III spectrometer (Pharmacia LKB) connected to a Commodore PC40 computer. The samples were dissolved at a concentration of 0.1 mM in double-distilled water and the spectra were recorded in quartz cuvettes with a path length of 1 cm at 20 °C.

Positive FAB spectra were recorded on a Varian MAT 711 instrument coupled with an SS 200 data system. The FAB spectra were measured from a matrix of nitrobenzyl alcohol. The ion source was at 50 °C.

Electro-spray mass spectra (API-MS) were measured on a triple quadrupole mass spectrometer Sciex API III (Sciex) equipped with an Aero-Spray source operating at atmospheric pressure. The samples were dissolved in methanol with the addition of 1% (w/v) formic acid (50 nmol l⁻¹) and injected with a continuous flow of 2–5 litres min⁻¹.

Siderophore assays. The supernatant of cultures grown under iron-limiting conditions was tested by the CAS assay as described by Schwyn & Neilands (1987). The assays for catechol or hydroxamate groups were performed as described by Arnow (1937), Csaky (1948) and Rioux *et al.* (1983).

Determination of glucose and ammonium. Glucose was determined on-line by HPLC as described by Plaga *et al.* (1989). For the determination of ammonium ions an NH₄⁺-selective electrode was used (type M701, Orion).

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 3×10^8 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 1×10^9 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-⁵⁵Fe, yersiniabactin was used at a concentration of 1 µM with 0.2 µM-⁵⁵Fe. 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-⁵⁵FeCl₃, 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 H1852 *fur* are shown in Table 1. For large-scale production of yersiniabactin, the constitutive siderophore producer *Y. enterocolitica* H1852 *fur* (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 H1852 *fur* 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 1. Production of yersiniabactin under different conditions of iron supply

FeCl ₃ added to the medium (µM)	Yersiniabactin (mg l ⁻¹) produced by:	
	Strain WA-C	Strain H1852 <i>fur</i>
0	101	112
0.05	34	92
1	36	94
5	0	96
10	0	93

Fig. 1. (a) Production of yersiniabactin (■) and growth (measured as OD₅₇₈; □) of strain H1852 *fur* 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.

Fig. 2. UV-visible spectrum of iron-loaded yersiniabactin. The insert shows a typical HPLC run of enriched yersiniabactin.

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 3×10^8 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

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

blotting and probing with specific antibodies against FyuA (Fig. 4b). The antibodies reacted with the FyuA band from WA-C and a smaller band was visible with the membrane from WA-1. No cross-reacting material was visible with strains WA-8 and WA-9. Good ferrioxamine B mediated iron uptake was observed with strain WA-8. However, no yersiniabactin iron was taken up (Fig. 3), indicating that the pesticin receptor FyuA is also the receptor for yersiniabactin.

There are only two strains of *E. coli* described in the literature which are pesticin sensitive, *E. coli* ϕ and CA42 (Ferber *et al.*, 1981). *E. coli* ϕ was able to grow in the bioassay for siderophore detection with yersiniabactin in contrast to *E. coli* K12. In addition, *E. coli* ϕ was able to transport ⁵⁵Fe-yersiniabactin with similar efficiency to *Y. enterocolitica* WA-C (data not shown). This gives additional evidence that the pesticin receptor is the receptor for yersiniabactin.

Fig. 4. (a) Outer membrane proteins from different *Yersinia* strains separated by SDS-PAGE. Lanes: 1, WA-C grown on TY medium (iron rich); 2, WA-C grown on nutrient broth with 20 μ M-EDDA; 3-5, strains H1852 *fur* (3), WA1 (4) and WA-8 (5) grown on nutrient broth with 10 μ M-EDDA. The position of FyuA is indicated by a triangle. (b) Immunoblot of outer-membrane proteins separated by SDS-PAGE and developed with anti-FyuA serum. The proteins were from (1) *Yersinia enterocolitica* WA-C, (2) WA-8, (3) WA-9 and (4) WA-1. Preparation of outer membranes, SDS-PAGE, blotting and the antiserum have been described by Heesemann *et al.* (1993).

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 *Yersiniae* (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* H5030 and *E. coli* ϕ 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* ϕ , 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.

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