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# Yersiniabactin from *Yersinia pestis*: biochemical characterization of the siderophore and its role in iron transport and regulation

Robert D. Perry, Paul B. Balbo, Heather A. Jones, Jacqueline D. Fetherston and Edward DeMoll

Author for correspondence: Robert D. Perry. Tel: +1 606 323 6341. Fax: +1 606 257 8994. e-mail: rperry@pop.uky.edu

Department of Microbiology and Immunology, MS415 Medical Center, University of Kentucky, Lexington, KY 40536-0084, USA

**A siderophore-dependent iron transport system of the pathogenic yersiniae plays a role in the pathogenesis of these organisms. The structure of the yersiniabactin (Ybt) siderophore produced by *Yersinia enterocolitica* has been elucidated. This paper reports the purification of Ybt from *Yersinia pestis* and demonstrates that it has the same structure as Ybt from *Y. enterocolitica*. Purified Ybt had a formation constant for $Fe^{3+}$ of $\sim 4 \times 10^{-36}$. Addition of purified Ybt from *Y. pestis* enhanced iron uptake by a siderophore-negative (*irp2*) strain of *Y. pestis*. Maximal expression of the Ybt outer-membrane receptor, Psn, in this strain was dependent upon exogenously supplied Ybt. Regulation of Psn expression by Ybt occurred at the transcriptional level. *Y. pestis* DNA was used to construct *irp2* and *psn* mutations in *Yersinia pseudotuberculosis*. The *irp2* mutant strain no longer synthesized Ybt and the *psn* mutant strain could not use exogenously supplied Ybt. As in *Y. pestis*, Ybt was required for maximal expression of Psn. Regulation by Ybt occurred at the transcriptional level. In contrast to *Y. pestis*, in which a *psn* mutation does not repress synthesis of Ybt siderophore or expression of the iron-regulated HMWP1 and HMWP2 proteins, the same mutation in *Y. pseudotuberculosis* partially repressed these products.**

Keywords: plague, iron affinity, iron transport, siderophore

## INTRODUCTION

Iron is an essential trace element for nearly all organisms. The exceedingly low solubility of ferric ions at neutral pH under aerobic conditions and the chelation of iron and haem by mammalian proteins requires microbial pathogens to elaborate high-affinity transport systems to acquire sufficient iron for growth. One solution to acquiring essential iron is the use of siderophore-dependent iron-transport systems. Siderophores are low-molecular-mass compounds with high affinities for ferric iron that are secreted into the environment, where they bind precipitated iron or remove it from host binding proteins. Uptake of the iron–siderophore complex provides the bacterial cell with the iron needed for growth (Braun *et al.*, 1998; Byers & Arceneaux, 1998; Guerinot, 1994; Mietzner & Morse, 1994).

The production of a siderophore-like activity by *Yersinia pestis* was reported over 20 years ago (Wake *et al.*, 1975). Since then there have been extensive studies on this siderophore-dependent iron-transport system, known as the yersiniabactin (Ybt) system. The Ybt system is encoded within a high-pathogenicity island and is present in the pathogenic strains of *Y. pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* as well as several types of enteropathogenic *Escherichia coli* (Buchrieser *et al.*, 1998; Carniel *et al.*, 1996; de Almeida *et al.*, 1993; Pelludat *et al.*, 1998; Rakin *et al.*, 1995; Schubert *et al.*, 1998). For *Y. pestis* and *Y. enterocolitica*, nearly all the genes of the Ybt system have been sequenced (Bearden *et al.*, 1997; Fetherston *et al.*, 1995, 1996; Gehring *et al.*, 1998a; Guilvout *et al.*, 1993; Pelludat *et al.*, 1998; Rakin *et al.*, 1994); the

**Abbreviations:** CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIP, 2,2′-dipyridyl; HMWP, high molecular weight (mass) protein; MALDI, matrix-assisted laser desorption; OM, outer membrane; TBA, tryptose blood agar base; Ybt, yersiniabactin.

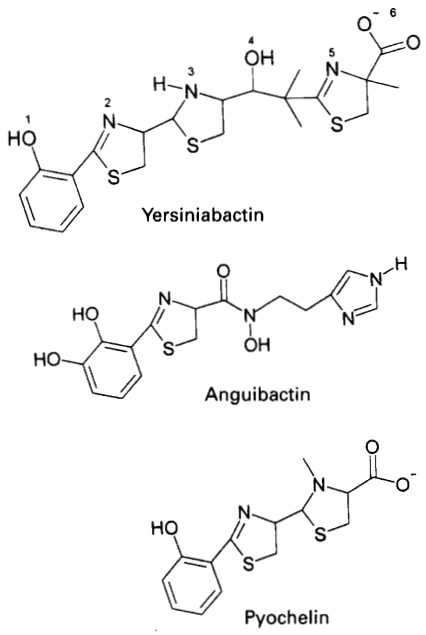


***Fig. 1.*** Structure of yersiniabactin and the related siderophores anguibactin and pyochelin. Ferric-yersiniabactin shows the proposed iron coordination sites (indicated by small numbers).

> 97% sequence identity suggests that the system is functionally identical in these two pathogens. A number of genes thought to be involved in the biosynthesis of Ybt have been identified based on sequence similarities to other siderophore biosynthetic genes (Bearden *et al.*, 1997; Gehring *et al.*, 1998a; Guilvout *et al.*, 1993; Pelludat *et al.*, 1998). However, the function of only a small number of the putative Ybt biosynthetic genes has been conclusively demonstrated. Mutational analyses have shown that *irp2* (encoding HMWP2, a non-ribosomal peptide synthetase), *ybtE* (encoding a salicyl-AMP ligase) and *ybtS* (probably encoding an enzyme required for salicylate synthesis) are essential for Ybt biogenesis (Bearden *et al.*, 1997; Gehring *et al.*, 1998a). In addition, biochemical functions, predicted from sequence analysis, have been demonstrated for HMWP2, HMWP1 (encoded by *irp1*) and YbtE (Gehring *et al.*, 1998a, b). Yersiniabactin produced by *Y. enterocolitica*, alternatively termed yersiniophore when the two activities were thought to be chemically distinct (Chambers & Sokol, 1994), has been isolated and structurally characterized (Chambers *et al.*, 1996; Drechsel *et al.*, 1995; Haag *et al.*, 1993). The Ybt molecule is composed of a phenolic group, probably derived from salicylate, as well as one thiazolidine and two thiazoline rings that are derived from the condensation of three cysteine residues (Gehring *et al.*, 1998a). As previously noted (Chambers *et al.*, 1996; Drechsel *et al.*, 1995), this structure contains significant similarities to pyochelin and anguibactin, siderophores produced by *Pseudomonas aeruginosa* and *Vibrio anguillarum* respectively (Cox *et al.*, 1981; Jalal *et al.*, 1989) (Fig. 1).

Here we describe the purification and structural characterization of Ybt produced by *Y. pestis*, the causative agent of plague. Ybt from *Y. pestis* is identical to that from *Y. enterocolitica* and has a high affinity for ferric iron. Purified Ybt enhances iron uptake and expression of the *psn* gene that encodes the outer-membrane (OM) receptor for Ybt. In addition, we have used mutations in *Y. pestis irp2* and *psn* genes to generate similar mutations in *Y. pseudotuberculosis*. These studies indicate that the Ybt systems in *Y. pestis* and *Y. pseudotuberculosis* are interchangeable and that Ybt also participates in regulating gene expression in *Y. pseudotuberculosis*.

## METHODS

**Bacterial strains, plasmids and media.** All relevant characteristics of strains and plasmids used in this study are presented in Table 1. *Y. pseudotuberculosis* strains with mutations in *irp2* and *psn* were constructed using suicide vehicles pPSN15 and pCIRP498.8 for allelic exchange as previously described for *Y. pestis* (Fetherston *et al.*, 1995). Mutations were confirmed by Southern blot analysis and by loss of pesticin sensitivity for the *Δpsn2045.1* mutation (Fetherston & Perry, 1994) or by loss of Ybt siderophore production (see below). *Y. pseudotuberculosis* strains cured of the low-calcium-response virulence plasmid were selected by growth at 37 °C on tryptose blood agar base (Difco) plates supplemented with 20 mM sodium oxalate and 20 mM $MgCl_2$ (Higuchi & Smith, 1961). Plasmids were purified from overnight heart infusion broth (Difco) cultures by alkaline lysis (Birnboim & Doly, 1979) and further purified when necessary by polyethylene glycol precipitation (Humphreys *et al.*, 1975). *Y. pestis* and *Y. pseudotuberculosis* cells were transformed by electroporation as previously described (Fetherston *et al.*, 1995). Where relevant, the haemin storage ($Hms^+$) phenotype of *Y. pestis* strains was determined on Congo red plates (Surgalla & Beesley, 1969). For studies under iron-starvation conditions, cells were cultivated at 37 °C in the defined medium PMH deferrated by extraction with Chelex 100 (Staggs & Perry, 1991). Precipitation of residual iron in deferrated PMH was achieved by supplementation with 0·5 mM $NaCO_3$, 0·01 mM $MnCl_2$ and 4·0 mM $CaCl_2$ (PMH-S) (Fetherston *et al.*, 1995). PMH-S was solidified with 1% agarose. For *Y. pseudotuberculosis* studies, PMH-S plates were supplemented with the iron chelator 2,2′-dipyridyl (DIP). For growth under various iron conditions, acid-cleaned glassware was soaked overnight in chromic/sulfuric acid (46·3 g $K_2Cr_2O_7$ per litre ~ 12 M sulfuric acid) or Dichrol (Baxter Diagnostics) to remove contaminating iron and copiously rinsed in deionized water.

**Protein, β-galactosidase and pesticin sensitivity analyses.** Whole cells of *Y. pestis* and *Y. pseudotuberculosis* strains, subjected to iron-deficient or iron-sufficient growth conditions in PMH for ~ 6 generations at 37 °C, were incubated for 1 h with $^{35}S$-labelled amino acids (DuPont NEN Research Products) as previously described (Fetherston & Perry, 1994) with or without addition of purified Ybt. Labelled proteins were resolved by SDS-PAGE. Equal amounts of trichloroacetic-acid-precipitable counts were loaded in each lane. Dried gels were exposed to Kodak BioMax MR film at room temperature.

Cells carrying either reporter plasmid pEUPP1 or pEUYbtP were cultivated at 37 °C for ~ 6 generations in PMH in the presence or absence of 10 μM iron. The β-galactosidase

***Table 1.*** Bacterial strains and plasmids used

| Strain or plasmid | Relevant characteristics* | Reference(s) or source |
|---|---|---|
| ***Y. pestis*** | | |
| KIM6+ | Hms$^+$ Ybt$^+$ Lcr$^-$ | Fetherston *et al.* (1995) |
| KIM6-2045.1 | Hms$^+$ Ybt$^-$(Δ*psn2045.1*) Lcr$^-$ | Fetherston *et al.* (1995) |
| KIM6-2045.6 | Hms$^+$ Ybt$^-$(Δ*psn*::*kan2045.6*) Km$^r$ Lcr$^-$ | Fetherston *et al.* (1996) |
| KIM6-2046.1 | Hms$^+$ Ybt$^-$(*irp2*::*kan2046.1*) Km$^r$ Lcr$^-$ | Fetherston *et al.* (1995) |
| ***Y. enterocolitica*** | | |
| WA-LOX | Ybt$^+$ Lcr$^-$ serotype O:8 | Fetherston *et al.* (1992); Perry & Brubaker (1983) |
| ***Y. pseudotuberculosis*** | | |
| PB1/+ | Ybt$^+$ Lcr$^+$ serotype 1 | Perry & Brubaker (1983) |
| PB1/0 | Ybt$^+$ Lcr$^-$ serotype 1 | Fetherston *et al.* (1992); Perry & Brubaker (1983) |
| PB1-2045/+ | *psn*::pPSN15 Lcr$^+$ Ap$^r$ Suc$^s$ type 1 | This study |
| PB1-2045.1/+ | Ybt$^-$ (Δ*psn2045.1*) Lcr$^+$ Ap$^s$ Suc$^r$ type 1 | This study |
| PB1-2045.1/0 | Ybt$^-$ (Δ*psn2045.1*) Lcr$^-$ Ap$^s$ Suc$^r$ type 1 | This study |
| PB1-2046/+ | *irp2*::pCIRP1 Lcr$^+$ Ap$^r$ Km$^r$ Suc$^s$ type 1 | This study |
| PB1-2046.1/+ | Ybt$^-$ (*irp2*::*kan2046.1*) Lcr$^+$ Ap$^s$ Km$^r$ Suc$^r$ type 1 | This study |
| PB1-2046.1/0 | Ybt$^-$ (*irp2*::*kan2046.1*) Lcr$^-$ Ap$^s$ Km$^r$ Suc$^r$ type 1 | This study |
| **Plasmids** | | |
| pCIRP498.8 | *irp2*::*kan2046.1* suicide vector, Ap$^r$ Km$^r$ Suc$^s$ | Bearden *et al.* (1997) |
| pEUPP1 | Low-copy-number *psn*::*lacZ* reporter plasmid, Spc$^r$; iron-, Fur – and YbtA-regulated expression of β-galactosidase | Fetherston *et al.* (1996) |
| pEUYbtP | Low-copy-number *ybtP*::*lacZ* reporter plasmid, Spc$^r$; iron-, Fur – and YbtA-regulated expression of β-galactosidase | Fetherston *et al.* (1999) |
| pPSN15 | Δ*psn2045.1* suicide vector, Ap$^r$ Suc$^s$ | Fetherston *et al.* (1995) |

* Ybt$^+$, a completely functional yersiniabactin biosynthesis and transport system. Lcr$^+$/Lcr$^-$, presence or absence, respectively, of the low-calcium-response virulence plasmid. Hms$^+$, functional haemin-storage phenotype. Ap$^r$, Km$^r$, Spc$^r$, Suc$^r$, resistant to ampicillin, kanamycin, spectinomycin and sucrose, respectively; Suc$^s$, sensitive to sucrose.

activities of cultures were determined spectrophotometrically by following cleavage of 4-nitrophenyl β-D-galactopyranoside. β-Galactosidase activities are expressed in Miller units (Miller, 1992).

A plate assay system was used to determine sensitivity or resistance of *Y. pseudotuberculosis* strains to the yersinial bacteriocin pesticin. From overnight liquid heart infusion broth cultures, ~ $5 \times 10^5$ cells were inoculated into 5 ml molten TBA containing 2·5 mM $CaCl_2$ and 2·5 mM EDTA and overlayered onto similarly supplemented TBA plates. Wells of ~ 1 mm were formed in seeded plates and 20 µl of a 1:50 dilution of partially purified pesticin was added; plates were then incubated for 10–18 h at 37 °C.

**Iron transport studies.** *Y. pestis* strains were cultivated at 37 °C in PMH for ~ 6 generations under iron-deficient conditions. For KIM6-2046.1, purified Ybt was added to yield a 1:16 titre 1 h prior to the start of transport assays. For energy-poisoned cell controls, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 100 µM 10 min prior to the start of transport assays. Assays were initiated in cultures at an $OD_{620}$ of ~ 0·4 by the addition of $^{55}FeCl_3$ to a concentration of 0·2 µCi (7·4 kBq) per ml of culture. For transport assays with KIM6-2046.1, additional purified Ybt (to a titre of 1:32) was added with the radioisotope. Aliquots of 0·5 ml were collected through 0·45 µm membrane filters (Gelman Sciences) at regular intervals and washed twice with PMH. Filters were presoaked in PMH containing 20 µM $FeCl_3$ to prevent nonspecific adsorption. Direct samples were taken to calculate the percentage uptake of radioisotope. All samples were counted in Bio-Safe II scintillation cocktail (Research Products International) on a Beckman LS3801 liquid scintillation counter using a 0–1000 window. Results are presented as percentage uptake of isotope per 0·4 $OD_{620}$ (corresponding to ~ $2{\cdot}8 \times 10^7$ c.f.u. $ml^{-1}$). Control experiments using uninoculated PMH showed that radioisotope was not retained by the filters (data not shown).

**Purification of yersiniabactin and determination of affinity for ferric iron.** Isolation of Ybt was achieved using methods modified from Chambers *et al.* (1996) and Drechsel *et al.* (1995). In a typical purification procedure Ybt was isolated from 3 litres of filtered supernatant from a *Y. pestis* KIM6-2045.1 culture grown at 37 °C in deferrated PMH for ~ 8 generations. Potassium phosphate was added to a concentration of ~ 1 M at pH 7·0 and the solution was extracted twice with 0·5 vol. ethyl acetate. Ethyl acetate fractions were

pooled, evaporated to near dryness, then brought up to ~ 20 ml in 70% ethanol. Two C-18 SEP-PAK cartridges were used in a preliminary purification step; the sample was applied, the column washed with 70% ethanol, and Ybt was eluted with 100% methanol. Methanol fractions were reduced in volume by evaporation to a volume of 1·6 ml. Water and ammonium formate, pH 8·0, were added to adjust to 10 mM ammonium formate in ~ 5 ml. This was applied to a preparative C-18 HPLC column and eluted with a gradient of 20% methanol, 10 mM ammonium formate, pH 8·0, to 100% methanol. Ybt eluted with the 100% methanol phase, and was detected by its absorbance maximum at 385 nm (Drechsel *et al.*, 1995) and by bioassay.

$Fe^{3+}$-saturated Ybt and EDTA were used in competition experiments to determine the formation constant for Ybt with ferric iron. Competition reactions containing 16 μM Ybt-$Fe^{3+}$ in a final volume of 1 ml were reacted with ten concentrations of EDTA over a range from 0 to 63·0 mM at a pH of 8·35. All reactions were allowed to reach equilibrium by incubating for 24 h at 37 °C and then were stored at –70 °C until analysis. Competition reactions were analysed by HPLC employing an analytical C-18 column, and the final Ybt-$Fe^{3+}$ concentrations for each reaction were calculated from $A_{385}$ values.

**Ybt bioassay.** *Y. pestis* KIM6-2046.1 cells were cultured and overlayered onto PMH-S plates as previously described (Fetherston *et al.*, 1995). The *irp2::kan2046.1* mutation in this strain prevents Ybt synthesis; consequently it cannot grow on PMH-S plates at 37 °C unless supplied with an exogenous source of Ybt (Fetherston *et al.*, 1995). Fractions from Ybt purification procedures or filtered culture supernatants from *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* strains were added to wells bored into the agar to test for growth simulation of KIM6-2046.1 at 37 °C.

## RESULTS

### Isolation and characterization of yersiniabactin

Ybt from *Y. pestis* KIM6-2045.1 was isolated as described in Methods. HPLC UV/visible diode array analysis indicated that the preparation of Ybt was free of detectable contaminants. Bioassays demonstrated that the purified Ybt preparation possessed growth-stimulatory activity for *Y. pestis* KIM6-2046.1 at a dilution of 1:64000 but did not support the growth of KIM6-2045.1, which lacks the Ybt OM receptor, on PMH-S plates. Supernatants from iron-deficient cultures of KIM6+ after ~ 8 generations of growth generally possess growth-stimulatory activity at dilutions of 1:8 or 1:16.

The iron-saturated form of Ybt was analysed by UV/visible spectroscopy and found to produce a spectrum at pH 7 indistinguishable from that published by Haag *et al.* (1993) and Chambers *et al.* (1996), with absorption maxima at approximately 255, 310 and 385 nm (data not shown). The high-resolution matrix-assisted laser desorption (MALDI) mass analysis (positive ion mode) of iron-saturated Ybt yielded a spectrum with major ion intensities at *m/z* 295·056, 489·029, 535·035 and 572·992 (Fig. 2). The 535·035 and 572·992 ions are consistent with the formula $C_{21}H_{24}N_3O_4S_3HFe^+$ (calculated 535·036) and $C_{21}H_{24}N_3O_4S_3KFe^+$ (calculated 572·992), respectively, and represent the $FeH^+$ and $FeK^+$ adducts of the Ybt molecule also seen in the purification

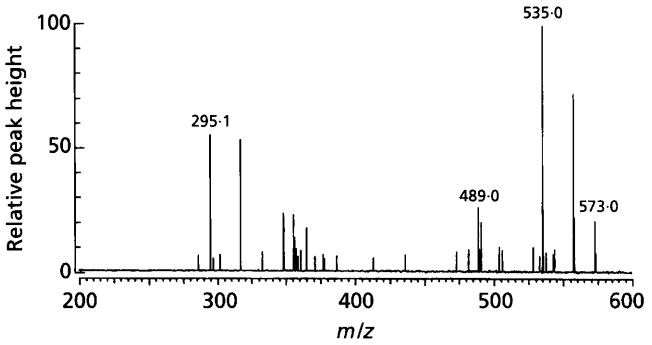


***Fig. 2.*** MALDI mass spectrum of Fe-yersiniabactin isolated from *Y. pestis*.

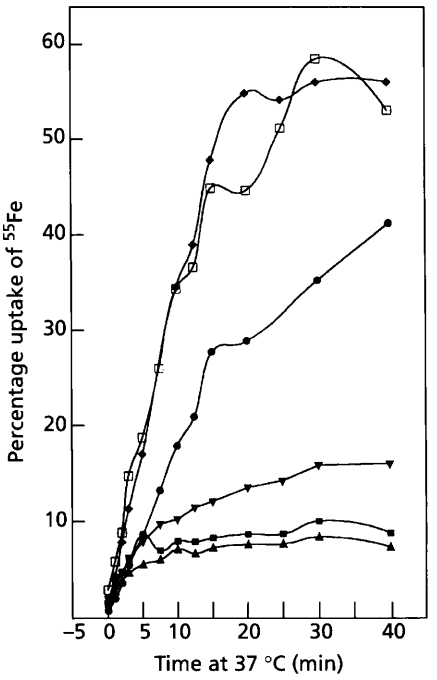


***Fig. 3.*** Uptake of iron by *Y. pestis* KIM cells. □, ■, Ybt+ KIM6+ without (□) and with 100 μM CCCP (■); ▼, ▲, Psn⁻ KIM6-2045.6 without (▼) and with 100 μM CCCP (▲); ●, ◆, Ybt⁻ KIM6-2046.1 without (●) and with exogenous Ybt siderophore (◆) (both without CCCP). Iron uptake is presented as percentage uptake per 0·4 $OD_{620}$ of cells to normalize for differences between cultures and for bacterial growth during the assay period.

of Ybt from *Y. enterocolitica* (Chambers *et al.*, 1996; Drechsel *et al.*, 1995). The *m/z* 295 ion was also seen in mass spectra of the siderophore from *Y. enterocolitica* and the Ybt cleavage product it represents was identified (Drechsel *et al.*, 1995); however, the 489 ion was not detected. This is possibly due to our use of MALDI rather than an electrospray interface. The 489 ion probably arises from decarboxylation of the molecular ion of the $FeH^+$ adduct. Decarboxylation of the $Al^{3+}$ adduct of Ybt from *Y. enterocolitica* was observed in high-resolution electron-impact mass spectrometry

***Table 2.*** *β*-Galactosidase activities of *Y. pestis* and *Y. pseudotuberculosis* strains grown at 37 °C to mid-exponential phase in PMH medium

| Strain | *β*-Galactosidase activity* of cells grown in PMH with: | | | |
|---|---|---|---|---|
| | 10 μM $FeCl_3$ | No added iron or Ybt | Ybt (1:16 titre) | Minus/plus ratio† |
| ***Y. pestis*** | | | | |
| KIM6(pEUPP1)+ | ND | 2035 (±467) | ND | ND |
| KIM6-2046.1 (pEUPP1) | ND | 616 (±372) | 3806 (±697) | 5·9 |
| KIM6(pEUYbtP)+ | 500 (±207) | 28606 (±11615) | ND | 57 |
| KIM6-2046.1(pEUYbtP) | 141 (±13) | 1787 (±351) | ND | 13 |
| ***Y. pseudotuberculosis*** | | | | |
| PB1/0(pEUYbtP) | 84 (±23) | 13629 (±4318) | ND | 162 |
| PB1-2046.1/0(pEUYbtP) | 73 (±13) | 505 (±112) | ND | 7 |

* Activity is expressed in Miller units; the data are means from two to five experiments, with standard deviations in parentheses. ND, Not determined.

† −Fe/+Fe ratio for all strains except KIM6-2046.1(pEUPP1) (−Ybt/+Ybt ratio).

***Table 3.*** Growth of *Y. pestis* and *Y. pseudotuberculosis* derivatives on PMH-S at 37 °C

| Strain | Growth on PMH-S | Cross-feeding of KIM6-2046.1 on PMH-S | Cross-fed by KIM6-2045.1 or PB1/0 on PMH-S-75 μM DIP |
|---|---|---|---|
| ***Y. pestis*** | | | |
| KIM6+ ($Psn^+$ $Ybt^+$) | + | + | ND |
| KIM6-2046.1 (*irp2::kan2046.1*) | − | − | ND |
| KIM6-2045.1 (Δ*psn2045.1*) | − | + | ND |
| ***Y. pseudotuberculosis*** | | | |
| PB1/0 ($Psn^+$ $Ybt^+$) | + | + | ND |
| PB1-2046.1/0 (*irp2::kan2046.1*) | − | − | + |
| PB1-2045.1/0 (Δ*psn2045.1*) | − | − | − |

ND, Not determined.

(Chambers *et al.*, 1996). Thus Ybt synthesized by *Y. pestis* appears to be identical to the siderophore synthesized by *Y. enterocolitica* (Chambers *et al.*, 1996; Drechsel *et al.*, 1995). The mass spectrum also supports the octahedral coordination of the ferric iron atom by a phenolate, three sets of electron pairs from the nitrogen atoms, an electron pair from the oxygen of the nonionized secondary alcohol, and a carboxylate (see Fig. 1).

## Formation constant for $Fe^{3+}$-yersiniabactin

Titration of Ybt in methanol with 0·5 M $FeCl_3$ reduced the $A_{385}$. In contrast, titration of purified Ybt with $FeSO_4$ under strictly anaerobic conditions did not alter the Ybt spectrum (data not shown), indicating that Ybt binds $Fe^{3+}$ with a much greater affinity than $Fe^{2+}$. Ybt was saturated with iron by titrating a small volume in methanol with 0·5 M $FeCl_3$ until the $A_{385}$ reached a minimum value. The $Fe^{3+}$-saturated Ybt preparation was used in competition reactions with EDTA to determine a formation constant for Ybt with $Fe^{3+}$.

A proton-independent formation constant ($K_{Ybt}^{PI}$) for Ybt with $Fe^{3+}$ was determined from the observed formation constant ($K_{obs,Ybt}$) and $a_{H1}$, the fraction of Ybt calculated to be in the singly protonated form. The values of the acid dissociation constants ($pK_1$ to $pK_6$) for Ybt were approximated from published values for compounds representing the various functional groups.

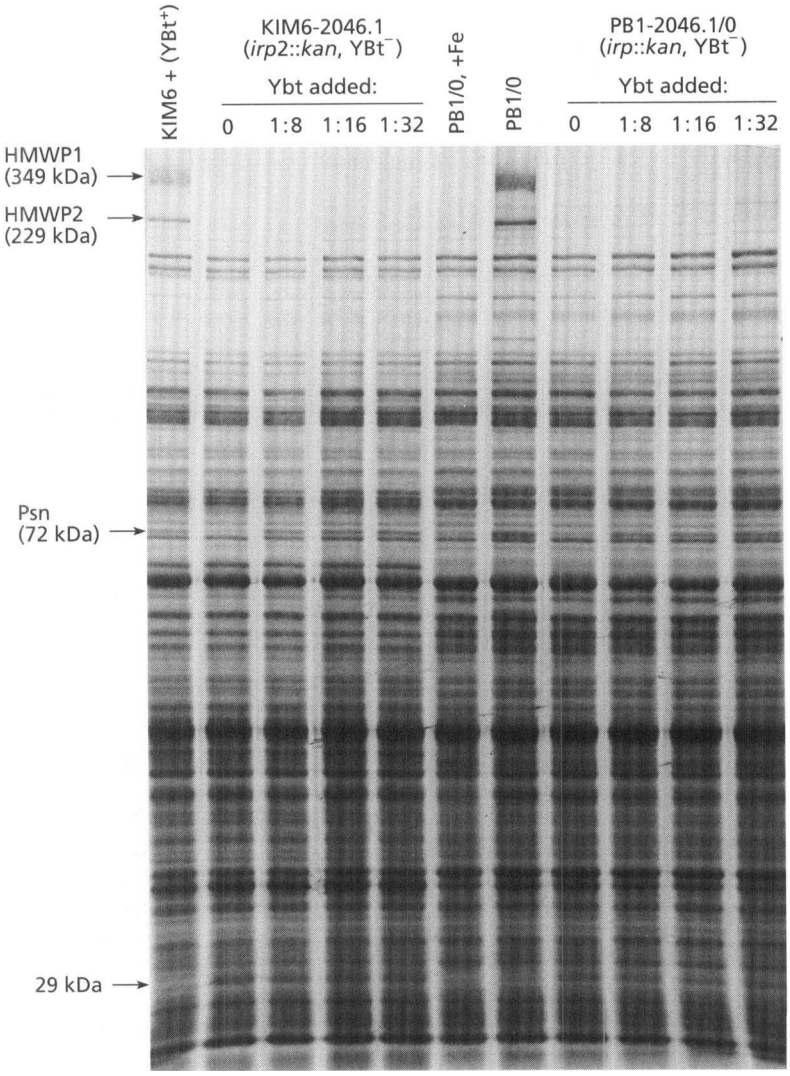


***Fig. 4.*** SDS-PAGE analysis of whole-cell proteins from *Y. pestis* and *Y. pseudotuberculosis* cells grown in PMH with or without added $FeCl_3$ (10 μM) or Ybt siderophore. Concentrations of Ybt added are given as growth-response titres (i.e. 1:32 is the highest concentration of added Ybt). Molecular masses of HMWP1, HMWP2 and Psn are from the deduced amino acid sequence and not estimates based on migration in SDS-PAGE.

These were $pK_1 = 3{\cdot}10$ (aliphatic-substituted thiazoline); $pK_2 = 3{\cdot}12$ (phenyl-substituted thiazoline) (Schmir, 1965); $pK_3 = 4{\cdot}80$ (carboxylic acid); $pK_4 = 9{\cdot}89$ (phenol) (Bird & Cheeseman, 1984); $pK_5 = 11{\cdot}3$ (thiazolidine) (Schmir, 1965); and $pK_6 = 16$ (secondary alcohol) (Bordwell, 1963), which was assumed to be protonated in the calculation. A similar calculation was performed for EDTA using p*K* values of 2·0, 2·7, 6·2 and 10·3 (Dawson *et al.*, 1987). Using these values, the proton-independent formation constant of $Fe^{3+}$-Ybt ($K_{Ybt}^{PI}$) was determined to be 36·6 (or $\sim 4 \times 10^{-36}$).

## Yersiniabactin enhances iron uptake

Fig. 3 shows uptake of iron by iron-starved *Y. pestis* cells possessing all the genes needed for Ybt synthesis and utilization (KIM6+) or with a mutation in either a Ybt biosynthetic operon (KIM6-2046.1, *irp2::kan2046.1*) or the OM receptor for Ybt (KIM6-2045.6, *Δpsn::kan2045.6*). Iron uptake by KIM6+ cells was energy-dependent and essentially complete after 15–20 min. Energy-independent binding of iron to KIM6+, KIM6-2045.6 and KIM6-2046.1 cells was nearly identical (Fig. 3 and data not shown). Energy-dependent uptake of iron by KIM6-2046.1 cells was significantly reduced relative to KIM6+ cells, while KIM6-2045.6 cells did not exhibit substantial energy-dependent uptake of iron. Addition of purified Ybt to KIM6-2046.1 cells stimulated iron uptake to levels similar to that observed in $Ybt^+$ KIM6+ cells (Fig. 3).

## Role of yersiniabactin in gene regulation

Previously we showed that a mutant of *Y. pestis* unable to syntheize Ybt (KIM6-2046.1) was defective in expression of Psn, the OM receptor for Ybt. Expression levels of Psn were enhanced when cells were incubated with filtered culture supernatant from a Ybt-producing *Y. pestis* strain (Fetherston *et al.*, 1995). To clearly demonstrate that the supernatant component responsible for this enhanced expression was Ybt, we performed similar experiments using purified Ybt. Fig. 4

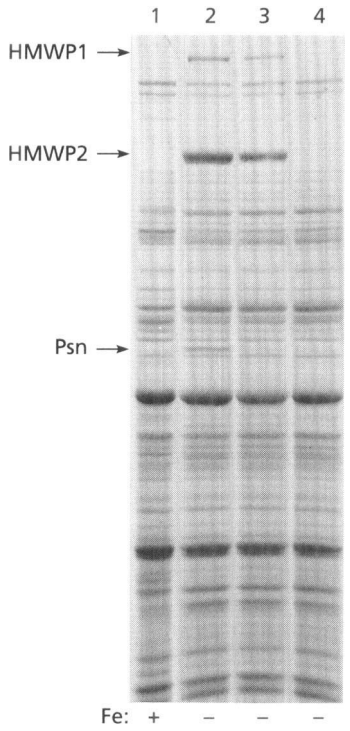


***Fig. 5.*** SDS-PAGE analysis of whole-cell proteins from *Y. pseudotuberculosis* cells grown in PMH with 10 μM $FeCl_3$ (lane 1) or without added iron (lanes 2–4). Lanes 1 and 2, *Y. pseudotuberculosis* PB1/0 (pEUYbtP); lane 3, *Y. pseudotuberculosis* PB-2045.1/0(pEUYbtP)(*Δpsn2045.1*); lane 4, *Y. pseudotuberculosis* PB-2046.1/0(pEUYbtP) (*irp2::kan2046.1*).

shows that expression of Psn by KIM6-2046.1 cells is enhanced by adding increasing concentrations of Ybt to the culture 1 h prior to harvesting cells for SDS-PAGE analysis. HMWP1, HMWP2, YbtE and other Ybt proteins encoded within this operon are not expressed in this mutant due to polar effects of the *irp2::kan* insertion. Addition of purified Ybt also enhanced expression of an ~ 29 kDa polypeptide which was not observed in iron-deficient cells of KIM6+. Within the high-pathogenicity island that encodes the Ybt system, only YbtA has a corresponding molecular mass (Fetherston *et al.*, 1996; Gehring *et al.*, 1998a). If this band does correspond to YbtA, the experimental conditions used may have caused an abnormally high level of expression of this regulatory protein.

We used a well-characterized reporter to demonstrate that Ybt exerts its regulatory effects at the level of gene transcription. Reporter plasmid pEUPP1 is a low-copy-number plasmid in which expression of *lacZ* is driven by the *psn* promoter (*psn::lacZ*) (Table 1). This construct was previously used to show that transcription from this promoter is iron and Fur regulated and requires YbtA for maximal expression (Fetherston *et al.*, 1996). Table 2 shows that *Y. pestis* cells unable to synthesize Ybt (KIM6-2046.1) reduced transcriptional activity from the *psn* promoter by a factor of three when compared to cells synthesizing Ybt (KIM6+). Addition of purified Ybt 1 h prior to collecting assay samples caused a sixfold increase in transcriptional activity.

### The yersiniabactin system and *Y. pseudotuberculosis*

Although there is now abundant evidence for the identity of the Ybt systems in *Y. pestis* and *Y. enterocolitica* (Bearden *et al.*, 1997; Fetherston *et al.*, 1995, 1996; Gehring *et al.*, 1998a; Guilvout *et al.*, 1993; Pelludat *et al.*, 1998), studies using *Y. pseudotuberculosis* are rare. Consequently, we constructed *Δpsn2045.1* and *irp2::kan2046.1* mutations in *Y. pseudotuberculosis*. As expected, *Y. pseudotuberculosis* PB1-2045.1/0 (*Δpsn2045.1*) cells were resistant to the bacteriocin pesticin, which uses the Ybt receptor. In addition, neither *Y. pseudotuberculosis* mutant was able to grow on PMH-S plates at 37 °C (Table 3), while the PB1/0 parental strain was able to grow at 37 °C on PMH-S containing 75 μM DIP. Similar results were obtained with parental and mutant strains of *Y. pestis* (Table 3 and data not shown).

We have previously shown that culture supernatants from *Y. pestis* KIM6-2045.1 (*Δpsn2045.1*) but not KIM6-2046.1 (*irp::kan2046.1*) cells grown under iron-deficient conditions can stimulate growth of KIM6-2046.1 cells on PMH-S plates (Fetherston *et al.*, 1995). While culture supernatants from *Y. pseudotuberculosis* PB1/0 parental cells stimulated the growth of *Y. pestis* KIM6-2046.1 on PMH-S plates, supernatants from both *Y. pseudotuberculosis* mutants failed to provide growth-promoting activity (Table 3). Supernatants from iron-deficient cultures of *Y. pestis* KIM6-2045.1 and *Y. pseudotuberculosis* PB1/0 stimulated growth of *Y. pseudotuberculosis* PB1-2046.1/0 but not PB1-2045.1/0 cells on PMH-S-75 μM DIP plates at 37 °C (Table 3). These results suggest that both *Y. pseudotuberculosis* mutants are unable to synthesize or secrete significant quantities of Ybt siderophore under iron-starvation conditions, and that only the $Irp2^-$ mutant is able to use exogenous Ybt siderophore for growth.

Fig. 4 shows that Ybt is also necessary for maximal expression of the Ybt OM receptor, Psn and the unidentified ~ 29 kDa polypeptide in *Y. pseudotuberculosis* PB1-2046.1 (*irp2::kan2046*). These results are essentially identical to those obtained with the same mutation in *Y. pestis* (Fig. 4). We also analysed transcription from a Ybt-regulated promoter fused to *lacZ* in the *Y. pseudotuberculosis* strains (Table 2). For this study we used a low-copy-number vector carrying *lacZ* fused to the *ybtP* promoter. YbtP is a cytoplasmic membrane permease required for transport of iron from Ybt into the cell. Like *psn*, transcription of the *ybtP* promoter in *Y. pestis* is iron, Fur and YbtA regulated and requires Ybt for maximum expression (Table 2; Fetherston *et al.*, 1998). The results indicate that transcription from this promoter in *Y. pseudotuberculosis* is tightly iron regulated. A mutation in the *irp2* gene completely repressed transcription (Table 2).

Although the Y. *pseudotuberculosis* strain carrying the Δ*psn* mutation showed slightly reduced levels of HMWP1 and HMWP2 expression (Fig. 5), β-galactosidase expression from pEUYbtP in this strain was inconclusive and statistically unreliable (data not shown).

## DISCUSSION

Given the high degree of sequence identities between the genes encoding the Y. *pestis* and Y. *enterocolitica* Ybt systems (Bearden *et al.*, 1997; Fetherston *et al.*, 1995, 1996; Gehring *et al.*, 1998a; Guilvout *et al.*, 1993; Pelludat *et al.*, 1998), we have assumed that the siderophores from these organisms would have identical or nearly identical structures. The UV/visible spectrum and mass analysis of Ybt from Y. *pestis* indicates that it is indeed identical to the siderophore produced by Y. *enterocolitica*. Like many other siderophores, Ybt has little or no affinity for binding ferrous iron but has a formation constant of $4 \times 10^{-36}$ for ferric iron. Thus Ybt has a higher affinity for ferric iron than a number of other siderophores such as ferrichrome, the ferrioxamines, aerobactin and pyoverdin (Albrecht-Gary & Crumbliss, 1998). It would be interesting to compare the binding constant of Ybt with pyochelin and anguibactin, siderophores produced by *P. aeruginosa* and *V. anguillarum*, respectively, that are structurally similar to Ybt (Fig. 1). Unfortunately, the affinities of pyochelin and anguibactin for iron apparently have not been experimentally determined.

As in Y. *enterocolitica* (Haag *et al.*, 1993), Ybt functions to transport iron into Y. *pestis* (Fig. 3). Thus KIM6-2046.1, a strain unable to produce Ybt, accumulates iron at reduced levels relative to KIM6+, which possesses an intact Ybt system. This residual iron transport in KIM6-2046.1 is presumably due to the presence of other low-affinity iron-transport systems in Y. *pestis*, such as the Yfe system (Bearden *et al.*, 1998). Addition of purified Ybt to KIM6-2046.1 cells restored iron transport to wild-type levels seen with KIM6+ cells. Iron uptake by KIM6-2045.6, a strain bearing a mutation in the OM receptor for Ybt, was substantially lower than for KIM6-2046.1. KIM6-2045.6 cells still synthesize and secrete Ybt, which then presumably binds iron in the medium, making it unavailable for transport by other iron-transport systems.

Ybt apparently plays a regulatory role in controlling its own synthesis as well as expression of the Ybt OM receptor, Psn. We previously reported that cell-free culture supernatants containing Ybt enhanced expression of HMWP1, HMWP2 and YbtE, proteins involved in Ybt biosynthesis (Gehring *et al.*, 1998a, b) as well as Psn. In addition, cells unable to synthesize Ybt show reduced levels of transcription from the *psn* and *ybtP* promoters (Bearden *et al.*, 1997; Fetherston *et al.*, 1995, 1999). Using Ybt purified from Y. *enterocolitica*, Pelludat *et al.* (1998) subsequently demonstrated regulation of Y. *enterocolitica* HMWP2 and Psn proteins. We have confirmed and extended our findings using purified Ybt. Addition of Ybt to iron-deficient cells of KIM6-2046.1 greatly increased expression of Psn (Fig. 4). Using a transcriptional reporter, we demonstrated that this regulation occurs at the level of transcription (Table 2).

The Ybt systems present in the three pathogenic *Yersinia* spp. are closely related and functionally interchangeable. DNA sequence similarities (Rakin *et al.*, 1995) and the ease of allelic exchange of a mutated Y. *enterocolitica irp2* gene into Y. *pseudotuberculosis* (Carniel *et al.*, 1992) suggest that the Y. *pseudotuberculosis* Ybt genes are nearly identical to those of Y. *enterocolitica*. We have extended these findings by showing that Y. *pseudotuberculosis* culture supernatants containing Ybt support the growth of Y. *pestis* and that mutated Y. *pestis psn* and *irp2* genes can be used to construct Y. *pseudotuberculosis* mutants. The Y. *pseudotuberculosis irp2* mutant failed to synthesize Ybt, while the *psn* mutant was unable to use Ybt for growth. In addition, Ybt also serves a regulatory role in Y. *pseudotuberculosis*. A mutant unable to synthesize Ybt had no detectable expression of Psn and partially repressed transcription from the *ybtP* promoter. Exogenous Ybt restored expression of the Ybt OM receptor Psn to this mutant.

Regulation of expression by small molecules involved in iron transport is not unprecedented; for example ferric citrate in *E. coli* and several of the siderophores produced by *Pseudomonas* species induce expression of their respective receptors (Braun *et al.*, 1998; Heinrichs & Poole, 1996; Venturi *et al.*, 1995). In most cases, the receptor is also needed for induction. However, deletion of *psn* in Y. *pestis* did not reduce expression of a *psn*::*lacZ* reporter gene or genes for Ybt biosynthesis (Bearden *et al.*, 1997; Fetherston *et al.*, 1996, 1999). This is in contrast to recent results with Y. *enterocolitica*, where a Ybt receptor mutant displayed reduced levels of HMWP2, suggesting that the receptor is important for regulation (Pelludat *et al.*, 1998). Although earlier studies from the same laboratory noted increased siderophore synthesis and secretion from the same mutant strain (Rakin *et al.*, 1994), this anomaly has not been addressed. In Y. *pseudotuberculosis*, a *psn* mutation resulted in a slight reduction in expression of HMWP1 and HMWP2 (Fig. 5). This strain also did not produce levels of Ybt siderophore detectable by bioassay. In contrast, the same mutation in *psn* in Y. *pestis* does not repress expression of HMWP1, HMWP2 or Ybt siderophore (Fetherston *et al.*, 1995).

Thus, identical mutations in Y. *pestis* and Y. *pseudotuberculosis* have slightly different regulatory effects. One possible explanation for the different regulatory effects of $Psn^-$ mutants in Y. *pestis* and the enteropathogenic yersiniae would be putative differences in Ybt permeation through the OM. Presumably the OM of Y. *pestis* allows sufficient diffusion of Ybt into the periplasm to serve as a regulatory signal while the OM of Y. *pseudotuberculosis* allows reduced diffusion and the Y. *enterocolitica* OM is essentially impermeable to

Ybt. In this model Psn is a 'signal transducer' only in the sense that it is translocating Ybt across the OM. It is intriguing that a recent study of the pathogenic yersinias concluded that the OM of *Y. pestis* was most permeable to small hydrophobic molecules while the *Y. enterocolitica* OM was least permeable to these molecules (Bengoechea *et al.*, 1998).

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