Yersiniabactin Production Requires the Thioesterase Domain of HMWP2 and YbtD, a Putative Phosphopantetheinylate Transferase

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One requirement for the pathogenesis of Yersinia pestis, the causative agent of bubonic plague, is the yersiniabactin (Ybt) siderophore-dependent iron transport system that is encoded within a high-pathogenicity island (HPI) within the pgm locus of the Y. pestis chromosome. Nine gene products within the HPI have demonstrated functions in the nonribosomal peptide synthesis (NRPS)/polyketide (PK) synthesis or transport of Ybt. NRPS/PK synthetase or synthase enzymes are generally activated by phosphopantetheinylation. However, no products with similarities to known phosphopantetheinyl (P-pant) transferases were found within the pgm locus. We have identified a gene, ybtD, encoded outside the HPI and pgm locus, that is necessary for function of the Ybt system and has similarities to other P-pant transferases such as EntD of Escherichia coli. A deletion within ybtD yielded a strain (KIM6-2085+) defective in siderophore production. This strain was unable to grow on iron-deficient media at 37°C but could be cross-fed by culture supernatants from Ybt-producing strains of Y. pestis. The promoter region of ybtD was fused to lacZ; β-galactosidase expression from this reporter was not regulated by the iron status of the bacterial cells or by YbtA, a positive regulator of other genes of the ybt system. The ybtD mutant failed to express indicator Ybt proteins (high-molecular-weight protein 1 [HMWP1], HMWP2, and Psn), a pattern similar to those seen with several other ybt biosynthetic mutants. In contrast, cells containing a single amino acid substitution (S2908A) in the terminal thioesterase domain of HMWP2 exhibited any ybt regulatory defects but did not elaborate extracellular Ybt under iron-deficient conditions.

To cause infections nearly all pathogenic bacteria must remove iron, an essential trace nutrient, from host iron- and/or heme-chelating proteins (14, 50, 75). Yersinia pestis, the causative agent of bubonic and pneumatic plague, possesses an ABC hemoprotein transport system (Hmu) that allows it to use a host of heme proteins as a source of iron (40, 73) as well as a Has/hemophore system that may not be functional (62). Analysis of the sequence of CO92 (Y. pestis biotype orientalis) (53) and KIM10+ (Y. pestis biotype mediaevalis) (21) revealed nine potentially functional inorganic iron transport systems; three of these have demonstrated iron acquisition ability. The Yfu system (35) belongs to a family of ABC iron transporters present in Yersinia enterocolitica (Yfu), neisseriae (Fbp), Haemophilus influenzae (Hit), Actinobacillus pleuropneumoniae (Afu), and Serratia marcescens (Sfu) (8). The Y. pestis Yfe system belongs to a family of cation-transporting ABC systems and transports both iron and manganese. This system acquires iron during the later stages of plague (5, 6).

The yersiniabactin (Ybt) iron transport system produces a siderophore composed of phenolate, thiazoline, and thiazolidine rings (16, 22, 55) via a nonribosomal peptide synthesis (NRPS)/polyketide (PK) synthesis scheme. The Ybt siderophore has considerable similarity to the siderophores pyochelin and anguibactin, produced by Pseudomonas aerugi-
nosa and Vibrio anguillarum, respectively (20, 42). The Ybt iron acquisition system is essential for the virulence of Y. pestis during the early stages of infection in mice (5) and appears to be the primary iron acquisition system of plague (56). Ybt biosynthetic, regulatory, and transport genes are encoded within a high-pathogenicity island (HPI) that is present in highly pathogenic isolates of Y. pestis, Yersinia pseudotuberculosis, and Y. enterocolitica, as well as several types of pathogenic Escherichia coli (10, 11, 15, 30, 38, 60, 66). In Y. pestis, the HPI resides within the pgm locus, a 102-kb region of chromosomal DNA subject to high-frequency deletion (11, 12, 27, 30, 37, 48).

Iron from Fe-Ybt is transported into the cell via a TonB-dependent OM receptor (termed Psn in Y. pestis and FyuA in Y. enterocolitica) in conjunction with an ABC transport system encoded by ybtP and ybtQ. Psn also binds the bacteriocin pesticin (24–26, 39, 45, 61). Both YbtP and YbtQ are necessary for use of iron from Ybt and resemble inner membrane permeases fused to an ATP-binding domain. No periplasmic-binding protein has been identified for the Ybt system (9, 24, 30). YbtA is a transcriptional regulator of the AraC family that activates transcription of ybt biosynthetic and transport operons and represses transcription from its own promoter (23).

Ybt biogenesis uses a mixed NRPS/PK synthesis mechanism that assembles the siderophore from salicylate, a linker group derived from malonyl coenzyme A, three molecules of cysteine, and three methyl groups donated by S-adenosylmethionine (30). The requirement of six gene products (high-molecular-weight protein 1 [HMWP1], HMWP2, YbtE, YbtS, YbtT, and YbtU) for in vivo Ybt synthesis has been clearly demonstrated. YbtS likely participates in salicylate biosynthesis (4, 30). YbtE adenylates salicylate and transfers this activated compound to HMWP2 (31). HMWP2, encoded by irp2,
possesses NRPS domains involved in the initial cyclization and condensation reactions involving salicylate and two cysteine molecules (30, 31, 36, 71). YbtU reduces the middle thiazoline ring to a thiazoline structure (51). HMWPI, encoded by irp1, contains PfK-fatty acid synthase and modified NRPS domains that add the branched isobutyl-alcohol linker and the last thiazoline moiety. YbtT contains a thioresterase (TE) domain (4, 30) and may be involved in removing aberrant structures from the enzymatic complex, while the terminal TE domain of HMWPI is hypothesized to remove the completed siderophore from the enzyme complex (33).

NRPS/PK synthetase or synthase enzymes are generally activated by phosphopantetheinylation. Phosphopantetheinyl (P-pant) transferases transfer the 4'-phosphopantetheine moiety of coenzyme A to a specific site on the NRPS or PK enzymes. Activated acyl groups or amino acids are subsequently added to these tethers in preparation for the assembly of the compounds. Phosphopantetheinylation of a peptidyl carrier protein domain of HMWPI (PCP3) has been demonstrated in vitro using E. coli EntD (30). No gene encoding an apparent P-pant transferase is present within the HPI or the pgm locus.

Here we report on genes involved in the first and last steps of Ybt biogenesis—activation of the NRPS/PK synthetase complex by phosphopantetheinylation and cleavage of the completed siderophore by the terminal TE domain of HMWPI.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. All relevant characteristics of strains used in this study are presented in Table 1. All the Y. pestis strains used in this study were derived from KIM6+, an avirulent strain that possesses all of the known Y. pestis virulence determinants except for pCD1, a 70.5-kb plasmid encoding the low-calcium response (Lcr) regulon (27, 68). The Lcr virulence regulon is unrelated to the Pgm+ phenotype and has no demonstrable role in iron metabolism (57, 59).

All strains were stored at -20°C in phosphate-buffered glycerol. Y. pestis cells were grown routinely at 30°C on Congo red (72) from glycerol stocks and then grown in heat inunction broth (Difco Laboratories) or on tryptose-blood agar base (Difco). For iron-deficient growth, Y. pestis cells were grown in the chemically defined medium PMH or PMH2 (33) which had been extracted prior to use with Chelex 100 resin (Bio-Rad Laboratories) (70). Growth of the cultures was monitored by determining the optical density at 620 nm with a Genesys spectrophotometer (Barnstead/Thermolyne Instruments). Residual iron not removed from deferred PMH or PMH2 by the resin can be precipitated by the addition of 0.5 M FeCl3.

All nucleotide sequence accession number. The ybtD gene (spanning the TE regions) in pPSN3 was amplified by primers TE-PCR1 (5'CGGTATGCATTGGTTAGGTGA-3') and TE-PCR2 (5'ACGACCGTTGTTATGCTG-3'). Reaction mixtures contained 0.2 mM deoxynucleoside triphosphates and 0.2 μM primers. Reactions consisted of 4 min at 94°C followed by 25 cycles of 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C and a single cycle at 72°C for 7 min. One cycle containing the desired insert was designated pYBTD1. A deletion encompassing most of the ybtD gene was made by removal of a 774-bp EcoRV/AstII fragment from pYBTD1 to yield pYBTD2 (see Fig. 2). A 3.7-kb Xbal/ApaI fragment from pYBTD2 was cloned into the XbaI/Apal sites of the suicide vector pKNG101 creating pYBTD3. The recombinant suicide plasmid was introduced into Y. pestis KIM6+ by electroporation. Y. pestis isolates with the plasmid recombinant into the chromosome were selected on tryptose-blood agar base plates containing 50 μg of streptomycin/ml. As previously described, cells grown overnight without antibiotics were used to select sucrose-resistant isolates that had completed allelic exchange (5). Isolates containing the chromosomal ybtD mutation were identified by PCR using Taq polymerase with primers ENTD1 (5'-GCCAAGTGTTAGTTAGGTGA-3') and ENTD2 (5'-ACGACCGTTGTTATGCTG-3'). Reaction mixtures contained 0.2 mM deoxynucleoside triphosphates and 0.2 μM primers. Reactions consisted of 3 min at 94°C followed by 25 cycles of 15 s at 94°C, 30 s at 55°C, and 90 s at 72°C and a single cycle at 72°C for 7 min. One isolate, strain KIM6-2085+, was selected for further characterization.

For trans complementation of the ybtD mutation, a 0.93-kb PCR product was amplified from pYBTD1 using ProStart Taq polymerase (Qiagen) with primers ENTD-1 (5'-CGGAGATCTCCTCAGTCCACAACC-3') and ENTD-2 (5'-GCTCTAGACTTCTTTCATATTCAGCCC-3'). Reaction mixtures contained 0.2 mM deoxynucleoside triphosphates and 0.2 μM primers. Reactions consisted of 3 min at 94°C followed by 25 cycles of 20 s at 94°C, 30 s at 55°C, and 90 s at 72°C and a single cycle at 72°C for 7 min. Following digestion with BamHI and XbaI, the product was ligated into the BamHI/XbaI sites of pACYC184 generating pYBTD4.

Plasmid HMPW1-TEmut (Table 1) was used to construct an HMWPI-TE+ mutant (KIM6-2086). This plasmid contains a 9.491-bp Ncol/XhoI fragment from irp1 in which a single base pair replacement results in the substitution of alanine for serine in the TE domain of HMWPI at residue 2908. A 1.372-bp PvuII/XhoI fragment of pET22b-HMPW1-TEmut was subcloned into the Smal/SalI sites of the suicide plasmid pNG101, generating pIRP1TE1. The mutation was introduced into Y. pestis KIM6+ by allelic exchange as described previously (5). Individual colonies were analyzed for the ability to grow at 37°C in iron-depleted PMH-DIP. One of the isolates that was unable to grow after 24 to 48 h of incubation was used for incorporation of the irp1 mutation. The irp1-TE region of this putative mutant was amplified by PCR using primers TE-PCR1 (5'-CTGTCGACTTCTCCAGTCCACAACC-3') and TE-PCR2 (5'-AGATGCGCGAATTCTGTCG-3'). Reaction mixtures consisted of 3 min at 94°C followed by 25 cycles of 20 s at 94°C, 30 s at 55°C, and 90 s at 72°C and a single cycle at 72°C for 7 min. Following digestion with BamHI and XbaI, the product was ligated into the BamHI/XbaI sites of pACYC184 generating pYBTD4.

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Ybt on protein synthesis, Ybt, in the form of KIM6/H11001 Tcr, and Cm r, resistance to ampicillin, kanamycin, spectinomycin, streptomycin, tetracycline, and chloramphenicol, respectively.

grown through two passages in PMH2, with or without 10\(^{-6}\) M FeCl\(_3\), for a total of six to nine generations at 37°C. Dried gels were exposed to Kodak BioMax MR film at room temperature.

Ybt bioassay. Culture supernatants were obtained from Y. pestis cells inoculated into deferrated PMH2 and grown for a total of six to nine generations at 37°C as previously described (25). Cells were pelleted by centrifugation, and the supernatant was filtered through a 0.2-μm-pore-size filter. For growth responses, PMH-S, PMH2-S, and/or PMH-DIP plates were overlaid with 0.04 optical density (at 620 nm) units of KIM6-2046.1 (irp2::kan2046.1) cells grown in deferrated PMH2 and 25 μl of filtered supernatants from iron-deicient cultures was added to wells in the plates.

The iron and Ybt locus containing the genes for hemin storage (pgm) and the Ybt system. All other Y. pestis strains contain a mutation within the ggn locus due to either a deletion or insertion of an antibiotic resistance cassette. Strains synthesizing the siderophore Ybt are designated Ybt°.

Strains that do not produce Ybt are unable to grow on PMH-S, PMH2-S, and PMH-DIP plates. Prior to streaking, the mutants were adapted to iron-deficient PMH-DIP and 25 μl of filtered supernatants from Ybt-producing strains were added to wells in the plates. The Ybt° mutants were also tested for their ability to promote growth of Ybt-producing strains (33). Lcr° indicates the absence of the low-calcium-response virulence plasmid pCD1. Abbreviations: Ap r, Km r, Sp r, Sm r, Tc r, and Cm r, resistance to ampicillin, kanamycin, spectinomycin, streptomycin, tetracycline, and chloramphenicol, respectively.


table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
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<td>Y. pestis DH5α (Apir)</td>
<td>Strain for propagating plasmids with R6K origins, derived from DH5α</td>
<td>S. C. Straley</td>
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<td>Y. pestis KIM6+</td>
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<td>Y. pestis KIM6-2085+</td>
<td>Hms° Ybt° (ΔybtD2085) Lcr°</td>
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<td>Hms° Ybt° (irp1-2086) Lcr°</td>
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<td>Y. pestis KIM6-2086.1+</td>
<td>Pgm° (Hms° Ybt°) Lcr°’ derived from KIM6-2086 by replacement of the irp1-2086 mutation with irp1°</td>
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**Plasmids**

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<th>Plasmid</th>
<th>Description</th>
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<td>pACYC184</td>
<td>4.2-kb cloning vector; Cm’ Tc’</td>
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<td>pEU730</td>
<td>15.2-kb low-copy-number vector with promoterless lacZ; Sp’ Sm’</td>
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<td>pEU/YbtP</td>
<td>15.4-kb low-copy-number ybtP::lacZ reporter plasmid; Sp’; iron-; Fur-; and YbtA-regulated expression of β-galactosidase</td>
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<td>pKNG101</td>
<td>6.8-kb suicide vector; sacB°, R6K origin; Suc’ Sm’</td>
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<td>pWSK29</td>
<td>5.4-kb low-copy-number cloning vector; Ap’</td>
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<tr>
<td>pET22-HMWPI1-TEmut</td>
<td>9.49-kb NcoI/XhoI fragment of irp1, with altered bp, in pET22b; Ap’, 15.0 kb, irp1-2086 (HMWP1-S2908A)</td>
<td>Z. Suo and C. T. Walsh</td>
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<td>pEU/YbtD1</td>
<td>15.4-kb low-copy-number ybtD::lacZ reporter plasmid; 182-bp AscI/KpnI fragment containing ybtD promoter region cloned into AscI/KpnI sites of pEU730; Sp’ Sm’; fusion of ybtD 167-bp promoter to pEU730</td>
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<td>pEU/YbtD2</td>
<td>15.6-kb low-copy-number ybtD::lacZ reporter plasmid; 355-bp AscI/KpnI fragment containing ybtD promoter region cloned into AscI/KpnI sites of pEU730; Sp’ Sm’; fusion of ybtD 342-bp promoter to lacZ</td>
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<td>pIrp1TE2</td>
<td>1.37-kb PvuI/XhoI fragment from pET22b-HMWPI1-TEmut cloned in SmaI/SalI sites of suicide vector pKNG101; Suc’ Sm’, 8.2 kb, irp1-2086 (HMWP1-S2908A)</td>
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<td>pKNG1R1</td>
<td>1.703-bp PCR product from pPSN3 ligated into the BamHI/XbaI site of pKNG101; sacB°, R6K origin; Suc’ Sm’, 8.5 kb; irp1-TE°</td>
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<td>4.44-kb XhoI/BglII fragment from Y. pestis KIM6+ genomic DNA cloned into XhoI/BglII sites of pWSK29; Ap’; 9.8 kb; ybtD°</td>
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<td>pYBTD2</td>
<td>774-bp EcoRV/AsuI fragment within ybtD removed from pYBTD1; Ap’; 9.0 kb; ΔybtD</td>
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<td>pYBTD3</td>
<td>3.7-kb XbaI/ApaI fragment from pYBTD2 cloned into XbaI/ApaI sites of suicide vector pKNG101; Suc’ Sm’; ΔybtD</td>
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<td>pYBTD4</td>
<td>0.93-kb BamHI/XbaI PCR product of ybtD and its putative promoter region ligated into BamHI/XbaI sites of pACYC184; Cm°; 10.5 kb; ybtD°</td>
<td>This study</td>
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</table>

\( Y. \) *pestis* strains with a plus sign possess an intact 102-kb pgm locus containing the genes for hemin storage (pgm) and the Ybt system. All other *Y. pestis* strains contain a mutation within the ggn locus due to either a deletion or insertion of an antibiotic resistance cassette. Strains synthesizing the siderophore Ybt are designated Ybt°, while those affected in Ybt production are Ybt°. Lcr° indicates the absence of the low-calcium-response virulence plasmid pCD1. Abbreviations: Ap r, Km r, Sp r, Sm r, Tc r, and Cm r, resistance to ampicillin, kanamycin, spectinomycin, streptomycin, tetracycline, and chloramphenicol, respectively.
YbtD2), primers EntD.pR-1 (5’-GGGGTACCCTACCAATCTTTA ATC-3’) and EntD.qqR-2 (5’-GGGTACCGGTTCGTCTGATCACTCCCCAG-3’) were used, respectively. Reactions for both products consisted of 3 min at 94°C, followed by 25 cycles of 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C and a single cycle at 72°C for 7 min. The cloned promoter regions were sequenced to confirm that no PCR errors had been introduced.

β-Galactosidase assays. Lysates were prepared from cells carrying the ybtD lacZ or ybtD lacZ reporter plasmid. The cells were grown in PMH2 in the presence or absence of iron through two transfers for a total of approximately six generations, as previously described (69). β-Galactosidase activities were measured spectrophotometrically with a Genesys5 spectrophotometer (Spectronic Instruments, Inc.) following cleavage of ONPG (4-nitro-phenyl-β-D-galactopyranoside). Activities are expressed in Miller units (52).

RESULTS

Mutation of ybtD causes a loss of Ybt siderophore production. The initiating step in assembling the siderophore on the NRPS/PKS complex is phosphopantetheinylation of carrier sites on HMWP1 and HMWP2. However, a P-pan transferase is not encoded within the HPI of the yersiniae. A BLAST search of the two Y. pestis genomes using the amino acid sequence of E. coli EntD identified two strong P-pan transferase candidates. One gene product was highly similar to E. coli ACPS (acyl carrier protein synthase; 77% identity and 91.3% similarity over the 126 amino acids of both proteins) and is likely the essential P-pan transferase for fatty acid synthesis (46). The other gene was designated ybtD and lies between two tRNA^Asn approximately 14.2 kb apart (Fig. 1). The ybtD open reading frame has multiple potential start sites encoding proteins ranging from 27.5 to 17.5 kDa (246 to 156 amino acids; Fig. 2). YbtD shows high similarities to Vibrio cholerae NgrA and VibB—both involved in siderophore biosynthesis (18, 76). Figure 3 shows an alignment of YbtD, using the first potential start site, with NgrA, VibB, and EntD, the E. coli P-pan transferase required for enterobactin biosynthesis (1, 19, 32). In addition to containing the two conserved domains found in P-pan transferases, (46) these proteins also display strong homology at the N terminus (residues 34 to 112 of YbtD in Fig. 3).

A ybtD deletion was constructed and crossed into Y. pestis KIM6+ using allelic exchange (see Materials and Methods). PMH-S, PMH2-S, and/or PMH-DIP were used to examine the effect of the mutation on the ability of this strain to grow at 37°C under iron-chelated conditions. KIM6-1519 (ΔybtD2085) did not grow at 37°C on the iron-chelated media (Table 2), indicating that the mutated strain lost the ability to either synthesize and secrete or utilize Ybt. Supernatants from iron-deficient cultures of KIM6-2085+ were unable to stimulate the growth of a Y. pestis strain (KIM6-2046.1) defective in Ybt synthesis (Table 2). However, culture supernatants from KIM6+, a Ybt-producing strain of Y. pestis, restored growth of KIM6-2046.1 cells under these conditions. In addition, KIM6+ was able to cross-feed KIM6-2085+ as well as KIM6-2046.1 cells (Table 2). This suggests that the YbtD^− mutant is defective in synthesis of the Ybt siderophore but is still able to use it.

For complementation analyses, a PCR product encompassing the ybtD gene was cloned into pACYC184 and designated pYBTD4. The recombinant plasmid restored the ability of KIM6-2085+ cells to grow on iron-chelated plates at 37°C. In addition, culture supernatant from the complemented strain was able to promote the growth of KIM6-2046.1 (irp2::kan2046.1) on PMH-DIP plates (Table 2). The ΔybtD2085 mutation results in decreased expression of ybt operons. Previously we showed that most mutations (e.g., ΔybtE, ΔybtU, Δirp2, and Δirp1::kan) which result in the loss of siderophore production lower the expression of other Ybt biosynthetic genes (HMWP1, HMWP2, and YbtE) as well as the Ybt receptor (Psn) (4, 23, 33). However, two mutations in genes encoding Ybt biosynthetic enzymes (ΔybtT and ΔybtS) did not affect the expression of these indicator proteins (33). To determine the effect, if any, of the ΔybtD2085 mutation on the expression of these indicator proteins, total 35S-labeled proteins synthesized by cells grown under iron-sufficient and iron-deficient conditions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The protein expression pattern of the YbtD^− mutant was similar to that of the ΔybtE, ΔybtU, Δirp2, and Δirp1::kan mutants that are defective in siderophore biosynthesis. In the absence of iron, the level of expression of HMWP1, HMWP2, and Psn proteins was greatly reduced in KIM6-2085+ (ΔybtD2085) cells (Fig. 4, lane 3) compared to that in the parental strain KIM6+ (Fig. 4, lane 1). In this experiment, YbtE was not detected due to inadequate separation of the polypeptides in this size range. The reduced level of Psn expressed in the YbtD^− mutant was similar to that observed in KIM6-2046.1 (Fig. 4, lane 2); HMWP1 and HMWP2 are not detected in KIM6-2046.1 cells because of the irp2::kan2046.1 mutation and its polar effects. Complementation of the YbtD^− mutant with pYBTD4, encoding ybtD, restored expression of HMWP1, HMWP2, and Psn (Fig. 4, lane 4). We have previously shown that addition of purified Ybt or supernatant containing Ybt to Y. pestis Ybt biosynthetic mu-
tants restores expression of HMWP1, HMWP2, YbtE, and Psn (4, 23, 33, 55). Likewise wild-type levels of HMWP1, HMWP2, and Psn were expressed by iron-deficient cultures of the YbtD/H11002 cells labeled in the presence of supernatant from KIM6/H11001 (expressing Ybt siderophore) but not with supernatant from the Ybt-biosynthetic mutant KIM6-2046.1 (Fig. 4, lanes 5 and 6).

We used pEUybTP, a ybtP promoter fusion to lacZ, to test the effect of the ybtD2085 mutation on gene transcription. Previous studies showed that the ybtP promoter is regulated by Fur, iron, YbtA, and the Ybt siderophore (23, 24, 33). Because our Y. pestis strains are phenotypically β-galactosidase negative, any β-galactosidase activity is due to the presence of the reporter plasmid (33, 70). The ybtP-galactosidase activities of cells bearing pEUybTP and grown in deferrated PMH2 in the presence of or absence of added iron, are presented in Table 3. As expected, expression of lacZ from the ybtP promoter was iron regulated in KIM6+/+, which contains all the genes needed for Ybt synthesis and utilization; there was an 18.4-fold repression of β-galactosidase activity in cells grown in the presence of surplus iron compared to those cultured under iron-deficient conditions. Expression of the ybtP::lacZ reporter in the YbtD− mutant, KIM6-2085+, was still somewhat iron regulated (14-fold repression); however, compared to KIM6++, the overall expression was greatly reduced (a 9.8-fold reduction in iron-starved cultures [Table 3]). Similar results were observed with the ybtP::lacZ reporter in KIM6-2046.1, an HMWP2− mutant unable to synthesize Ybt (Table 3). As in previous studies (24), lacZ expression from pEUybTP in a pgm mutant was even lower, likely due to the absence of the YbtA transcriptional activator (Table 3). These studies suggest that loss of expression of HMWP1, HMWP2, and Psn in the YbtD− mutant is the result of decreased transcription from the relevant ybt promoters.

Transcription of ybtD is not affected by YbtA or iron status of the cell. The promoters of other ybt genes encoding siderophore biosynthetic and transport functions are repressed by iron through the action of Fur and are activated by YbtA and

![Fig. 2. Genetic organization of the Y. pestis ybtD region showing restriction sites used. (A) Dashed line indicates the region deleted in the ΔybtD mutant. The PCR product used in complementation studies is also indicated. The BamHI and XbaI sites in parentheses are artificial restriction sites introduced by PCR. (B) Putative −10 and −35 regions, potential ribosomal binding sites (RBS), and a region with similarity to a Fur binding site (underlined nucleotides), as well as the potential protein start sites, are indicated (underlined and in boldface type). Arrows show the two promoter regions tested in expression studies.](http://iai.asm.org/)
the siderophore (4, 23, 24, 69). Due to the multiple possible protein start sites and the two potential regions (Fig. 2B), we constructed two transcriptional reporters to examine expression from the ybtD promoter. Both constructs start with the same upstream site (left-pointing arrow in Fig. 2B) and include both and the potential Fur binding site; the 167-bp promoter fragment (pEUYbtD1) ends at the first potential valine start site, while the longer 342-bp construct (pEUYbtD2) ends at the third potential valine start (two right-pointing arrows in Fig. 2B) to ensure that all potential transcriptional regulatory elements were included. These regions were cloned into the lacZ transcriptional fusion vector, pEU730 (28), which contains an RNase III site upstream of lacZ (29). Processing of the message at this site removes any sequences which might alter message stability or affect translational efficiency (47). The level of β-galactosidase activity from Y. pestis KIM6(pEUYbtD2) cells grown under iron-sufficient or iron-deficient conditions was 2-fold lower than that determined in Y. pestis KIM6(pEU730)+ cells (Table 3). Since the amino acid similarities between YbtD and other P-pant transferases start upstream of the third potential valine start, we used pEU730 to further characterize the ybtD promoter region. The ybtD::lacZ reporter failed to show any repression under iron-sufficient growth conditions (Table 3). In addition, the level of β-galactosidase activity was similar in YbtA+ and YbtA− strains of Y. pestis bearing pEU730 (Table 3). Thus, the ybtD promoter does not appear to be regulated by iron or YbtA.

A single amino acid substitution in the TE domain of HMWP1 causes loss of siderophore production. HMWP1 contains an internal TE domain which is hypothesized to be involved in the final step of siderophore biogenesis: release of the siderophore from the biosynthetic machinery. Previously we showed that YbtT, which contains a TE domain, is required for siderophore synthesis in Y. pestis (33) and likely serves an editing function to release aberrant intermediates on carrier sites of HMWP1 and HMWP2. To determine if the internal TE domain in HMWP1 is required for Ybt synthesis, we con-

FIG. 3. Amino acid sequence alignment of YbtD from Y. pestis, NgrA from P. luminescens, VibD from V. cholerae, and EntD from E. coli. Residues with identity to YbtD are in white with a solid black background. Conservative and semiconservative amino acid substitutions are shaded. The consensus line shows identical residues in all four proteins (uppercase letters) and identical residues in two or more proteins (lowercase letters). The identity and similarity of YbtD to each of these proteins are 34 and 65.3% (NgrA), 31.3 and 60.2% (VibD), and 27.2 and 58.5% (EntD). The residues below the consensus line indicate conserved (lowercase) and highly conserved (uppercase) amino acids within the proposed P-pant transferase domain derived from comparison of 22 P-pant transferases (46).
HMWP1 contains one acyl and one peptidyl carrier domain, synthesized via a mixed NRPS/PK synthetase mechanism. Ybt siderophore is likely released from the completed molecule, in siderophore producing transferase activity of KIM6-2086 cells bearing pEUYbtP and grown with KIM6+ cells containing the Ybt biosynthetic mutant, KIM6-2086.1; i.e., HMWP1, HMWP2, and Psn were highly expressed in the mutant (Fig. 6). In addition, the β-galactosidase activity of KIM6-2086 cells grown under iron-deficient conditions were analyzed by SDS-PAGE. The protein expression pattern of the HMWP1-TE mutant (KIM6-2086) was similar to that of Ybt+ strain KIM6+.1; i.e., HMWP1, HMWP2, and Psn were highly expressed in the mutant (Fig. 6). In the β-galactosidase activity of KIM6-2086 cells bearing pEUYbtP and grown in iron-starved KIM6-2086 cells did not allow the growth of the Ybt-biosynthetic mutant, KIM6-2046.1, on PMH2-S plates (Table 2). Finally, culture supernatant from iron-starved KIM6-2086 cells did not allow the growth of the Ybt-biosynthetic mutant, KIM6-2046.1, on PMH2-S plates (Table 2). These results indicate that the mutation in the TE domain of irp1 caused a loss of siderophore production and/or secretion.

**The irp1-2086 mutation does not affect the expression of ybt operons.** To determine whether expression of Ybt proteins was affected by the irp1-2086 mutation, total radioactivity of KIM6-2086 cells grown under iron-deficient conditions were analyzed by SDS-PAGE. The protein expression pattern of the HMWP1-TE mutant (KIM6-2086) was similar to that of Ybt+ strain KIM6+.1, and Ybt siderophore. Growth on PMH2-S plates at 37°C was also restored when the chromosomal mutation was replaced by the wild-type sequence (strain KIM6-2086.1+). Finally, culture supernatant from iron-starved KIM6-2086 cells did not allow the growth of the Ybt-biosynthetic mutant, KIM6-2046.1, on PMH2-S plates (Table 2). These results indicate that the mutation in the TE domain of irp1 caused a loss of siderophore production and/or secretion.

**DISCUSSION**

PK synthases, fatty acid synthases, and nonribosomal peptide synthetases all require post-translational modification of the acyl, aryl, and/or peptidyl carrier protein domains for catalytic activation, the first step in the biogenesis of their products. The last step in this process is release of the product from the enzyme complex by a TE. In this study we have examined the roles of YbtD, a putative P-pant transferase for the Ybt synthetase complex, and the TE domain of HMWP1, which likely releases the completed molecule, in siderophore production.

P-pant transferases activate the enzyme complex by catalyzing transfer of P-pant moieties from coenzyme A molecules to the carrier domains of PK synthases, fatty acid synthases, and nonribosomal peptide synthetases. Ybt siderophore is synthesized via a mixed NRPS/PK synthetase mechanism. HMWP1 contains one acyl and one peptidyl carrier domain.
suggesting that it is required to activate the carrier domains of a ybtD::lacZ reporter plasmid. However, in CO92, these same two tRNA genes are separated by 285 bp. This difference between KIM10/H11022 and CO92 probably results from an inversion involving the IS35 element. This inversion has also been found in the vicinity of ybtD to the region containing the ybtD gene.

The region of KIM10/H11022 chromosomal DNA that contains ybtD is essential for normal siderophore production in KIM10 (2086). In KIM6-2086 (irp2::kan2086), the two tRNAAsn genes flank the ybtD gene. Sequences adjacent to the ybtD gene in KIM6 and KIM6-2086 are homologous to the sequences flanking these tRNA genes. Thus, it is possible that in some strains of Y. pseudotuberculosis and Y. enterocolitica the HPI is located close to the region containing ybtD. In KIM10+, the two tRNAAsn genes are separated by >179 kb. (53) This difference between KIM10+ and CO92 probably results from an inversion involving the IS285 element.

We have demonstrated that ybtD is essential for normal production of the Ybt siderophore. Although the authentic start site for YbtD remains to be determined, this protein contains two conserved P-pant transferase domains (Fig. 3), suggesting that it is required to activate the carrier domains of the siderophore.

### Table 3. β-Galactosidase activities of Y. pestis strains containing either a ybtP::lacZ or a ybtD::lacZ reporter plasmid

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Strain</th>
<th>Mean β-galactosidase activitya (Miller units) ± SD of cells grown:</th>
<th>Ratio (growth without Fe/ growth with Fe)</th>
<th>Ratio (WT/Mutant) for cells grown:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without iron</td>
<td>With iron</td>
<td>Without iron</td>
</tr>
<tr>
<td>pEUYbtP (ybtP::lacZ)</td>
<td>KIM6+ (ybt+)</td>
<td>29,929 ± 1,582</td>
<td>1,626 ± 249</td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>KIM6-2046.1 (irp2::kan2046.1)</td>
<td>4,351 ± 1,705</td>
<td>292 ± 20</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>KIM6-2085+ (ybtD::2085)</td>
<td>3,044 ± 249</td>
<td>216 ± 26</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>KIM6-2086 (irp1-2086)</td>
<td>34,004 ± 4,963</td>
<td>2,058 ± 580</td>
<td>16.5</td>
</tr>
<tr>
<td>pEUYbtD1 (ybtD::lacZ; 167-bp promoter region)</td>
<td>KIM6+ (ybt+)</td>
<td>4,252 ± 1,415</td>
<td>4,674 ± 1,236</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>KIM6-2055 (ybtA::kan2055)</td>
<td>4,811 ± 982</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pEUYbtD2 (ybtD::lacZ; 342-bp promoter region)</td>
<td>KIM6+ (ybt+)</td>
<td>2,637 ± 446</td>
<td>2,072 ± 307</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a Cells were harvested during exponential growth at 37°C after approximately six generations in PMH2 containing either no added iron or 10 μM FeCl3.

b Enzyme activities are expressed in Miller units (52). The values ± standard deviations represent an average of three to four individual reactions from two or more independent cultures.

c ND, not determined or not applicable.

The HPI of Y. pseudotuberculosis can insert at any one of three different tRNAAsn genes; only one HPI insertion site has been identified in three different strains of Y. pestis (10, 30, 38, 53). The region of KIM10+ chromosomal DNA that contains ybtD is flanked by two tRNAAsn genes (Fig. 1). Sequences adjacent to the Y. pseudotuberculosis and Y. enterocolitica HPIs (3, 10, 38, 60) are homologous to the sequences flanking these tRNA genes. Thus, it is possible that in some strains of Y. pseudotuberculosis and Y. enterocolitica the HPI is located close to the region containing ybtD. In KIM10+, the two tRNAAsn genes in the vicinity of ybtD are approximately 14.2 kb apart (Fig. 1). However, in CO92, these same two tRNA genes are separated by >179 kb. (53) This difference between KIM10+ and CO92 probably results from an inversion involving the IS285 element (Fig. 1).

We have demonstrated that ybtD is essential for normal production of the Ybt siderophore. Although the authentic start site for YbtD remains to be determined, this protein contains two conserved P-pant transferase domains (Fig. 3), suggesting that it is required to activate the carrier domains of the siderophore.
HMWP1 and HMWP2. Bioassays using the Δybtd mutant indicate that it is defective in Ybt siderophore production (Table 2). Our bioassay detects Ybt in iron-deficient culture supernatants diluted 1:16 (data not shown). Thus, if any Ybt siderophore is present in culture supernatants of the YbtD− mutant it is present at <6% of wild-type levels.

Surprisingly, transcription from the ybtD promoter is not regulated by the iron status of the cell or by YbtA (Table 3). This suggests that ybtD may have been recently converted for use in the Ybt system or that this P-pant transferase is used to activate more than one system. The Y. pseudotuberculosis KIM10+ (21) and CO92 genomes (53) both contain two NRPS systems in addition to Ybt that would require activation by a P-pant transferase. One system encodes enzymes with homologies to Bordetella siderophore biosynthetic enzymes as well as an OM receptor and an ABC transporter related to similar components in other iron transport systems (34, 43, 56). The second putative NRPS system contains open reading frames showing similarities to Yersinia HMWP1 and HMWP2 proteins and to YbtP, a fused function permutate/ATP hydrolase ABC transporter component. To be functional, both these systems would require activation by a P-pant transferase, possibly YbtD. It is unlikely that the Y. pseudotuberculosis ACPS would work since in E. coli, the ACPS P-pant transferase activity is specific for fatty acid biosynthesis (46). However, it is unknown whether either of these putative NRPS systems is functional.

The Ybt system, like many bacterial NRPS and/or PK synthase systems, possesses a C-terminal TE domain as part of the putative NRPS systems is functional. ACPS P-pant transferase activity is specific for fatty acid biosynthesis (46). However, it is unknown whether either of these putative NRPS systems is functional.

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