Cupric Yersiniabactin Is a Virulence-Associated Superoxide Dismutase Mimic

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Supporting Information

ABSTRACT: Many Gram-negative bacteria interact with extracellular metal ions by expressing one or more siderophore types. Among these, the virulence-associated siderophore yersiniabactin (Ybt) is an avid copper chelator, forming stable cupric (Cu(II)-Ybt) complexes that are detectable in infected patients. Here we show that Ybt-expressing E. coli are protected from intracellular killing within copper-replete phagocytic cells. This survival advantage is highly dependent upon the phagocyte respiratory burst, during which superoxide is generated by the NADPH oxidase complex. Chemical fractionation links this phenotype to a previously unappreciated superoxide dismutase (SOD)-like activity of Cu(II)-Ybt. Unlike previously described synthetic copper-salicylate (Cu(II)-SA) SOD mimics, the salicylate-based natural product Cu(II)-Ybt retains catalytic activity at physiologically plausible protein concentrations. These results reveal a new virulence-associated adaptation based upon spontaneous assembly of a non-protein catalyst.

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athogenic Gram-negative bacteria secrete chemically diverse low molecular weight virulence factors called siderophores. These small molecules solubilize host ferric iron for import as a bacterial nutrient source and transport it back to the bacteria by means of high affinity transporters (see review, ref 1). The virulence of pathogenic Enterobacteriaceae, including highly virulent strains of Yersinia pestis (agent of the black plague) and uropathogenic Escherichia coli (UPEC) is strongly linked to expression of the salicylate-based siderophore yersiniabactin (Ybt).2−6 Ybt’s biosynthetic (ybtT, ybtE, ybtS), transport (fyuA, ybtP, ybtQ), and regulatory (ybtA) genes are encoded by a chromosomal locus designated the Yersinia high-pathogenicity island (HPI).7 Ybt’s iron scavenging activity conforms to the canonical Gram-negative siderophore process in which it binds ferric iron, is actively transported through the outer membrane via a TonB-dependent β barrel protein (FyuA), and subsequently delivers iron to the cytosol through ATP cassette proteins (YbtPQ). Recently, we have shown that Ybt can protect bacteria from metal toxicity independently of transporter proteins by sequestering cupric ions (Cu(II)) as stable extracellular complexes (Cu(II)-Ybt) in patients infected with uropathogenic Escherichia coli (UPEC).8

Physiologic studies have demonstrated that infection is accompanied by systemic changes in copper concentration within the host (see review, ref 9). The plasma concentration of ceruloplasmin, the primary host copper-transporting protein, increases during inflammation or infection, leading to copper accumulation at sites of inflammation.10−12 Elemental analysis and radiotracer studies have shown concentrations of copper up to several hundred micromolar within granulomatous lesions in lungs infected by Mycobacterium tuberculosis and at sites of inflammation such as wound exudates and burns where macrophages congregate.13−16 Genes encoding mammalian copper uptake are upregulated in macrophages infected by Mycobacterium tuberculosis, Salmonella typhimurium, and other intracellular pathogens.17−20 Phagocytes such as macrophages represent one of the first lines of defense against invading microbial pathogens and rely on high local concentrations for copper(II) for their bactericidal action.21,22 Impairment of copper transport to the macrophage phagosome disrupts normal immune function in these cells and permits increased bacterial survival following phagocytosis.

In this study we used cellular, chemical, and computational approaches to evaluate the hypothesis that Ybt’s previously documented copper-binding activity protects UPEC during phagocytosis. Our results indicate that Ybt expression confers a copper-dependent intracellular survival advantage in multiple phagocytic cell types. This survival advantage is minimized in phagocytes with pharmacologic or genetic deficiencies in NADPH oxidase-derived superoxide production. The chemical basis for these findings is a SOD-like activity attributed to Cu(II)-Ybt complexes. Together, these studies provide new...
insights into how pathogenic bacteria use secondary metabolites to survive innate host defenses.

**RESULTS AND DISCUSSION**

**Ybt Promotes *E. coli* Survival in RAW264.7 Cells.** To determine whether Ybt expression protects phagocyted bacteria from the copper-dependent bactericidal activity of macrophage-like RAW264.7 cells, we compared intracellular survival of the model uropathogen UTI89 to its isogenic Ybt-deficient mutant UTI89ΔybtS (Figure 1a). As described in the copper-dependent *E. coli* bactericidal system by White et al., we infected RAW264.7 cells with or without overnight preincubation in copper-containing media with UTI89 or UTI89ΔybtS at a multiplicity of infection (MOI, ratio of bacteria to mammalian cells) of 10. Following gentamycin treatment to ensure assessment of intracellular bacteria only, viable bacteria were determined 1 h after infection by colony forming unit (CFU/mL) determination. The number of internalized bacteria were unaffected by either RAW264.7 cell copper availability or bacterial strain. In copper-replete RAW264.7 cells, wild type UTI89 exhibits significantly greater (~2 log CFU/mL, p = 0.001) survival than UTI89ΔybtS. The survival difference between wild type UTI89 and UTI89ΔybtS survival is eliminated in copper-deficient RAW264.7 cells. These results show that Ybt expression promotes UPEC intracellular survival in copper-replete RAW264.7 cells.

Ybt may benefit intracellular pathogens by sequestering copper outside the bacterial cell or mediating metal ion import through the outer membrane ferric-yersiniabactin importer FyuA. To distinguish between these possibilities, we compared the intracellular survival of isogenic FyuA-deficient strains with and without Ybt biosynthetic activity (UTI89ΔfyuA and UTI89ΔfyuAΔybtS, respectively, Supplemental Figure S1). Even in the absence of FyuA, the Ybt-expressing strain (UTI89ΔfyuA) still displayed a significant intracellular survival advantage (approximately 1.77 log CFU/mL, p = 0.001) in copper-replete RAW264.7 cells compared to the double mutant. This survival advantage was eliminated in copper-deficient RAW264.7 cells. Ybt’s protective effect in copper-replete RAW264.7 cells thus persists even when its value as a ferric ion siderophore is negated by a null mutation of the Ybt importer. These observations show that Ybt expression can promote UPEC intracellular survival independently of its cognate outer membrane transporter.

**Ybt-Dependent Survival Is Maximal during the Respiratory Burst.** UTI89 exhibited greater intracellular survival in copper-replete RAW264.7 cells (p = 0.018 when compared to RAW cells cultured without copper, t test). No such advantage was observed with non-pathogenic K12 cells in this (Supplemental Figure S2) or a prior study. One possible explanation for this copper-dependent gain of function is that the Cu(II)-Ybt complexes forms within the phagosomal membrane of copper-replete RAW264.7 cells catalyze superoxide dismutation similarly to synthetic Cu(II)-salicylate (Cu(II)-SA) complexes. To evaluate this hypothesis we compared wild type UTI89 and UTI89ΔybtS survival in RAW 264.7 cells treated with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI, Figure 1b). DPI treatment substantially diminished UTI89’s survival advantage over UTI89ΔybtS (p = 0.0044 when compared to DPI untreated UTI89, t test) and became statistically insignificant. Without copper repletion, UTI89 and UTI89ΔybtS survival was indistinguishable in DPI-treated RAW 264.7 cells (Supplemental Figure S3). These findings show that Ybt’s copper-dependent intracellular survival advantage is maximal in the presence of respiratory burst-derived superoxide.

**UTI89 Exhibits a Competitive Survival Advantage in RAW 264.7 Cells.** To more directly compare intracellular survival of Ybt-expressing and non-expressing bacteria, RAW 264.7 cells were co-infected with a 1:1 mixture of wild type UTI89::kan and UTI89ΔybtS. Ybt-specific intracellular fitness was determined by comparing each strain’s relative intracellular survival (calculated as log competitive survival indices; see Methods). In copper-replete RAW264.7 cells, Ybt-expressing UTI89 exhibited a significant survival advantage over UTI89ΔybtS (competitive index > 0, p = 0.0039, Wilcoxon signed-rank test) (Figure 2a). This competitive advantage was abolished in copper-depleted RAW 264.7 cells. These results
show that Ybt expression selectively promotes UPEC intracellular survival in copper-replete RAW264.7 cells during identical culture conditions.

**Ybt Affects Survival in Respiratory Burst-Competent Peritoneal Macrophages.** To determine whether a genetic respiratory burst deficiency in macrophages impacts Ybt’s survival advantage, we compared competitive intracellular survival in murine resident peritoneal macrophages derived from wild type C57BL/6 to those from X-CGD (gp91phox−/−) mice. X-CGD (gp91phox−/−) mice have a disruption in the gene encoding the 91 kDa subunit of oxidase cytochrome b and therefore lack phagocyte superoxide production.26 Competitive infection with Ybt-expressing and non-expressing UTI89 strains (UTI89::kan vs UTI89ΔybtS) was assessed in resident peritoneal macrophages from wild type C57BL/6 and X-CGD (gp91phox−/−) mice (Figure 2b). In copper-replete wild type peritoneal macrophages, Ybt-expressing wild type UTI89 exhibited a significant survival advantage over ΔybtS (log CI 2, \( p = 0.0041 \), Wilcoxon signed-rank test) (Figure 2b). This competitive advantage was significantly diminished when copper was omitted in wild type C57BL/6 macrophages or when copper-replete X-CGD macrophages were used (CI of 0.4 and 0.4, \( p = 0.0036 \) and 0.0041, respectively, Wilcoxon signed-rank test). These findings further support a role for Cu(II)-Ybt in resisting superoxide-derived host defenses in mouse peritoneal macrophages. Without copper supplementation, an elevated CI (CI of 1.7, \( p < 0.0039 \), Wilcoxon signed-rank test) was noted in X-CGD macrophages, consistent with a copper-independent Ybt function that facilitates UTI89 survival in peritoneal macrophages but not RAW 264.7 cells.

To determine whether the survival differences noted in murine macrophages require Ybt import, we determined competitive indices for UTI89 and its transport-deficient ΔfyuA mutant (UTI89::kan vs ΔfyuA). Unlike UTI8ΔybtS, UTI89ΔfyuA and wild type UTI89 survival were indistinguishable in all conditions (\( p = 0.7712 \), Wilcoxon signed-rank test), consistent with a critical role for Ybt biosynthesis, not uptake, in intracellular survival (Supplemental Figure S4). In summary, although peritoneal macrophages and RAW264.7 cells have different cellular origins and deploy different antimicrobial effectors,27–30 Ybt-expressing *E. coli* exhibit an intracellular survival advantage in copper-replete phagocytes during the respiratory burst that is independent of the ferric-Ybt transporter FyuA.

**Cu(II)-Ybt Exhibits Superoxide Dismutase-Like Activity.** We hypothesized that Ybt or related products protect intracellular pathogens from the respiratory burst within copper-containing phagosomes by forming Cu(II) complexes that mimic superoxide dismutases. Cu(II)-SA complexes with superoxide dismutase (SOD)-like activity in the presence of copper, although peritoneal macrophages and RAW264.7 cells have different cellular origins and deploy different antimicrobial effectors,27–30 Cu(II)-Ybt or related products might mimic superoxide dismutases. Cu(II)-SA complexes with superoxide dismutase-like activity have been described extensively and were once proposed for pharmaceutical use.23,31 To determine whether wild type UTI89 culture supernatants exhibit superoxide dismutase (SOD)-like activity in the presence of copper, minimal media culture supernatants from UTI89 and UTI89ΔybtS were fractionated and screened for SOD activity (Figure 3a and b) using the xanthine/xanthine oxidase reaction WST-formazan-based superoxide assay.38,39 Activities are expressed relative to 100 μM bovine Cu,Zn-SOD standard. Maximal SOD activity was observed in the 80% methanolic extract of copper-supplemented wild type UTI89 (87.6% of total supernatant activity). This active fraction was absent in copper-deficient or UTI89ΔybtS fractions.

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H2O2 is a superoxide dismutation reaction product that would hydrogen peroxide produced during this reaction (Figure 4c). Catalytic cycling through reactions 1 and 2, we quantified versus the presence of Cu(II)-Ybt at 80% methanol fraction from copper-treated UTI89 supernatant reveals of copper. (c) A representative full scan mass spectrum of the active not observed in UTI89, but not the activity is observed in the 80% methanolic extracts of copper-replete rather than consumptive mode of superoxide degradation.

Figure 3. UTI89 supernatants exhibit superoxide dismutase activity following copper addition. Copper-treated and untreated culture supernatant fractions of UTI89 and UTI89ΔybtS were tested for superoxide dismutase (SOD) activity. SOD activity is expressed as inhibition of xanthine oxidase-generated superoxide levels relative to 100 μM Cu2Zn-SOD standard (defined as 100% inhibition). (a) SOD activity is observed in the 80% methanolic extracts of copper-replete UTI89, but not the ΔybtS culture supernatant. The data are presented as means ± SD of three independent experiments. (b) SOD activity is not observed in UTI89 or ΔybtS culture supernatants in the absence of copper. (c) A representative full scan mass spectrum of the active 80% methanol fraction from copper-treated UTI89 supernatant reveals the presence of Cu(II)-Ybt at m/z 543.

changed between controls and the complete xanthine/xanthine oxidase system. These findings are consistent with a catalytic rather than consumptive mode of superoxide degradation.

To further differentiate between superoxide consumption versus catalytic cycling through reactions 1 and 2, we quantified hydrogen peroxide produced during this reaction (Figure 4c). H2O2 is a superoxide dismutation reaction product that would not be generated if Cu(II)-Ybt is irreversibly consumed during the reaction with superoxide anion, as might conceivably occur if Cu(I)-Ybt were a stable product. In the xanthine/xanthine oxidase system, H2O2 concentrations were significantly higher (p = 0.0067, t test) in the reaction with Cu(II)-Ybt (0.04 μM) than with apo-Ybt or control. This result further supports the proposed catalytic mode of superoxide degradation in which a second superoxide oxidatively regenerates Cu(II)-Ybt.

Ybt’s SOD-Like Activity Requires a Complexed, Redox Active Metal. To determine the effect of omitting copper or substituting other Ybt metal ligands, we assessed the superoxide dismutase-like activity of apo-Ybt, ferric-Ybt (Fe(III)-Ybt), and gallium-Ybt (Ga(III)-Ybt) and compared it to that of Cu(II)-Ybt (Figure 4b). Catalytic activity was in the order Cu(II)-Ybt > Fe(III)-Ybt ≫ Ga(III)-Ybt, a trend that reflects the activities for equimolar quantities of the corresponding metal salts. The negligible activity of Ga(III)-Ybt is consistent with gallium’s inability to participate in biological redox cycling reactions. Cu(II)-Ybt exhibited the highest activity, suggesting that Ybt-secreting bacteria not only sequester phagolysosomal copper but also use it to help resist the respiratory burst.

Ybt’s Heterocyclic Ring System Maintains SOD-Like Activity. Cu(II)-Ybt SOD-like activity may parallel that described for synthetically generated Cu(II)-salicylate complexes.23 To determine the structure—function relationship between Ybt’s salicylate and non-salicylate constituents, we compared superoxide dismutase-like activity of Cu(II) in the presence of salicylate (SA) or Ybt (Figure 5a). Both SA and Ybt enhanced the SOD-like activity of Cu(II) to a similar degree, consistent with activity enhancement from phenolate coordination. Competitive Cu(II) binding by physiologically plausible protein concentrations greatly attenuates the SOD-like activity of Cu(II)-SA complexes. To determine if Cu(II)-Ybt’s activity is similarly limited, we repeated the above experiments in the presence of 1.0 mg/mL bovine serum albumin (BSA). Whereas BSA strongly attenuated the SOD-like activity of Cu(II)-SA as previously reported, Cu(II)-Ybt activity was unaffected (Figure 5b and c).

Proposed Cu(II)-Ybt Catalytic Model. Superoxide dismutation by copper-based catalysts involves two sequential reactions (see eqs 1 and 2 above. In this instance, M = Cu, n = 1). To develop a model for superoxide dismutation catalyzed by Cu(II)-Ybt, we used density function theory (DFT) to simulate Cu(II)-Ybt complexes and their interactions with superoxide. Simulated Cu(II)-Ybt structures predict two closely related linkage isomers (Figure 6a) with a common square planar metal coordination core, which is in agreement with experimental ion fragmentation data (Supplemental Figure S5). Cu(II) coordination by the salicylate oxygen and the ring 2 thiazole nitrogen is common to both linkage isomers, and these bonds are retained upon superoxide binding (Figure 6b). Subsequent Cu(II) reduction by superoxide (accompanied by the loss of dioxygen) yields species A0Ybt, a tridentate cuprous complex with two long-bond metal interactions. Complexation with the second superoxide displaces all interactions except those with the salicylate oxygen and ring 2 thiazole nitrogen coordinating groups to form the tridentate unit A0Cu. Superoxide reduction and protonation releases H2O2 and restores the original complex. Stepwise complexation, superoxide dismutation, and subsequent H2O2 dissociation are accompanied by a favorable net negative total enthalpic contribution (predicted \( \Delta H_{\text{total}} = -31.3 \text{ kcal/mol} \)). These simulations support Ybt as a
hemilabile ligand able to retain its association with a redox cycling Cu(II) ion while interacting with superoxide anions. Our results demonstrate that Ybt expression protects intracellular uropathogenic E. coli from the respiratory burst following phagocytosis in two different macrophage cell types. This protective function requires copper ions, which spontaneously form stable Cu(II)-Ybt complexes with superoxide dismutase-like activity (Figure 7). Key features of this complex are its redox-active copper center, a phenolate-metal interaction, and an extended heterocyclic chain that permits catalytic activity while maintaining copper-coordination in protein-rich environments. Ybt emerges from these studies as a multifunctional virulence-associated secondary metabolite capable of forming a non-protein, copper-centered catalyst that helps bacteria resist the respiratory burst of activated phagocytes.

The intraphagosomal space surrounding internalized E. coli confines secreted host and pathogen molecules within a very small volume (≈1.2 × 10^{-15} L), resulting in local molar concentrations much higher than those achieved in our in vitro experimental systems. While peak steady state superoxide concentrations in the xanthine/xanthine oxidase system are ≈1 μM, steady state phagosomal superoxide concentrations have been determined to exceed 100 μM. Product levels in minimal media cultures suggest that mid- to high-millimolar Ybt may accumulate intraphagosomally within 1 h. As a second order disproportionation reaction with respect to superoxide, intraphagosomal Cu(II)-Ybt-catalyzed dismutation is expected to proceed at a far greater rate than in vitro xanthine/xanthine oxidase-based reactions. Although Cu,Zn-SOD is the most efficient (k_{cat}/K_{m}) enzyme known and exhibits an approximately 20-fold higher molar activity advantage than Cu(II)-Ybt, the Ybt-based catalyst offers several potential advantages to pathogens. Its biosynthetic cost is lower (0.5 kD complex vs >15 kD protein), resulting in lower metabolic costs to a nutritionally stressed pathogen. Furthermore, because Cu(II)-Ybt’s peptide bonds are condensed to protease-resistant thiazoline and thiazolidine rings, it would be expected to survive the protease-rich phagosome far longer than a protein-based catalyst. Indeed, Kim et al. showed that a Cu,Zn-SOD with greater protease resistance more effectively protected Salmonella from phagosomal killing than protease-sensitive Cu,Zn-SOD. Ybt expression may thus be a specialized defense against phagocytic killing.

The precise nature of microbial protection from extracellular superoxide remains unclear. NADPH-derived superoxide anions do not cross membranes, suggesting that the relevant interactions occur outside the bacterial cytoplasm and are unaffected by cytoplasmic SODs. In the presence of phagosomal copper ions, it is possible that NADPH oxidase-derived superoxide reduces Cu(II) (eq 3), generating a more freely diffusible toxic cuprous (Cu(I)) species that can displace...
Cu(II)-Ybt’s SOD activity may facilitate bacterial survival in multiple pathologically significant environments. Protein superoxide dismutases are recognized virulence-associated factors in an array of bacterial and fungal pathogens known to occupy intracellular locations in the host. Phagocyte-rich lymphatic tissue is a common pathophysiologic niche for pathogenic Yersinia, which carry Ybt biosynthetic genes. Although not classically recognized as an intracellular pathogen, uropathogenic E. coli exhibit enhanced macrophage survival relative to non-pathogenic strains, which may facilitate intracellular persistence. Less clear is whether Ybt influences intracellular survival in non-professional phagocytic cells such as bladder epithelial cells, where UPEC have been described to establish intracellular reservoirs. Although urinary epithelial cells express the non-phagocyte-associated NADPH oxidase Duox1, its impact on urinary tract pathogenesis or bacterial colonization remains unclear.

It is notable that synthetic SOD mimics have been the goal of multiple synthetic efforts for several decades (see review, ref 54). Although they span a broad range of ligands and metals, these mimics share the same metal-centered redox cycling catalytic mechanism identified for protein SODs. As with Cu(II)-Ybt, early SOD mimics were based on Cu(II)-SA complexes. These complexes were limited by low formation constants and instability in the presence of competitive metal chelators, which are highly abundant in physiologic systems. The natural products Ybt and methanobactin appear to have solved this limitation by incorporating multiple heteroaromatic rings that maintain copper coordination while permitting superoxide interactions. Natural products such as these may provide useful insights for synthetic SOD mimics.

Ybt’s ability to bind copper and act as a catalyst shows how the chemical diversity characteristic of microbial siderophores may manifest not only in ferric ion binding and acquisition, but also as additional, idiosyncratic interactions with other metal ions and host factors. Other microbial siderophores and natural products may perform additional enzymatic or catalytic functions that offer important advantages over enzymes in certain host microenvironments. A greater understanding of microbial secondary product chemistry and the environments associated with their expression may uncover previously unappreciated virulence-associated functions.

### METHODS

#### Bacterial Strains and Cultivation

UTI89, a well-characterized and fully sequenced uropathogenic E. coli strain, was used as the prototypic pathogen in this study. UTI89-kan was constructed by inserting a kanamycin resistance cassette into the HK phage attachment site using previously described methods. UTI89 mutant strains used in this study are listed in Supplemental Table 1. Bacterial cultures were grown from a single colony in Difco Luria–Bertani broth, Miller (LB) (Beckton Dickinson) for 3 h and subsequently diluted 1:100 into M63 medium supplemented with 0.2% v/v glycerol and 10 mg/mL niacin (Sigma). Bacterial cultures were incubated for 18 h at 37 °C in a rotary shaker. UTI89 with a kanamycin resistance cassette was grown in 50 μg/mL antibiotic when appropriate. Antibiotic resistant strains were selected on LB-kanamycin (100 μg/mL) plates.

#### Deletion-Strain Construction

In-frame deletions in UTI89 were made using the lambda Red recombinase method, as previously described, using pKD4 or pKD13 as a template. To confirm the appropriate deletions, we performed PCR with flanking primers. Antibiotic resistance insertions were removed by transforming the mutant strains with pCP20 expressing the FLP recombinase.

Figure 5. SOD activity of Cu(II)-Ybt is selectively retained in the presence of protein. (a) Salicylate is modified by nonribosomal peptide transferase/polyketide synthase proteins in bacterial pathogens to synthesize Ybt. (b) Superoxide-dismutase activities of Cu(II), salicylate, and Ybt alone and in combination were determined. SOD activity determined for Cu(II)-salicylate complexes is 61.6%, similar to the 65% determined for Cu(II)-Ybt complexes. (c) SOD activities of these complexes were measured in the presence of 1.0 mg/mL protein [bovine serum albumin (BSA)] to determine whether activity is retained in a more physiologically relevant environment with high concentration of protein. BSA quenched the superoxide dismutase activity associated with Cu(II)-salicylate complexes but not that associated with Cu(II)-Ybt complexes. This functional preservation suggests an additional biochemical rationale for the extended salicylate modification by yersiniabactin biosynthetic proteins.
Chemicals and Reagents. Methanol (HPLC grade) and water (HPLC grade) were purchased from Fisher Scientific (Fisher Scientific). Salicylic acid, cupric sulfate, ferric chloride, gallium nitrate, diphenyleneiodonium chloride (DPI), and bovine serum albumin (BSA) were purchased from Sigma (Sigma-Aldrich Corporation). Superoxide assay kit (CAT19160) and catalase assay kits (CAT 100) were purchased from Sigma (Sigma-Aldrich Corporation).

Yersiniabactin Isolation and Characterization. Ferric chloride or copper sulfate (1.0 M) was added to UTI89\Delta\text{entB} cell culture supernatants to a final concentration of 50 mM and metal-Ybt complexes were purified as described previously. The supernatant from
Figure 7. Model for the interaction between Cu(II) and Ybt within the phagosome. In activated macrophages, host ATP7A secretes copper into the phagosomal compartment enclosing internalized bacteria. Superoxide anions (O$_2^−$) are generated within this compartment by NADPH oxidase. Ybt secreted by intraphagosomal bacteria spontaneously interacts with Cu(II) to form Cu(II)-Ybt. Cu(II)-Ybt’s SOD-like activity diminishes steady state superoxide concentration. By complexing free copper and minimizing superoxide levels, yersiniabactin reduces levels of toxic Cu(I) or other redox-active toxins.

**this precipitation reaction was clarified by centrifugation and subsequently subjected to preparative chromatography, eluted with 100% methanol. The presence of cupric- and ferric-yersiniabactin was confirmed by LC−MS detection of these complexes at m/z 543 and 535, respectively.**

**Tissue Culture.** RAW264.7 cells were obtained from the American Type Culture Collection and maintained in Gibco’s RPMI 1640 medium (Invitrogen) containing 10% v/v fetal bovine serum (Invitrogen) in 5% CO$_2$ at 37 °C.

**Bacterial Survival within RAW 264.7 Macrophages.** RAW264.7 macrophages were detached from TPP cell culture flasks by scraping into ice-cold medium containing 10% v/v FBS, washed twice, and resuspended in 24-well plates at 10$^5$ cells/well. The seeded wells were treated in the following order: 24 h in (i) ice-cold medium containing 10% v/v FBS, followed by a 24 h incubation in (ii) serum-free media, followed by a 24 h incubation in (iii) serum-free media either with or without 20 μM CuSO$_4$. Wild type E. coli (strain UTI89), isogenic mutants ΔfyuA, ΔfyuAΔybtS, E. coli harboring a resistance cassette to kanamycin (strain UTI89::kan), or MG1655 were grown for 18 h to stationary phase in M63 minimal media and added to the RAW264.7 macrophages at a multiplicity of infection (MOI; macrophage/bacteria ratio) of 1:10 or 1:1. Each experimental condition was set up in triplicate wells. Bacterial phagocytosis was allowed to proceed for 30 min at 37 °C.

**Superoxide Dismutase Activity.** Superoxide dismutase (SOD) activity was measured indirectly in multwell plates, using xanthine/xanthine oxidase as the superoxide-generating system and the reduction of Dojindo’s highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) to produce a water-soluble formazan dye as the detector (Sigma-19160). Reduction of WST-1 was monitored at 440 nm in a microtiter plate. Controls for the SOD assay included ensuring that the compound did not affect the superoxide-generating reaction, testing solvent alone, and ensuring that the compound does not react independently with WST-1. To
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Mass Spectrometry and Spectrophotometry of Yersiniabactin in the Presence of Superoxide Anion. The SOD activity assay reagents (Sigma) described above was adapted to determine the effects of superoxide anion on the yersiniabactin backbone and the Cu(II)-Ybt complex. Aliquots (200 μL) of 10 μM copper sulfate, apo-Ybt, or Cu(II)-Ybt were exposed to the xantine/xanthine oxidase reaction system. Since WST-1 reacts with free superoxide anions, it was not added to any reaction mixture. The reaction was allowed to proceed for 20 min at 37 °C. Following incubation, 10 μL of 13C-labeled internal standard was added to a 150-μL aliquot of each sample, and the samples were subjected to preparative chromatography. The ratio of labeled to unlabeled Cu(II)-Ybt was determined in each sample as described previously by Chaturvedi et al.

Hydrogen Peroxide Quantification. Aliquots (1 mL) of 10 μM copper, Cu(II)-Ybt, and appropriate SOD reaction substrates (defined above) were allowed to incubate for 15 min, and the reaction was stopped using the catalase assay stop solution (15 mM sodium azide in buffer). H2O2 levels in each reaction vial were determined by adapting a catalase assay reagents (Cat-100, Sigma). This assay is normally deployed to determine H2O2 levels following catalase activity using a colorimetric substrate. This colorimetric method uses a substituted phenol (3,5-dichloro-2-hydroxybenzene-sulfonic acid), which couples oxidatively to 4-aminonitropririne in the presence of H2O2 and horseradish peroxidase (HRP) to give a red quinonimine dye (N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine) that absorbs at 520 nm. H2O2 standard (Sigma) was used to generate a standard curve to calibrate this reaction (Supplemental Figure S6).

High-Resolution Liquid Chromatography—Mass Spectrometry. High-resolution mass spectrometry analyses of cupric- and ferric-yersiniabactin complexes were conducted using a Bruker Mass Q-ToF operated in positive ion mode as previously described. The samples were directly infused at a flow rate of 0.3 μL/min. The ion spray voltage was set to 4500 V for positive ion and −551 V for negative ion mode, respectively. The nebulizer gas (air) and turbo gas (air) were set in the sample and reagent gas modes, respectively. The heater temperature was set to 350 °C. The ion source and cone gas were both set in the heated nebulizer mode as described previously by Chaturvedi et al.

Theoretical Calculations. Theoretical calculations were performed to characterize the potential-energy surface (PES) associated with fragmentation and reaction. Conformer spaces for precursors (cupric and ferric complexes with Ybt), and intermediates were explored by Monte Carlo/MMFF molecular mechanisms/dynamics methods. From these results, structures of precursors, intermediates, and scans for associated transition states were explored by using the PM3 semiempirical algorithm, both in Spartan06 for Linux v. Two (Wave function, Inc.). DFT (Density Functional Theory, part of Gaussian 03 and 09 suites, Gaussian, Inc.) calculations were performed by using the PBE0 functional (PBE1PBE in Gaussian parlance) with basis sets De2-SVP and De2-TZVP.88 Minima and transition states were optimized at the level B3LYP/6-31G(d,p).92–94 Solvent-based interactions were calculated at the same level by using the CPCM polarizable conductor calculation model for water and using the Universal Force Field for atomic radii. The hybrid functional and basis sets were chosen on the basis of performance with transition metal complexes.93–95 DFT was selected for high-level calculations on pragmatic reasons because it requires overall less computational overhead than ab initio methods and performs adequately.72–74 All results are reported in kcal/mol as enthalpies of formation relative to a selected, suitable precursor. The complexes are all radicals because of the transition-metal cation involved. The Cu(II) complexes are spin 1/2 with the Cu(II) in low-spin.

Statistical Analyses. Statistics and graphs were generated using GraphPad Prism 4 (GraphPad software). Student’s t test was used to compare growth differences between paired strains. Analyses of paired intracellular survival differences in competitive co-infections were performed using the Wilcoxon signed-rank test for significance.

ASSOCIATED CONTENT

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The authors declare no competing financial interest.

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