Enterobacteria secrete an inhibitor of *Pseudomonas* virulence during clinical bacteriuria

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Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases treated by physicians and are increasingly associated with antibiotic-resistant bacteria (1, 2). Although bacteriuria, the presence of bacteria in the urine, is a key feature of UTI, it also occurs frequently in the absence of symptoms. Bacteriuria seeded from the intestinal microbiome may self-resolve, progress to UTI, or persist asymptptomatically, in which case antibiotic treatment is not recommended (3). Indeed, recent studies support the existence of urinary tract microbiomes in otherwise healthy individuals (4). Multiple lines of evidence suggest that the microbiome can prevent infections. Paradoxically, antibiotic treatment has been observed to increase the risk of subsequent UTIs (5) and to increase the risk of UTIs caused by atypical uropathogens such as *Pseudomonas* (6). Members of the family Enterobacteriaceae, particularly *Escherichia coli*, inhabit the intestinal microbiome and are most commonly associated with asymptomatic bacteriuria and UTI. The early bacteriuric state from which UTIs can develop is poorly understood and challenging to study, but it is known that UTI-associated *E. coli* isolates frequently possess nonconserved genetic systems related to virulence (7, 8).

Prominent among virulence-associated adaptations in uropathogenic *E. coli* (UPEC) isolates are siderophores, specialized metabolites able to bind extracellular Fe(III) ions for nutritional use in low-iron conditions (7, 9). High-affinity Fe(III) binding by siderophores helps bacteria resist host-mediated iron deprivation, a prototypical example of nutritional immunity (10, 11). Siderophores are widespread among medically important bacteria, which often express chemically distinctive siderophore types. A recent quantitative metabolomics study of *E. coli* isolates from patients with UTI revealed that the resident intestinal strain in each patient that successfully infected the urinary tract preferentially secreted the siderophores yersiniabactin (Ybt) and salmochelin (12). These results suggest that the early stages of ascending UTI are competitive and favor isolates with specific siderophore profiles. In this and other studies, Ybt, a siderophore encoded by the *Yersinia* high pathogenicity island (HPI), was associated with urinary tract virulence (7, 13–17).

The *Yersinia* HPI is a complex, 4-operon mobile genetic element encoding proteins involved in Ybt biosynthesis, import, and transcriptional regulation. It is highly prevalent among clinical extraintestinal pathogenic *E. coli* isolates and is also found with varying frequencies in other Enterobacteriaceae, including the eponymous *Yersinia pestis* (18). *Yersinia* HPI genes are highly upregulated during UTI, and Ybt is detectable in patients’ urine specimens (12, 14, 17). Ybt, the recognized product of *Yersinia* HPI-positive bacteria to date, has been distinguished from other *E. coli* siderophores by its ability to form stable Cu(II) complexes. This broad-spectrum metal-binding property enables Ybt to scavenge Fe(III) for nutritional use, detoxify copper, and catalyze superoxide dismutation (14, 15, 19). The multiple functions of Ybt illustrate the importance of diverse metal ion interactions during microbial pathogenesis.

A hybrid nonribosomal peptide synthetase/polypeptide synthase (NRPS/PKS) pathway encoded on the *Yersinia* HPI directs the biosynthesis of Ybt. There is remarkable natural diversity in microbial
NRPS/PKS systems, which are notable in infectious diseases as the source of antibiotics such as mupirocin, tetracyclines, and macrodides, but also the source of many virulence-associated siderophores such as pyochelin in *Pseudomonas aeruginosa* and mycobactin in *Mycobacterium tuberculosis* (20–22). NRPS and PKS pathways are characterized by their thiotemplateing, assembly line mechanism, whereby all substrates and intermediates are covalently attached to phosphopantetheinylated carrier protein (CP) domains as they proceed through the catalytic domains of large, multimodular biosynthetic enzymes. The Ybt biosynthetic substrates are chorate, 3 molecules of cysteine, malonate from malonyl-CoA, and methyl groups derived from S-adenosyl-methionine (SAM). During Ybt biosynthesis, YbtS converts chorismate to salicylate (23), which is activated and transferred onto the first NRPS, HMWP2 (irp2), by YbtE. After condensing salicylate and 2 molecules of cysteine, the last intermediate synthesized by HMWP2 is transferred onto the hybrid NRPS/PKS HMWP1 (irp1), where malonate, another molecule of cysteine, and methyl groups are incorporated. At this point, YbtU catalyzes a NADPH-dependent reduction of an HMWP1-bound intermediate. Once the molecule is complete, Ybt is released from the final CP domain of HMWP1 by a terminal thioesterase domain (20, 24). Although Ybt biosynthesis has been elegantly characterized in vitro, an untargeted survey of this pathway’s products in bacterial culture has not been reported.

In this study, comparative metabolomic profiling revealed escherichelin, a newly appreciated cellular product of the Ybt biosynthetic pathway in *E. coli* and other urinary Enterobacteriaceae isolates. Structural characterization identified escherichelin as 2-(2-hydroxyphenyl)-4,5-dihydro-[2,4]bisthiazolyl-4-carboxylic acid, (abbreviated as HPTT-COOH from hydroxyphenylthiazolyl-thiazolinyl-carboxylic acid), an Fe(III) chelator that was previously synthesized and found to inhibit pyochelin-mediated iron uptake in *P. aeruginosa* (25). Escherichelin purified from *E. coli* lacks the siderophoric activity of Ybt, but inhibits *P. aeruginosa* growth in a pyochelin-dependent culture condition. Consistent with this activity, we observed that *P. aeruginosa* minimizes escherichelin release compared with *E. coli*, despite generating the same biosynthetic precursors to escherichelin during pyochelin biosynthesis. This bioactivity and its production during human *E. coli* bacteriuria raises the possibility that escherichelin may help human hosts avoid developing symptomatic UTIs from pyochelin-producing organisms. Escherichelin was detectable in a human subject who was experimentally colonized with a derivative of the bacterial interference strain *E. coli* 83972 that has been studied in several human trials as a potential agent to prevent UTI (26, 27). These findings reveal how a second metallophore product of the *Yersinia HPI* biosynthetic system may participate in interspecies competition during polymicrobial bacteriuria.

**Results**

**Metabolomic profiling identifies multiple *Yersinia* HPI–associated metabolites.** To identify secreted metabolites associated with the *Yersinia* HPI, we used liquid chromatography–mass spectrometry (LC-MS) to compare culture supernatants from UTI89, a Ybt-producing model UPEC strain (28), and its isogenic mutant UTI89Δ*ybtS* that cannot perform the first committed step of Ybt biosynthesis, salicylate synthesis (12, 23). As a control, the biosynthetic block in UTI89Δ*ybtS* was experimentally overcome by chemical complementation with salicylate-supplemented medium. The metabolomic profiles from Ybt producers (WT and UTI89Δ*ybtS* plus salicylate) and the Ybt nonproducer (UTI89Δ*ybtS*) were compared using supervised principal component analysis–discriminant analysis (PCA-DA) (Figure 1A). Uniform separation of these 2 groups along the y axis of the PCA-DA plot confirmed that extracellular metabolites can differentiate Ybt-producing from nonproducing strains (Figure 1A).

To identify the specific extracellular metabolites that distinguish Ybt producers from nonproducers, we performed a loadings plot analysis (Figure 1B). In this plot, features with the highest D1 loading scores are most strongly associated with Ybt producers and are, therefore, candidates for follow-up analysis. Ybt in the metal-free form (apo) and bound to Al(III) were among the molecular features with the highest loading scores, as expected (Table 1) (29). We also observed dihydroaeruginoic acid (Dha), a known metabolite of pyochelin-producing strains of *P. aeruginosa* (Table 1 and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI92464DS1) (30, 31).

Dha is the free form of an early phosphopantetheine-bound intermediate in both the pyochelin and Ybt biosynthetic pathways (20, 32). Unexpectedly, the molecular feature with the highest loading score was an ion with m/z 307, which is lower than apo-Ybt (m/z 482) (Figure 1B and Table 1). Like Ybt, the molecular feature at m/z 307, herein called escherichelin, was present in the WT and salicylate-complemented UTI89Δ*ybtS* supernatants but entirely absent in Δ*ybtS* supernatants. These results are consistent with the production of a previously unappreciated secreted metabolite by *E. coli* with an intact *Yersinia* HPI.

**Clinical Enterobacteriaceae isolates coproduce escherichelin and Ybt.** To determine whether escherichelin production is specific to UTI89 or is present in other bacteria encoding the *Yersinia* HPI, we measured its production in a series of clinical urinary isolates and model strains of the Enterobacteriaceae family (Figure 1C). The UPEC model strains UTI89 and NU14 produced both Ybt and escherichelin, whereas CFT073, a widely used UPEC model strain with known mutations in the *Yersinia* HPI, and the K12 strain MG1655 produced neither (33). *Klebsiella pneumoniae* and *Citrobacter diversus* isolates that produced Ybt also produced escherichelin (Figure 1C). These observations suggest that escherichelin production is pervasive among clinically significant Ybt-producing Enterobacteriaceae family members.

**Escherichelin production is associated with early Ybt biosynthetic enzymes.** The *Yersinia* HPI and other siderophore systems are regulated by Fur, an iron-responsive transcription factor, such that Ybt biosynthesis is downregulated in iron-rich conditions. To determine whether escherichelin is similarly regulated, we measured its secretion in a high-iron medium (complete M63 supplemented with 16.2 mg/l ferric chloride) (34). The high-iron medium abolished escherichelin production, suggesting that it is regulated by iron in a manner similar to that of Ybt (Figure 2A).

To better understand the relationship between escherichelin secretion and Ybt biosynthetic genes (Figure 2B), we screened a panel of *Yersinia* HPI gene deletion mutants for altered escherichelin production (Figure 2C). Mutants lacking the biosynthetic genes *ybtS*, *ybtE*, *irp1*, or *ybtU* do not produce Ybt (Supplemental
The observation that late biosynthetic mutants (ΔUTI89

HPI product. lin production is consistent with it being a

Yersinia

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iron-restricted medium (38). Among Ybt-null strains, extracellu-

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a member of the AraC transcription factor

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remains unclear, it has been proposed that

this altered gene expression in the mutants

observed with analogous

irp2

mutants (UTI89

Δ

irp1

and

Δ

ybtU, Figure 2C) mirror the diminished irp2 expression observed with analogous Y. pestis mutants (36, 37). Although the precise reason for this altered gene expression in the mutants remains unclear, it has been proposed that feedback regulation from a late biosynthetic gene product is reduced, leading to the downregulation. The feedback regulation is believed to be mediated by YbtA, a member of the AraC transcription factor family whose members typically respond to allosteric activation by metabolites.

Figure 2) (23, 35–38). A mutant lacking the Yersinia HPI transcrip-
tional regulator ybtA also produces no Ybt (38, 39). Deletion of

ybtT, a putative editing enzyme, exhibits an intermediate pheno-
type with reduced Ybt levels (Supplemental Figure 2) (37, 38,

40). These isogenic mutants are not attenuated for growth in

restriction medium (38). Among Ybt-null strains, extracellular

escherichelin was undetectable in the culture supernatants of

UTI89

Δ

ybtS, ΔybtE, and ΔybtA (the 2 early biosynthetic genes and

transcriptional regulator, respectively). In contrast, escherichelin

was detectable in supernatants of UTI89

Δ

irp1 and ΔybtU, the late Ybt biosynthetic pathway deletion mutants (Figure 2C). Intracellular escherichelin levels followed a trend nearly identical to that of the extracellular levels between strains (Supplemental Figure 3). Overall, these patterns favor biosynthesis, rather than cel-

lar export, as the major determinant of escherichelin secretion.

The requirement of early Ybt biosynthetic genes for escherichelin production is consistent with it being a Yersinia HPI product. The observation that late biosynthetic mutants (UTI89

Δ

irp1 and

Δ

ybtU) produce escherichelin, but not Ybt, further suggests that escherichelin is a de novo metabolite and not a Ybt breakdown product. The diminished, but not absent, escherichelin levels in late biosynthesis mutants (UTI89

Δ

irp1 and

Δ

ybtU, Figure 2C) mirror the diminished irp2 expression observed with analogous Y. pestis mutants (36, 37). Although the precise reason for this altered gene expression in the mutants remains unclear, it has been proposed that feedback regulation from a late biosynthetic gene product is reduced, leading to the downregulation. The feedback regulation is believed to be mediated by YbtA, a member of the AraC transcription factor family whose members typically respond to allosteric activation by metabolites.

Table 1. Molecular features with the highest loading scores are candidate small molecules associated with Ybt biosynthesis

<table>
<thead>
<tr>
<th>Rank</th>
<th>Loading score</th>
<th>m/z</th>
<th>Retention time (min)</th>
<th>Identification</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.393</td>
<td>307.0</td>
<td>15.8</td>
<td>escherichelin</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.234</td>
<td>224.1</td>
<td>12.8</td>
<td>Dha</td>
<td>30–32</td>
</tr>
<tr>
<td>3</td>
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<td>295.0</td>
<td>17.1</td>
<td>apo-Ybt peak 2</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
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<td>188.1</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.187</td>
<td>498.1</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.169</td>
<td>482.1</td>
<td>17.1</td>
<td>apo-Ybt peak 2</td>
<td>14, 19</td>
</tr>
<tr>
<td>7</td>
<td>0.152</td>
<td>333.0</td>
<td>7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.143</td>
<td>506.1</td>
<td>11.6</td>
<td>All(III)-Ybt</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>0.137</td>
<td>482.1</td>
<td>16.3</td>
<td>apo-Ybt peak 1</td>
<td>14, 19</td>
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<td>0.129</td>
<td>295.1</td>
<td>16.4</td>
<td>apo-Ybt peak 1</td>
<td>14</td>
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</tbody>
</table>

*The loading scores come from the loading plot of the positive-ion LC-MS data (Figure 1B).

1Source-decay fragment of apo-Ybt.

Escherichelin is HPTT-COOH. Multiple structural analyses were used to determine the chemical structure of escherichelin. These analyses were guided in part by previous in vitro investigations of Ybt biosynthesis that noted a product with a positive-ion m/z of 307 that was identified as 2-(2-hydroxyphenyl)-4,5-dihydro-[2,4] bisthiazolyl-4-carboxylic acid (abbreviated as HPTT-COOH from hydroxyphenyl-thiazolyl-thiazolinyl-carboxylic acid) (24, 41–44). HPTT-COOH is a truncated version of Ybt that arises when a biosynthetic intermediate (Figure 2B, top structure) derived from salicylate and 2 cysteines is released from the first NRPS protein, HMWP2 (irp2), and spontaneously oxidized. High-resolution MS of escherichelin yielded an exact mass of 307.0216 for the [M+H]+ ion, supporting an empiric formula of C13H11N2O3S2 (calculated mass: 307.0206 for C13H11N2O3S2). To determine whether escherichelin is synthesized from salicylate, we chemically complemented UTI89

Δ

ybtS with 13C6-salicylate and analyzed the supernatant by LC-MS. Upon 13C6-salicylate complementation, escherichelin at m/z 307 disappeared, and a new molecular feature at m/z 313
respectively (Supplemental Figure 4). Together, the molecular formula from high-resolution MS, the MS/MS fingerprint, and the 1H-NMR chemical shifts support HPTT-COOH as the escherrichelin structure. Escherichelin corresponds to an oxidized form of a Ybt biosynthetic intermediate and is structurally similar to the \textit{P. aeruginosa} siderophore pyochelin. Escherichelin and pyochelin differ in the oxidation state of their first heterocyclic ring, and pyochelin is also further modified by the reduction of the second thiazoline to a thiazole and by N-methylation (45).

Escherichelin binds Fe(III). Given that escherichelin is produced by a siderophore biosynthetic pathway, regulated by Fur, and retains some of the functional groups required for metal binding by Ybt and pyochelin, we assessed the ability of purified escherichelin to form an Fe(III) complex in aqueous solution with UV-visible spectroscopy. As reported in previous studies, Fe(III) addition caused notable shifts in the UV-visible absorption spectra of Ybt and pyochelin, consistent with the formation of Fe(III) complexes (19, 46, 47). The escherichelin UV-visible absorption spectrum was similarly shifted following exposure to equimolar Fe(III) (Figure 3D). Cu(II) addition caused a qualitatively distinct shift from Fe(III) (Figure 3D). These results demonstrate that escherichelin, like Ybt and pyochelin, is a bacterial metallophore that forms Fe(III) and Cu(II) complexes.

To determine whether escherichelin binds metal ions in a manner similar to Ybt and pyochelin, we conducted quantum-based calculations using density functional theory (DFT). These calculations predict stable 1:1 and 1:2 complexes of Fe(III)-escherrichelin in a hexacoordinate octahedral geometry (Figure 3, E and F). In the 1:1 complex, Fe(III) is bound by the salicylate oxygen, the 2
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ion coordination in the Fe(III)- and Cu(II)-escherichelin complexes is similar to that observed for metal-Ybt complexes and Fe(III)-Pch (15, 19, 48, 50). These results support the idea that escherichelin is an Fe(III)-binding molecule.

Escherichelin does not support iron-dependent E. coli growth.

To determine whether escherichelin, like Ybt, can promote iron-dependent growth in E. coli possessing Yersinia HPI-encoded transport genes, we measured UTI89 growth in a previously described siderophore-dependent growth assay (Figure 3G) (16, 19).

Pyochelin binds Fe(III) in similar 1:1 and 1:2 complexes with the same ligands (48, 49). As with Ybt, escherichelin is also predicted to form a stable Cu(II) complex (Supplemental Figure 5). Overall, the calculated metal

Figure 3. Identification of escherichelin as HPTT-COOH, a metallophore inhibitor of Pseudomonas iron uptake. (A) Multiple chemical characterizations identify escherichelin as HPTT-COOH, a compound shown to inhibit pyochelin-mediated iron uptake in Pseudomonas. The positions of ¹³C salicylate incorporation are indicated in green, while MS/MS neutral losses are indicated as dashed lines. (B) Mass spectra at the retention time for escherichelin from WT UTI89, UTI89:ΔybtS, and ¹³C₆-SA-supplemented UTI89:ΔybtS culture supernatants revealed that 6 carbons in escherichelin are derived from salicylate. (C) MS/MS product ion spectrum of escherichelin. The 46-Da neutral loss indicates the presence of a carboxylic acid group. (D) UV-visible absorption spectra of purified escherichelin incubated with equimolar FeCl₃ or CuSO₄. (E and F) DFT simulations of escherichelin bound to Fe(III) in a 1:1 (E) and 2:1 (F) complex. The simulations predict stable Fe(III)-escherichelin complexes with hexacoordinate octahedral geometry. (G) UTI89 growth curve in the presence or absence of a ferric ion chelator (0.5 mM EDDHA) and/or ferric ion supplementation (1 μM FeCl₃). Growth was monitored by OD at 600 nm (OD₆₀₀). Data represent the mean of 3, with SD plotted. (H) PWS011 (pvdA-E02::ISlacZ/hah), a P. aeruginosa strain MPAAO1 transposon mutant (55, 56) expressing pyochelin as its sole siderophore, was grown in succinate medium for 20 hours in the presence of increasing escherichelin concentrations relative to vehicle control. Bacterial growth was quantified by enumerating CFU/ml and is expressed as a percentage of the vehicle control. Data represent the mean of 3, with the SEM plotted. *P < 0.01 and **P < 0.001, by 1-way ANOVA with Dunnett’s multiple comparisons test.

heterocyclic nitrogens, and the carboxylic acid group in the equatorial positions and by 2 water molecules in the axial positions. In the 1:2 complex, the carboxylic acids of the 2 escherichelin molecules and water do not participate as ligands. Instead, the nitrogen atoms and phenolic oxygen atoms of 2 the escherichelins occupy all of the liganding positions. Pyochelin binds Fe(III) in similar 1:1 and 1:2 complexes with the same ligands (48, 49). As with Ybt, escherichelin is also predicted to form a stable Cu(II) complex (Supplemental Figure 5). Overall, the calculated metal coordination in the Fe(III)- and Cu(II)-escherichelin complexes is similar to that observed for metal-Ybt complexes and Fe(III)-Pch (15, 19, 48, 50). These results support the idea that escherichelin is an Fe(III)-binding molecule.

Escherichelin does not support iron-dependent E. coli growth. To determine whether escherichelin, like Ybt, can promote iron-dependent growth in E. coli possessing Versinia HPI-encoded transport genes, we measured UTI89 growth in a previously described siderophore-dependent growth assay (Figure 3G) (16, 19). Addition
The iron chelator EDDHA to the test medium causes substantial growth restriction. As previously observed, addition of Ybt that has been preincubated with equimolar Fe(III) restores growth to a density comparable to that of the unchelated medium. In contrast, addition of escherichelin that has been preincubated with equimolar Fe(III) resulted in no significant growth enhancement (Figure 3G). Although both escherichelin and Ybt bind Fe(III), these results show a nonequivalent ability to support iron-limited growth. This discrepancy may arise from differences in Fe(III) affinity or from differential recognition of the complexes by bacterial transport proteins.

The deficient siderophoric activity of escherichelin raises the possibility that this molecule performs an alternative function. In prior work by Mislin et al., escherichelin (previously designated HPTT-COOH) was synthesized as a pyochelin analog (51) and found to act as a competitive inhibitor of pyochelin-mediated iron import through the TonB-dependent outer membrane transporter FptA in P. aeruginosa (25, 51, 52). Escherichelin did not mediate iron uptake and inhibited Fe(III)-pyochelin transport through FptA with a $K_i$ of 27 nM. In silico docking simulations could rationalize these results through occupancy of the Fe(III)-pyochelin binding site of FptA by a 1:2 Fe(III)-escherichelin complex [Fe(III):escherichelin] (25, 48). Given that escherichelin can bind Fe(III) in solution but does not possess canonical siderophoric activity, we hypothesized that it may be produced by UPEC to inhibit pyochelin uptake by competing P. aeruginosa strains in the urinary tract.

To determine whether escherichelin can inhibit ferric pyochelin uptake, we examined the effect of escherichelin on growth of the P. aeruginosa strain PAO1. Although pyochelin enhances P. aeruginosa growth during infections (53, 54), it is functionally redundant with pyoverdin, a second siderophore, during in vitro growth in standard culture media. Escherichelin addition to a defined, iron-restricted minimal medium caused a dose-dependent reduction in growth of a pyoverdin-deficient P. aeruginosa mutant strain, PW5011 (Figure 3H) (55–57). These results are consistent with the escherichelin-mediated inhibition of iron uptake in P. aeruginosa noted by Mislin et al. (25).

P. aeruginosa minimizes escherichelin production. Production of a P. aeruginosa iron uptake inhibitor from the enzyme-bound precursor hydroxyphenyl-thiazolinyl-thiazolinyl-S-ppant (Figure 2B, top structure) presents a potential paradox, because this enzyme-bound species is also a biosynthetic intermediate of pyochelin (20). Escherichelin release from this intermediate in P. aeruginosa would be expected to autoantagonize pyochelin-mediated iron uptake. To determine whether P. aeruginosa minimizes escherichelin release relative to E. coli, we compared escherichelin production between UTI89 and PAO1 in low-iron medium. Despite comparable growth of both strains and substantial pyochelin production by PAO1, extracellular and intracellular levels of escherichelin were markedly lower in PAO1 than in UTI89 (Figure 4 and Supplemental Figures 6 and 7). This may reflect selective pressure on the P. aeruginosa pyochelin biosynthetic pathway to avoid...
inhibiting pyochelin-mediated iron uptake, as well as an opposing selective pressure on E. coli to suppress competing Pseudomonas strains in polymicrobial settings.

Escherichelin is produced during human E. coli cystitis. Enterobacterial bacteriuria may be a frequent occurrence in the human urinary tract and is frequently accompanied by other flora (58–60). When native enterobacteria in the microbiome are suppressed or eliminated by antibiotic treatment, P. aeruginosa UTIs become more frequent (6). Therefore, we hypothesize that escherichelin release during enterobacterial bacteriuria prevents P. aeruginosa UTI. To determine whether Enterobacteriaceae produce escherichelin in the human urinary tract, we used LC-MS/MS to detect escherichelin in urine from 18 patients with uncomplicated cystitis caused by Ybt-producing E. coli. Escherichelin was detectable in 8 of the 18 samples (Figure 5A and Table 2). In four escherichelin-positive specimens, Ybt was undetectable. As expected, escherichelin was undetectable in patients with cystitis caused by Gram-positive species (Staphylococcus saprophyticus or Enterococcus sp.) or Ybt-null E. coli. These results demonstrate that E. coli can produce escherichelin in the human bladder.

Escherichelin is produced during experimental bladder colonization with an E. coli 83972 strain. The concept that one bacterial strain or species can prevent infection by another — termed bacterial interference — has been of longstanding interest in infectious diseases (61–63). In this context, an avirulent strain or species that outcompetes more pathogenic strains may be useful as a probiotic to prevent infections. E. coli 83972, which asymptptomatically colonized a young Swedish girl for over 2 years (64), has been used in multiple UTI prevention studies (26, 27, 63, 65–70). Among these studies are anecdotal reports of protection against symptomatic P. aeruginosa UTIs, including 1 patient with asymptomatic cocolonization of 83972 and P. aeruginosa that progressed to UTI following loss of 83972 (26, 66). E. coli 83972 possesses the Yersinia HPI (71, 72) and was observed to produce escherichelin in iron-restricted medium (data not shown).

To determine whether 83972 secretes escherichelin in the human urinary tract, we analyzed urine specimens following experimental colonization of catheter-dependent patients with an 83972-derived E. coli strain lacking the virulence-associated papG adhesin (E. coli HU2117) (27). Bladder colonization was established by inserting urinary catheters that had been precoated with E. coli HU2117 (Figure 5B). P. aeruginosa was detected by culture in the urine on day 7 but was unassociated with symptoms and became undetectable in subsequent specimens (27). On day 76, HU2117 colonization had ended, and the subject developed a febrile UTI caused by another strain of E. coli on day 76, after colonization had ended. On day 7, P. aeruginosa was also present in this subject’s urine. Escherichelin was detectable throughout the colonization period and during the symptomatic E. coli UTI. The noncolonized subject did not show a presence of HU2117 after day 0, when the study catheter was inserted, and escherichelin was not detected in the longitudinal samples. P. vulgaris, Proteus vulgaris; A. faecalis, Alcaligenes faecalis; O. anthropi, Ochrobactrum anthropi; B. bronchiseptica, Bordetella bronchiseptica; S. epidermidis, Staphylococcus epidermidis.

Discussion

In this study, we used isogenic mutants in the Yersinia HPI and untargeted LC-MS–based metabolomics to identify a previously unappreciated biosynthetic product of bacteria carrying the Yersinia HPI. Escherichelin had previously been observed during the in vitro reconstitution of Ybt biosynthesis (24), but we now identify it as a physiologically relevant product of the Ybt biosynthetic pathway. The name escherichelin was chosen to recognize its identification as a natural product from pathogenic E. coli (escheri-) and its
metal chelating properties (-chelin). Escherichelin may have eluded discovery in the 20 years since the identification of Ybt because its reverse-phase chromatographic and UV-visible spectroscopic characteristics are nearly identical to those of Ybt, making them difficult to resolve. Although escherichelin lacks the siderophoric activity of Ybt, the fortuitous synthesis of escherichelin prior to the present discovery (51) and the demonstration of its ability to inhibit pyochelin-mediated iron uptake by P. aeruginosa (25) suggests an alternative functional rationale for escherichelin’s secretion by Enterobacteriaceae: to inhibit iron uptake in a competing species. This niche exclusion function is consistent with clinical observations regarding P. aeruginosa UTI, asymptomatic bacteriuria, and protective influences of a healthy human microbiome.

Other microbes have demonstrated an ability to interfere with pyochelin function. Pseudomonas fluorescens can synthesize an enantiomeric form of pyochelin (enantio-pyochelin), which preserves the coordinating properties of pyochelin while resisting an enantiomeric form of pyochelin (enantio-pyochelin), which can synthesize with pyochelin function.

Table 2. Escherichelin and Ybt prevalence in urine samples from patients with uncomplicated UTI

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Causative species</th>
<th>Ybt-producing in culture</th>
<th>Escherichelin in urine sample</th>
<th>Ybt in urine sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>S. saprophyticus</td>
<td>+</td>
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<td>5</td>
<td>Enterococcus spp.</td>
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ND, strain did not grow in complete M63, so Ybt production in culture could not be determined.

Whereas the Ybt and pyochelin biosynthetic machineries both generate the immediate enzyme-bound precursor to escherichelin (Figure 2B, top structure), the pyochelin machinery in P. aeruginosa releases less escherichelin than does the Ybt machinery in E. coli. This is consistent with the evolutionary hypothesis that the pyochelin biosynthetic pathway evolved to avoid auto-inhibition of Fe(III)-pyochelin uptake. The architectures of these pathways provide a potential explanation for this difference in escherichelin production. Specifically, the escherichelin precursor in the Ybt pathway forms at the terminus of the first NRPS protein (HMWP2), where it must be transferred in trans to the second NRPS/PKS protein (HMWP1). In the pyochelin pathway, this same intermediate is instead passed in cis to a subsequent domain on the same protein (PchF). In the Ybt biosynthetic pathway, this handoff between proteins appears to be particularly susceptible to spontaneous hydrolysis, thereby allowing it to become a branch point in which the intermediate can proceed to form either escherichelin or Ybt. In contrast, this immediate precursor to escherichelin may be less vulnerable to hydrolysis off the pyochelin biosynthetic machinery, because it does not occur at a handoff between proteins. During pyochelin biosynthesis, Dha is passed in trans from PchE to PchF, and it is Dha, rather than escherichelin, that accu-
mulates in *P. aeruginosa*. Intriguingly, Dha has been shown to possess antimicrobial activity against fungal and bacterial species (31). Applied generally, this suggests that the NRPS/PKS pathways may evolve to segregate modules onto separate proteins at steps in the pathway where a “leak” yields a product that benefits the organism. Whether the branch point in Ybt biosynthesis that yields escherichelin and the similar branch points in other NRPS/PKS pathways reflect uncontrolled “leaks” or regulated processes is unclear. Further biochemical and genetic characterization of these pathways may address this possibility.

Advances in MS and the application of powerful computational and statistical analyses to the data have led to the fruitful application of metabolomics to secondary metabolism (78). MS-based metabolomics is enabling the rapid discovery of secondary metabolites from newly sequenced bacterial strains as well as novel products from well-studied pathways (79). This approach moves beyond the functional screens that were historically used to discover siderophores and other natural products. With this unbiased approach, one is open to discovering any molecule that is chemically compatible with the instrumentation, regardless of the molecule’s function. Continued application of LC-MS-based metabolomics to other siderophore biosynthetic pathways will further our understanding of iron acquisition by pathogens.

**Methods**

**Bacterial strains and culture conditions.** The well-characterized, cystidis-thedrisis model UPEC strain UTI89 was used for these studies (28). The creation of the isogenic mutants in *ybtS*, *ybtE*, *irp1*, *ybtU*, *ybtT*, and *ybtA* was previously described (12, 38). UTI89 Δirp1*entB::kan* was generated in the same manner, using the lambda red recombinase method (80, 81). For growth under iron-restricted conditions, strains were first grown in Luria-Bertani (LB) broth (BD Biosciences) overnight at 37 °C under shaking conditions. They were then back-diluted 1:1,000 into M63 minimal medium supplemented with 10 mg/ml nia-cin and 0.2% (v/v) glycerol (Sigma-Aldrich; complete M63). For salicylate complementation experiments, 50 μM salicylate or 13C6-salicylate (Sigma-Aldrich) was also added. To achieve iron-replete conditions, 16.2 mg/l FeCl3 (Sigma-Aldrich) was added to the complete M63. For comparison of *E. coli* and *P. aeruginosa* metabolites, the model *P. aeruginosa* strain PAO1 was used (82). In these experiments, UTI89 and PAO1 were grown in complete M63 supplemented with 1% (w/v) casamino acids (BD Biosciences). For *E. coli* siderophore-dependent growth assays, the iron-limited and nutrient-rich yeast extract-casamino acids (YESCA) medium was used (1% casamino acids and 0.12% yeast extract; BD Biosciences). All cultures were grown at 37 °C under shaking conditions for approximately 18 hours. Other model *E. coli* strains (MG1655 [ref. 83], CFT073 [ref. 84], NU14 [ref. 85], and 83972 [ref. 64]) as well as clinical isolates of UPEC, *K. pneumoniae*, and *C. diversus* were grown in the same manner.

**Metabolic analysis.** For metabolomics experiments, 5 biological replicate cultures were cultivated from 5 distinct single colonies under iron-restricted conditions, as described above. Cultures were centrifuged at 7,142 g for 10 minutes to pellet the cells. The spent supernatants were collected and filtered through 0.22-μm filters (EMD Millipore). A quality control sample was generated by combining an equal volume of each sample. The samples were analyzed in technical triplicates and in a randomized order on a Shimadzu Prominance UFLC-coupled AB Sciex 4000 QTRap mass spectrometer with a Turbo V electrospray ionization (ESI) source. The LC separation was performed on an Ascentis Express phenyl-hexyl column (100 × 2.1 mm, 2.7 μm; Sigma-Aldrich) with a 32-minute linear gradient from 2% A (HPLC-grade water plus 0.1% [v/v] formic acid; Sigma-Aldrich) to 98% B (90% acetonitrile plus 0.1% [v/v] formic acid; EMD Millipore) at 0.35 ml/min. The MS was operated in positive ion–enhanced MS mode, scanning from 50 to 1,200 m/z. The quality control sample was injected first to precondition the column and every 10 samples thereafter to assess instrument stability.

MarkerView, version 1.2.0 (Sciex) was used for the generation of an aligned peak list and statistical analysis. The triplicate runs were averaged, and the data were Pareto scaled for supervised PCA-DA with 2 groups: (a) UTI89 and *AbytS* plus salicylate; and (b) *AbytS*. Candidate molecular features were identified on the PCA-DA loading plots and then verified by visual inspection of the LC-MS data.

**Metabolite detection and quantification in bacterial culture.** Escherichelin, Dha, pyochelin, and Ybt were detected in culture supernatants and cellular extracts from UTI89, PAO1, and clinical Enterobacteriaceae strains by a multiple reaction monitoring (MRM) method on the Shimadzu Prominance UFLC-coupled AB Sciex QTRap 4000 with MS/MS transitions for escherichelin (307.0/219.9), Dha (224.0/178.0), pyochelin (325.0/190.0), apo-Ybt (482.2/295.1), and Fe-Ybt (535.6/349.5) and a collision energy of 37 V. The above column, mobile phases, and flow rate were used with the following LC gradients: solvent B was held constant at 5% for 2 minutes, increased to 56% over 8 minutes, and then increased to 98% over 2 minutes, all with a flow rate of 0.4 ml/min.

Stable isotope dilution was used to quantify escherichelin levels. A 13C6-escherichelin internal standard (IS) was generated by culturing UTI89 *AbytS* in complete M63 minimal medium supplemented with 50 μM 13C6-salicylate. For quantification of extracellular escherichelin, strains were grown under iron-restricted conditions, as described above, and 1 ml of culture supernatant was spiked with 25 μl 13C6-escherichelin IS and 6 μl FeCl3. The samples were incubated at room temperature for 10 minutes, centrifuged at 15,996 g for 3 minutes, and excised on C18 solid-phase extraction 96-well plates (United Chemical Technologies) with 80% methanol (Sigma-Aldrich). Samples were then analyzed by the escherichelin-specific MRM, and escherichelin levels were assessed by taking the peak area ratio of the native isotope escherichelin to the IS 13C6-escherichelin. Viable CFU were measured to ensure that all strains grew to an equal final density.

Intracellular escherichelin was extracted from 50-ml cultures grown under iron-restricted conditions, as described above. The cultures were centrifuged at 7,412 g for 10 minutes to pellet the cells. The supernatant was discarded, and the cells were washed 3 times with 25 ml PBS (Sigma-Aldrich). The pellets were then resuspended in 1.2 ml ice-cold 80% methanol, briefly vortexed, and incubated on dry ice for 30 minutes. After thawing, the samples were centrifuged at 15,996 g for 10 minutes to pellet the cell debris. The supernatant was retained, and 800 μl acetonitrile was added. The mixture was incubated on ice for 15 minutes and then centrifuged at 15,996 g for 2 minutes to remove any remaining debris. 13C6-escherichelin (10 μl) IS was added to 250 μl of each sample, and escherichelin levels were quantified by LC-MRM, as described above.

**MS for escherichelin identification.** A Shimadzu UFLC-coupled Agilent 6550 Q-TOF mass spectrometer with a Dual AJS ESI ion source was used to determine the accurate mass, and therefore molecular for-
mula, of escherichelin in UTI189 supernatant that had been prepared as described above. LC separation was performed on an Ascentis Express phenyl-hexyl column (100 × 2.1 mm, 2.7 μM) with the same mobile phases as described above. The gradient was as follows: solvent B was held constant at 2% for 1 minute, then increased to 50% over 6 minutes, then increased to 98% over 2 minutes, and then held constant at 98% for 1 minute, all with a flow rate of 0.6 ml/min. The MS was operated in positive-ion mode, scanning a mass range from 75 to 1,100 Da. The calculated mass for HPTT-COOH is [M+H]+ (C93H8N10O7S2) = 307.0206, and 307.0216 was found.

MS/MS was performed on the Shimadzu Prominence UFLC-coupled AB Sciex QTrap 4000. Supernatant from UTI189 grown under iron-restricted conditions was prepared as described above for detection and quantification. Data were collected in the positive-ion, enhanced product ion (EPI) mode, with the parent ion m/z set at 307 and the collision energy set at 37 V.

Escherichelin purification. Escherichelin was purified from UTI189Δirp1/entB::kan grown under iron-restricted conditions, as described above, with the addition of 20 μM 2,2′-dipyridyl (Sigma-Aldrich) and 0.1% casamino acids (w/v) to the complete M63. Culture supernatant was applied to a methanol-conditioned C18 silica column (Sigma-Aldrich). The column was washed with water and then eluted with 80% methanol. The eluate was dried down with a lyophilizer (Labconco), resuspended in 20% methanol, and further purified on a Bio-Rad BioLogic DuoFlow 10 system equipped with a QuadTec UV-Vis detector and a Kromasil Eternity-5-phenylhexyl column (Akzo-Nobel). The column was run at 0.7 ml/min with water plus 0.1% formic acid (solvent A) and acetonitrile plus 0.1% formic acid (solvent B) and the following gradient: solvent B held steady at 0% for 2.5 ml, then increased to 30% over 2 ml, then increased to 70% over 20 ml, and finally increased to 100% over 2 ml. Fractions containing escherichelin were identified via UV-Vis detection at 318 nm, pooled together, and dried down by lyophilization. Purification was confirmed by LC-MS.

NMR. Purified escherichelin was resuspended in DCDCl. One-dimensional H and two-dimensional H COSY spectra were collected at 20°C on a Varian Unity Inova-500 MHz instrument.

UV-Vis spectroscopy. Purified escherichelin was reconstituted to 1 mM in 50% ethanol (Sigma-Aldrich). For experiments to monitor metal binding, escherichelin was diluted to 100 μM in water and combined with an equimolar amount of FeCl3, or CuSO4 (Sigma-Aldrich). The samples were incubated at room temperature for 2 hours, and then a wavelength scan was collected from 200 to 800 nm on a UV-Vis spectrophotometer (Beckman Coulter; DU800). For each escherichelin-plus-metal scan, the instrument was blanked with a metal-only control. For escherichelin alone, the instrument was blanked with 5% ethanol.

E. coli siderophore-dependent growth assay. The assay was adapted from a previously published Fe(III)-Ybt-dependent growth assay, and apo-Ybt was purified as previously described (16, 19). UTI189 was grown overnight in LB broth at 37°C under shaking conditions and then washed 3 times by centrifugation and resuspension in a volume of PBS equal to the culture volume. The washed bacteria were inoculated 1:100 into YESCA or YESCA supplemented with 0.5 mM ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid) (EDDHA; Complete Green Company). Prior to their addition, apo-Ybt and escherichelin were preincubated for 2 hours with an equimolar amount of FeCl3, to allow for complex formation. The Fe(III) complexes were added at a final concentration of 1 μM, and the cultures were grown at 37°C under shaking conditions. Bacterial growth was monitored over time by measuring the OD at 600 nm.

Escherichelin activity assay on P. aeruginosa. PW5011 (pvdA-E02::ISlacZ/hah) (55, 56) was grown in LB broth overnight at 37°C under shaking conditions and then back-diluted into succinate medium (25) at 106 CFU/ml. The growth experiments were performed in a final volume of 100 μl in 96-well plates. Escherichelin, solubilized in ethanol, was added to the cultures at increasing concentrations. The final ethanol (vehicle) concentration in all cultures was 5% (v/v). The cultures were grown at 37°C under shaking conditions for 20 hours, at which point they were serially diluted in PBS and plated on LB agar for CFU quantification.

Theoretical calculations. The theoretical calculations to identify thermodynamically stable escherichelin-metal complexes were performed with the DFT method as previously described (15, 19). This DFT approach was previously used to model a structure of Fe(III)-Ybt that matched the published crystal structure (50). The simulated Fe(III)-escherichelin complexes agree with a previously described model of Fe(III)-escherichelin bound to FptA (25).

Escherichelin detection in urine. The clinical uncomplicated cystitis isolates were grown under iron-restricted conditions, and their supernatants were analyzed for Ybt by LC-MRM. Seventeen of the nineteen selected isolates were Ybt producers. The 2 Ybt nonproducers were used as controls. Two additional strains (Staphylococcus saprophyticus and Enterococcus sp.) were selected from the cohort to serve as non-E. coli, Ybt-negative controls. After determining whether the causative strain was a Ybt producer, the corresponding urine samples were thawed on ice and then centrifuged at 15,996 g for 3 minutes to remove debris. To each 500 μl of urine, 12.5 μl 13C6-escherichelin IS and 3 μl 0.5 M FeCl3 were added. The samples were incubated at room temperature for 15 minutes and then centrifuged at 15,996 g for 5 minutes. The supernatant was then extracted on 96-well C18 solid-phase extraction plates with 80% methanol. Escherichelin was detected by LC-MRM using the method described above. Escherichelin was detected in the bacterial interference trial specimens using the same extraction and LC-MRM methods.

Statistics. GraphPad Prism 4 (GraphPad Software) was used to perform statistical analysis and generate graphs for this study. All P values were determined by unpaired, 2-tailed t test or 1-way ANOVA with Dunnett’s multiple comparisons test. A P value of 0.05 or less was considered statistically significant.

Study approval. The urine and bacterial specimens from patients with uncomplicated cystitis were collected with the approval of the Human Subjects Review Committee of the University of Washington. All participants provided written informed consent for the sample collection and following analysis, prior to inclusion in the study. Study design, inclusion and exclusion criteria, and sample preparation were previously described (14, 86). β-Hemolytic E. coli isolates from urine cultures with 105 or more CFU/ml were selected from the cohort for analysis.

The HU2117 experimental human colonization study protocol was approved by the FDA (investigational new drug [IND] 140077), the IRB of Baylor College of Medicine, and the Houston VA Research and Development committee and registered as a clinical trial (ClinicalTrials.gov identifier: NCT00584996). All participants provided written informed consent prior to inclusion in the study. The study design, inclusion and exclusion criteria, and specimen preparation were previously reported (27).
Author contributions
SIO and JPH conceived and designed the experiments. SIO, DEG, and DAD performed the experiments. AES and WBT collected human specimens. SIO, DEG, DAD, and JPH analyzed the data. SIO and JPH wrote the manuscript.

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