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PNAS 2005;102;571-576; originally published online Dec 14, 2004; doi:10.1073/pnas.0408463102

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Notes:
In vitro characterization of IroB, a pathogen-associated C-glycosyltransferase

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Contributed by Christopher T. Walsh, November 15, 2004

Pathogenic strains of Escherichia coli and Salmonella enterica modify the tricaticholic siderophore enterobactin (Ent) by glycosylation of three aryl carbon atoms, a process controlled by the iroA locus [Hantke, K., Nicholson, G., Rabsch, W. & Winkelmann, G. (2003) Proc. Natl. Acad. Sci. USA 100, 3677–3682]. Here, we report the purification of the IroB protein and its characterization as the Ent C-glycosyltransferase. IroB transfers glucosyl groups from uridine-5’-diphosphoglucose to C5 of one, two, or three of the 2,3-dihydroxybenzoyl units of Ent to yield monoglucoyl-Ent (MGE), diglucoyl-Ent (DGE), and triglucoyl-Ent (TGE). DGE, also known as salmochelin S4, and macrolactone-opened derivatives have been isolated from the culture broths of S. enterica and uropathogenic E. coli [Bister, B., Bischoff, D., Nicholson, G. J., Valdebenito, M., Schneider, K., Winkelmann, G., Hantke, K. & Sussmuth, R. D. (2004) Biometals 17, 471–481], but MGE and TGE have not been reported previously. IroB has a kcat of ~10 min⁻¹ for the first C-glycosylation and is distributive, with sequential conversion and buildup of MGE and then DGE. The C5 to C1 regioselectivity of the 2,3-dihydroxybenzoyl-glucose linkage at all three rings of TGE suggests a C5 carbanion, para to the C2 phenolate oxygen, as the carbon nucleophile in this novel enzymatic C-glycosylation.

Bacterial growth in mammalian hosts is often limited by the availability of iron. FeIII is both insoluble and efficiently sequestered by mammalian proteins, such as hemoglobin, ferritin, transferrin, and lactoferrin, leaving a remarkably low available iron concentration of 10⁻²⁴ M (1, 2). Under iron-limiting conditions, bacteria up-regulate the transcription of a specific set of genes that encode the assembly, export, and import of iron-chelating small molecules, or siderophores (3).

Enterobactin (Ent) (Fig. 1), a prototypic siderophore of enteric bacteria (e.g., Escherichia coli and Salmonella enterica) (1), is a trimeric macroalcohol of N-(2,3-dihydroxybenzoyl)serine (DHB-Ser). The three catecholic rings form a hexadentate ligand for coordinating FeIII with a Kₚ estimated at 10⁻⁵² M (4). Ent is biosynthesized from DHB and Ser by using nonribosomal peptide synthetase assembly logic (5–12). The DHB-Ser monomers are tethered through a phosphopanteineyl arm to the peptidyl carrier protein domain of EntF (6) and undergo cyclotrimerization to generate the 12-membered macroalcohol scaffold (9). Once Ent is secreted and complexes FeIII, the FeIII-Ent complex is internalized by specific receptors (13–15). After transport through the inner membrane, FeIII is released in the cytoplasm upon enzyme-catalyzed hydrolysis of the macroalcohol, yielding linear DHB-Ser trimers, dimers, and monomers (16).

A intriguing set of Ent structural variants known as salmochelins were recently isolated from S. enterica and uropathogenic E. coli (17, 18). The salmochelins consist of Ent that has been glucosylated at one or two of the 2,3-dihydroxybenzoyl (DHB) rings. The macroalcohol is intact in salmochelin S4 but is hydrolyzed in salmochelin S2. Salmochelin S1 is a monoglucoylated linear DHB-Ser dimer, whereas salmochelin SX is a monoglucoylated DHB-Ser monomer (18) (Fig. 1). It has been proposed that Ent glucosylation may prevent sequestration of the siderophore by serum albumin (19) and siderocalin, a neutrophil lipocalin (20), leading to increased virulence of the salmochelin-producing bacteria.

Remarkably, the salmochelins are C-glycosides in which C1’ of glucose is bonded to C5 of the DHB ring (18). The 9.6 kb iroA gene cluster encodes five genes that collaborate with those of the Ent gene cluster to biosynthesize salmochelins (17). The DNA sequence of the iroA operon is known (21, 22), and its iroB gene is predicted to be a glucosyltransferase (17). Here, we report the overexpression, purification, and characterization of IroB. We show that IroB is indeed a C-glycosyltransferase that transforms Ent and uridine-5’-diphosphoglucose (UDP-Glc) into monoglucoyl-Ent (MGE), diglucoyl-Ent (DGE), and triglucoyl-Ent (TGE). Characterization of IroB therefore represents an unusual opportunity to examine a C–C bond forming glycosylation catalysis.

Materials and Methods

Materials, General Methods, and Instrumentation. E. coli TOP10 and BL21(DE3) chemically competent cells were purchased from Invitrogen. Plasmids pET-24b and pET-28b were purchased from Novagen. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Herculase DNA polymerase was purchased from Stratagene. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. H-Ser(tBu)-OMe hydrochloric acid and O-benzotriazole-N,N,N’,N’-tetramethyluronium hexafluorophosphate were purchased from Advanced ChemTech. All other chemicals were purchased from Sigma–Aldrich. All moisture- or oxygen-sensitive reactions were performed under a nitrogen atmosphere. Analytical and preparative RP-HPLC were performed on a Beckman System Gold (Beckman Coulter) by using a Vydec (Hesperia, CA) small pore C18 (4.6 × 220 mm) column at 4 mL/min (analytical) and a Vydec C18 (22 × 250 mm) column at 10 mL/min (preparative). Liquid chromatography (LC)-MS analysis was carried out on a Shimadzu LCMS-QP8000a by using a Higgin’s Analytical (Mountain View, CA) Sprite Targa C18 column (2.1 × 20 mm) at 0.8 mL/min. MALDI-TOF data were collected on a Applied Biosystems Voyager mass spectrometer. NMR spectra were recorded on Varian 200, 400, or 500 MHz Fourier Transform NMR spectrometers.

Cloning, Expression, and Purification of IroB. The iroB gene was amplified from E. coli CFT073 genomic DNA by using the

Abbreviations: TCEP, Tris(2-carboxyethyl)phosphine; LC-MS, liquid chromatography-MS; Ent, enterobactin; MGE, monoglucoyl-Ent; DGE, diglucoyl-Ent; TGE, triglucoyl-Ent; UDP-Glc, uridine-5’-diphosphoglucose; DHB-Ser, N-(2,3-dihydroxybenzoyl)serine; DHB, 2,3-dihydroxybenzoyl; Gtf, glycosyltransferase.

See Commentary on page S19.

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Enterobactin (Ent)  
Diglucosyl-enterobactin (DGE)  
(Salmochelin S4)  
Monoglucosyl-enterobactin (MGE)  

Diglucosyl-(DHB-Ser)_3  
(Salmochelin S2)  
Triglucosyl-enterobactin (TGE)  
(Monoglucosyl-(DHB-Ser)_2  
(Salmochelin S1)  
Monoglucosyl-DHB-Ser  
(Salmochelin SX)  

**Fig. 1.** Structures of Ent, DHB-Ser, and C-glucosylated derivatives.

forward primer 5′-GGAATTCATATGCGTATTTGTTTTTAGGCCCGC-3′ and the reverse primers 5′-GATCGAATTCTCGGCCCCTTTCTGTACCATGGATCAGG-3′ (pET-24b) or 5′-GATCGAATTCTACCCCTTTCTGTACCATGGATCAGG-3′ (pET-28b). The forward primer introduced a NdeI restriction site, and the reverse primers introduced EcoRI restriction sites (underlined above). PCR reactions were performed with Herculase DNA polymerase. The amplified gene sequences were digested with NdeI and EcoRI, then ligated into the above expression vectors and transformed into *E. coli* TOP10 cells. The identities of the resulting pET-24b-*iroB* and pET-28b-*iroB* constructs were confirmed by DNA sequencing. Expression constructs were transformed into *E. coli* BL21(DE3) cells, grown to saturation in 2× YT medium (1.6% tryptone/1% yeast extract/0.5% NaCl) supplemented with kanamycin (50 μg/ml) at 37°C and diluted 1:100 into 2× YT medium supplemented with kanamycin (30 μg/ml). The expression of N- and C-terminal His<sub>6</sub> fusion proteins was induced at OD<sub>600</sub> 0.5–0.6 with 0.5 mM DTT to saturation in 2/1 H<sub>2</sub>O/100 mM MgCl<sub>2</sub> or 5/1 H<sub>2</sub>O/1100 mM NaCl, then was subjected to hydrogenation using 10% Pd on carbon protected DHB-Ser (492 mg, 1.0 mmol) in methanol (3.0 ml), and the protected DHB-Ser was eluted with 4:1 hexanes/EtOAc; 1H NMR (200 MHz, CDCl<sub>3</sub>): δ 7.21 (d, J = 9.0 Hz, 3H), 6.94 (d, J = 8.0 Hz, 3H), 6.69 (d, J = 8.0 Hz, 3H), 5.01 (d, J = 5.0 Hz, 3H), 4.62 (t, J = 4.5 Hz, 6H); 13C NMR (75 MHz, CDCl<sub>3</sub>): δ 169.52, 148.26, 146.02, 118.96, 118.57, 115.64, 114.23, 101.3 ppm. The protected DHB-Ser (492 mg, 1.0 mmol) in methanol (3.0 ml) then was subjected to hydrogenation using 10% Pd on carbon (300 mg) and 1 atmosphere of H<sub>2</sub> (1 atm = 101.3 kPa). The reaction mixture was filtered and concentrated. Trifluoroacetic acid (TFA; 3 ml) was added to the residue, and the solution was stirred at 25°C for 3 h. The reaction mixture was loaded directly to a silica gel column, and the protected DHB-Ser was eluted with 4:1 hexanes/EtOAc to afford 1,250 mg (77%) of product.

**Synthesis of Ent.** The synthesis of Ent followed the method developed by Gutierrez and coworkers (23). The intermediates required for the synthesis, 2,3-dibenzyloxybenzyl chloride and 2,2-dibutyl-1,3,2-dioxastannolane, were prepared as described in refs. 24 and 25. 1H NMR (500 MHz, CD<sub>3</sub>OD): δ 7.21 (d, J = 9.0 Hz, 3H), 6.94 (d, J = 8.0 Hz, 3H), 6.69 (d, J = 8.0 Hz, 3H), 5.01 (d, J = 5.0 Hz, 3H), 4.62 (t, J = 4.5 Hz, 6H); 13C NMR (75 MHz, CD<sub>3</sub>OD): δ 169.52, 148.26, 146.02, 118.96, 118.57, 115.64, 114.23, 101.3 ppm. The protected DHB-Ser (492 mg, 1.0 mmol) in methanol (3.0 ml) then was subjected to hydrogenation using 10% Pd on carbon (300 mg) and 1 atmosphere of H<sub>2</sub> (1 atm = 101.3 kPa). The reaction mixture was filtered and concentrated. Trifluoroacetic acid (TFA; 3 ml) was added to the residue, and the solution was stirred at 25°C for 3 h. The reaction mixture was loaded directly to a silica gel column, and the protected DHB-Ser was eluted with 4:1 hexanes/EtOAc to afford 1,250 mg (77%) of product.

**Synthesis of DHB-Ser Methyl Ester.** We dissolved 2,3-dibenzyloxybenzyl acid (1,100 mg, 3.29 mmol), H-Ser(tBu)-OMe hydrochloride acid (700 mg, 3.30 mmol), O-benzotriazolone-N,N,N',N′-tetramethyluronium hexafluorophosphate (1,878 mg, 4.95 mmol), and N,N-diisopropylethylamine (1,150 μl, 6.6 mmol) in dichloromethane (10 ml). The reaction was stirred at 25°C for 3 h. The reaction mixture was loaded directly to a silica gel column, and the protected DHB-Ser was eluted with 4:1 hexanes/EtOAc to afford 1,250 mg (77%) of product. The product was flash-frozen in liquid N<sub>2</sub> and stored at −80°C. The concentrations of purified IroB N- and C-His<sub>6</sub> were determined spectrophotometrically at 280 nm by using calculated extinction coefficients of 56,000 M<sup>−1</sup>·cm<sup>−1</sup> for both proteins.
Enzymatic Preparation of DGE and TGE. A solution containing 75 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM Tris(2-carboxyethyl)phosphine (TCEP), 3 mM UDP-Glc, 500 µM Ent, and 1 µM IroB N-His₆ was incubated at 25°C, and reaction progress was monitored by LC-MS (0–40% CH₂CN in 0.1% formic acid/H₂O over 8 min, monitored at 220 nm) to follow the formation and subsequent disappearance of MGE (calculated [M+H]+ = 256.2, observed [M+H]+ = 256.1, calculated 256.1, observed 256.2).

Time Course and Kinetic Characterization of IroB Activity with Cosubstrates UDP-Glc and Ent. Reaction mixtures contained UDP-Glc and Ent in 75 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2.5 mM TCEP, 150 µM DHB-Ser methyl ester, 600 µM UDP-Glc, and 5 µM IroB C-His₆. Reactions were initiated by the addition of IroB and terminated by the addition of 3 volumes of MeOH and analyzed after 10 min, 50 min, and 24 h as described above.

Analysis of Ent (DHB) C5 Carbanion Formation by Deuterium Exchange. Ent (5.0 mg) was dissolved in DMSO-d₆ (240 µl) to give a 31 mM stock solution. All other stock solutions (1 M Mes, pH 6.5; 1 M Heps, pH 7.5; 1 M Heps, pH 8.0; 40 mM UDP; 1 M MgCl₂; and 100 mM TCEP) were made in D₂O. Deuterium exchange reactions were carried out at pH 6.5, 7.5, and 8.0 with 3.1 mM Ent, 75 mM Mes or Heps, 600 µM UDP, 5 mM MgCl₂, and 2.5 mM TCEP, with or without 1 µM IroB N-His₆, in D₂O with a total volume of 600 µl. The reaction was monitored by ¹H NMR and MALDI-TOF MS at different time points.

Results

Overexpression and Purification of IroB. The five genes of the iroA cluster, iroB, iroC, iroD, iroE, and iroN, have been shown previously to be necessary and sufficient for (i) glucosylation of Ent, (ii) export of apo glucosyl-Ent, and (iii) import of holo (Fe³⁺-bound) glucosyl-Ent (17, 18). The protein encoded by the iroB gene is homologous to glycosyltransferases (Gtfs) from the naganalycin (27) and erythromycin (28) gene clusters. To characterize the enzymatic C-glycosylation of Ent, we isolated genomic DNA from E. coli CFT073, a uropathogen containing the iroA gene cluster (21). By using this genomic DNA as a template, we subcloned the iroB gene into pET-24b and pET-28b expression vectors, from which IroB was heterologously overexpressed in E. coli BL21(DE3) cells as C- and N-terminal His₆ fusions, respectively. IroB fusion proteins (42 kDa) were over-
produced (26 mg/liter) and purified by immobilized nickel affinity chromatography to >90% homogeneity (Fig. 2A).

**IroB Catalyzes the Monoglucosylation of Ent.** When IroB was incubated with Ent and UDP-Glc for 5 min at 25°C, RP-HPLC analysis of the reaction mixture revealed a new peak (Fig. 2B and C). The identity of the corresponding compound was consistent with MGE by LC-MS ([M+H]+ m/z: calculated 832.2, observed 832.5).

**IroB Catalyzes the Successive Monoglucosylation, Diglucosylation, and Triglucosylation of Ent on C5 of DHB.** The isolation of DGE from _S. enterica_ harboring the _iroA_ gene cluster (18) suggested the possibility that IroB could catalyze multiple glucosylations of the same Ent substrate. To analyze this possibility, we followed the course of IroB-catalyzed glucosylation in the presence of UDP-Glc and Ent over a longer duration (80 min) than our initial experiment. RP-HPLC analysis of the reaction products (Fig. 3B) revealed two new products. After LC-MS analysis, we assigned their identities as DGE ([M+H]+ m/z: calculated 994.3, observed 994.0) and TGE ([M+H]+ m/z: calculated 1156.3, observed 1157.0).

To confirm the C-glycosidic nature of the glucosyl linkage to Ent in the DGE and TGE reaction products, a large-scale IroB-catalyzed glucosylation of Ent was performed, yielding ~20 mg of DGE and ~30 mg of TGE. Three lines of evidence from our subsequent NMR analysis of DGE and TGE strongly suggest a β-configured C-glycosidic bond between C1' of glucose and C5 of DHB (Fig. 3C).

First, the aromatic proton region of the 1H-NMR spectrum of Ent (Fig. 3D) consists of three clearly defined peaks: two doublet peaks corresponding to the C4 and C6 protons and a triplet corresponding to the C5 proton. As expected, this portion of the DGE 1H-NMR spectrum (Fig. 3D) resembles a 2:1 mixture of the TGE and Ent spectra. Taken together, the 1H NMR data suggest that DHB C5 is the site of glucosyl attachment to the catechol ring.

Second, the heteronuclear multiple bond correlation spectrum of TGE (Fig. 3E) confirms several important C–H adjacencies: (i) a 3J coupling between DHB C5 and glucose H1', (ii) 3J couplings between DHB C4/C6 and glucose H1', (iii) a 3J coupling between DHB C5 and glucose H2', and (iv) 3J couplings between glucose C1' and H4/H6 of DHB. Collectively, the
heteronuclear multiple bond correlation data indicate the presence of a C–C bond between C1 of glucose and C5 of DHB. Third, the coupling constant between the C1′ and C2″ protons (J = 10 Hz) implies the β-conformation of the glycosidic linkage. Our data are also in agreement with the recently reported NMR characterization of DGE (salmochelin S4) (18).

**Kinetic Analysis of IroB Activity with Cofactors UDP-Glc and Ent.** We measured the basic kinetic parameters for IroB-catalyzed monoglucosylation of Ent. To determine $k_{cat}$ and $K_m$ for IroB, the concentrations of the donor and acceptor cofactors, UDP-Glc and Ent, were varied under initial velocity conditions in which no conversion of MGE to DGE was detected (see Fig. 5, which is published as supporting information on the PNAS website). At $100 \mu M$ Ent, the $K_m$ for UDP-Glc is $13.3 \pm 2.1 \mu M$, and $k_{cat}$ is $9.7 \pm 1.6 \text{ min}^{-1}$. With the UDP-Glc concentration held constant at $100 \mu M$, the $K_m$ for Ent is $5.2 \pm 1.6 \mu M$, with a comparable $k_{cat}$ of $11.2 \pm 1.9 \text{ min}^{-1}$. These results demonstrate that Ent is a robust substrate for glucosylation by IroB with UDP-Glc as the glycosyl donor.

**DHB-Ser Is Not a Substrate for IroB.** In addition to DGE, Hantke and coworkers (18) isolated Glo-DHB-Ser (salmochelin SX), Glc-(DHB-Ser)$_2$ (salmochelin S1), and Glc$_2$-(DHB-Ser)$_3$ (salmochelin S2) from iroA-harboring strains of *S. enterica.* The presence of these C-glycodies in culture broth suggested that linear DHB-Ser derivatives might be substrates for IroB. To test this hypothesis, we synthesized DHB-Ser methyl ester and incubated it with IroB and UDP-Glc under conditions in which Ent is efficiently glucosylated. RP-HPLC analysis showed no detectable glycosylation (data not shown). Because our RP-HPLC analysis is sufficiently sensitive to detect conversion of 0.1% of the substrate under the assay conditions, we estimate that IroB glucosylates Ent at least $2 \times 10^4$-fold more efficiently than DHB-Ser methyl ester. This result suggests that DHB-Ser is not likely to be a physiological substrate of IroB.

**The C5 Carbocation Does Not Form Spontaneously.** To investigate the mechanistic requirements for formation of the presumed C5 carbocation on each DHB moiety, we incubated Ent in D$_2$O under assay conditions in which UDP-Glc was replaced with UDP. In the absence of the electrophilic UDP-Glc C1′, deuterium exchange at C5 of the DHB ring would suggest the formation of a transient carbocation at this position. However, both in the presence and absence of IroB, NMR and MALDI-MS analysis of the reaction products demonstrated the absence of deuterium incorporation at C5 (data not shown). We conclude that the presumed DHB C5 carbocation does not form spontaneously under these conditions (e.g., through deprotonation of the C2 phenolic hydroxyl group). Although deuterium exchange at C5 was not observed in the presence of IroB, this result does not exclude the possibility of IroB-catalyzed C5 carbocation formation in the presence of the glycosyl moiety of the UDP-Glc donor cosubstrate.

**Discussion**

Bacteria make a wide variety of nonribosomal peptide natural products that are subsequently tailored by dedicated Gtfs as the final step in their biosynthesis (29, 30). The antibiotics vancomycin (31–33) and ramoplanin (34) as well as the antitumor drug bleomycin (35) are examples of nonribosomal peptides in which the peptide scaffold is matured by specific antibiotic Gtfs (29). The siderophore Ent is made analogously by nonribosomal peptide synthetase logic (11), on a two-module, three-protein assembly line. The first module, comprising EntE and EntB, generates 2,3-DHB-S-pantetheinyl-EntB, whereas the second module, EntF, activates L-Ser and condenses the DHB donor to yield DHB-Ser-S-EntF. The unanticipated feature of EntF its ability to elongate DHB-Ser to the linear trimmer, still covalently tethered to EntF, and then catalyze its release by intramolecular cyclization to generate Ent (5–10, 12).

The IroB protein now can be classified as a dedicated tailoring Gtf for Ent. The iroA-encoded Ent glucosylation activity imparts a functional advantage on the siderophore that appears to arise from (i) reduction of the hydrophobic character of the DHB groups to attenuate binding to serum albumin or other host proteins (19), and (ii) installation of a steric impediment to recognition by siderocalin, a neutrophil-derived antibacterial lipocalin (20). Sequestration by serum albumin and siderocalin decreases the concentration of both apo- and Fe$^{III}$-loaded Ent in the serum. Glycosylation on one or more of the DHB rings inhibits this sequestration, increases the availability of the siderophore in the serum, and leads to increased virulence of the salmochelin-producing bacteria.

As anticipated, we found that UDP-Glc is the glycosyl donor and Ent is a robust substrate for IroB-catalyzed glucosylation to generate MGE, DGE, and TGE. The glucosyl transferase clearly acts distributively, because MGE builds up and is then converted to DGE, which, after some accumulation, then serves as substrate for the third C-glucosylation to produce the fully glucosylated TGE. The structure of the novel siderophore TGE was established both by MS and by the disappearance of the HS proton signal on the DHB rings in its $^1$H NMR spectrum, in agreement with the connectivity recently assigned (18) in the DGE (salmochelin S4) isolated from *S. enterica* growth medium. The catalytic efficiency ($k_{cat}/K_m$) of the second and third glucosylation steps catalyzed by IroB has yet to be elucidated in this complex A→B→C→D kinetic sequence, but qualitatively, the third C-glucosylation appears slower than the first two, allowing sufficient DGE to be isolated for NMR characterization.

This transient accumulation of DGE may be related to the observation that DGE, but not MGE or TGE, was detected in growth medium from *S. enterica* (18). It is possible that DGE forms rapidly from MGE and is exported from the bacterial cell as quickly as it is produced, precluding a third glucosylation. Alternatively, MGE and TGE might not be efficient substrates for export, leaving them to suffer degradation in the cytoplasm. Linear forms of monoglucosylated and diglucosylated DHB-Ser (salmochelins SX, S1, and S2) also were isolated from *S. enterica* growth medium (18). DHB-Ser methyl ester was not a substrate for C-glucosylation, however, supporting the notion that intact Ent, rather than a linear DHB-Ser fragment, is the relevant substrate for IroB in *vivo*. This hypothesis is consistent with our previous findings that the Ent synthetase module EntF is a robust elongation and cyclotrimerization catalyst and does not leak significant quantities of linear DHB-Ser intermediates (6, 9). Studies with strains deficient in one or more genes of the *iroA* cluster would shed more light on the steady-state distribution of glucosylated Ent derivatives and their relative rates of assembly and breakdown.

Perhaps the most remarkable attribute of IroB is that it catalyzes formation of a C–C bond during Ent glucosylation, unlike the other antibiotic Gtfs noted above, which glycosylate oxygen atoms. Very few examples of C-glycoside-bearing natural products have been described; among these are the myasin-family maize flavones (36) and the polyketides granaticin (37), simocyclinone D8 (38), and the angucycline antibiotics (27, 39–41). All of the C-Gtfs must generate a carbon nucleophile to attack C1′ of the glycosyl moiety of the donor cosubstrate NDP-hexose (Fig. 2B). During elaboration of the natural products listed above and in IroB catalysis, the acceptor cosubstrate is an electron-rich phenol ring. In the angucyclines and granaticin, the observation that the glycosylated carbon is *ortho* to the phenol oxygen has raised the question of whether the reaction sequence consists of (i) O-glycosylation followed by a nonenzym
matic O → C glycosyl migration (42) or (ii) direct C-glycosylation.

During IroB catalysis, C5 of the 2,3-DHB ring is the site of glycosylation. This carbon is meta to the C3–OH and para to the C2–OH. Although mechanistic analysis remains to be performed, if the C2–OH is deprotonated in the IroB active site, the resulting phenolate anion can be partially delocalized to C5 by the para-quinonoid resonance contributor (Fig. 4). The absence of deuterium exchange at C5 in the presence of IroB and UDP suggests that IroB-catalyzed C5 carbocation formation may require the presence of the glucosyl moiety of the UDP-Glc donor cosubstrate, which may serve as a gating mechanism to prevent capture of the C5 nucleophile by an adventitious electrophile.

During C-glycosylation by the enzyme, rather than O-glycosylation followed by rearrangement, is most likely because the latter mechanism would require an intramolecular regioselective multistep glycosyl transfer. The orientation of Ent relative to UDP-Glc in the IroB active site must position the C5-anion for nucleophilic attack on C1 of the glucosyl group. Furthermore, because this reaction sequence can occur up to three times, the IroB substrate-binding pocket must be able to accommodate the additional bulk of the glucosyl moieties of MGE and DGE. In contrast, DHB-Sep monomers are not C-glucosylated, suggesting architectural requirements for the Ent scaffold in the IroB active site.

The ability to use IroB to prepare tens of milligrams of MGE, DGE, and TGE provides substrates that will allow investigation of the three-step kinetics of IroB action on Ent to produce TGE. These substrates also will open the possibility of investigating the catalytic activities of other enzymes from the iroA cluster such as IroD and IroE (17).

Once DGE has undergone secretion and scavenged Fe(III) from the host, the Fe(III)-DGE complex is internalized by the outer membrane transporters Fiu, CepA, and IroN (13–15). The iroA cluster also encodes IroD (17), predicted to be an esterase that cleaves the ester linkages in DGE to release the iron that has been imported to the bacteria. Inhibition of any of the enzymes in the Ent assembly line and the iroA cluster should reduce the virulence and pathogenicity of enteric bacteria.

We thank Roberto Kolter (Harvard Medical School) for providing E. coli CFT073, Paul Straight (Harvard Medical School) for help with genomic DNA preparation, and Hiromi Seike (Harvard University) for help with genomic DNA isolation.

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