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A β-(1,2)-Glycosynthase and an Attempted Selection Method for the Directed Evolution of Glycosynthases

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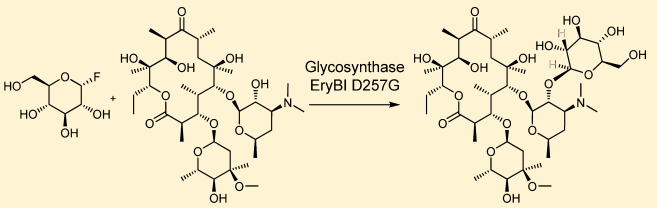
Supporting Information

ABSTRACT: Understanding how enzymes mediate catalysis is a key to their reprogramming for biotechnological applications. The family 3 retaining glycosidase postulated to be involved in erythromycin self-resistance was cloned, recombinantly expressed in *Escherichia coli*, purified, and characterized. Bioinformatics analysis allowed the identification of the acid/base and nucleophile residues, and mutation of these residues resulted in hydrolytically inactive proteins. One mutant was able to synthesize a glycosidic linkage using α-glucosyl fluoride as a donor and macrolide antibiotics as acceptors. This shows an unprecedented application of glycosynthase technology in accomplishing a challenging β-(1,2)-glycosylation of an amino sugar. This work also provides the first biochemical characterization of the EryBI protein and supports its role in the self-resistance mechanism involved in erythromycin biosynthesis. An in vivo selection approach was used in an attempt to spur evolution of the glycosynthase, and the results from the attempted selection method provide insight into the requirements for in vivo directed evolution of glycosynthases.



It is difficult to overestimate the importance of carbohydrates and variously glycosylated molecules in cells: they are mediators of communication, structural elements, energy stores, and the backbone of DNA and RNA. Methods for the construction of the glycosidic linkage are similarly critical in developing molecular approaches to mediating the roles played by carbohydrates in biology or medicine.[1] Glycosynthases are mutant glycosidases developed as tools to facilitate the synthesis of the glycosidic linkage.[2−10] The enzymes are designed through mutation of the catalytic nucleophile in a retaining glycosidase and, in the presence of a glycosyl fluoride with an inverted anomeric stereochemistry, are able to construct the glycosidic linkage through a transglycosylation mechanism as first proposed by Withers and co-workers (Figure 1A).[11] A wide variety of glycosynthases have been developed, but enhancement of glycosynthase activity is challenging.[12] It is highly dependent upon directed evolution approaches and the development of screening or selection methods to identify particularly active mutant enzyme catalysts. Such methods are challenging to devise because of the need to be able to monitor the synthesis of a glycosidic linkage;[12] nevertheless, several complementary methods have been devised, including an endocellulase coupled assay,[13,14] chemical complementation using a yeast three-hybrid system,[15] a pH screen,[16] and an enzyme-linked immunosorbent assay-based approach.[17] As part of a quest to broaden glycosynthase applicability, we sought to develop a glycosynthase capable of utilizing amino sugar acceptors, as part of an approach to the construction of amino sugar-containing glycoconjugates, for selective targeting or solubility enhancement of pharmaceuticals, and as a selection method for the directed evolution of glycosynthases.

The family 3 retaining glycosidase involved in extracellular reactivation of erythromycin was considered as a suitable candidate for the development of a glycosynthase able to form β-(1,2) glycosidic linkages. This was based on the biochemical activity for the homologous glycosidase OleR involved in oleandomycin self-resistance and extracellular reactivation[18] (Figure 1B and Supporting Information for protein alignment). In addition, our study would confirm the role of this gene product, as it has yet to be characterized biochemically, and there remains some conjecture in the literature about its function.[19] This subset of family 3 glycosidases is twice the size of the set of well-characterized β-*N*-acetylglucosaminidases within the same glycosidase family.[20]

EXPERIMENTAL PROCEDURES

Cloning of *eryBI*. Amplification of the *eryBI* gene encoding residues 29−808 of the native protein was accomplished with the AccuPrime (Invitrogen) high-fidelity polymerase. The DNA was blunt-end cloned into a TOPO vector (Invitrogen) and sequenced. The gene was excised by restriction digestion and ligated into a correspondingly digested pET28 vector using NdeI and EcoRI restriction enzymes.

Expression of EryBI. Recombinant EryBI and site-directed mutants thereof were produced with an N-terminal His_6 tag

Received: September 15, 2011
Revised: October 21, 2011
Published: October 28, 2011

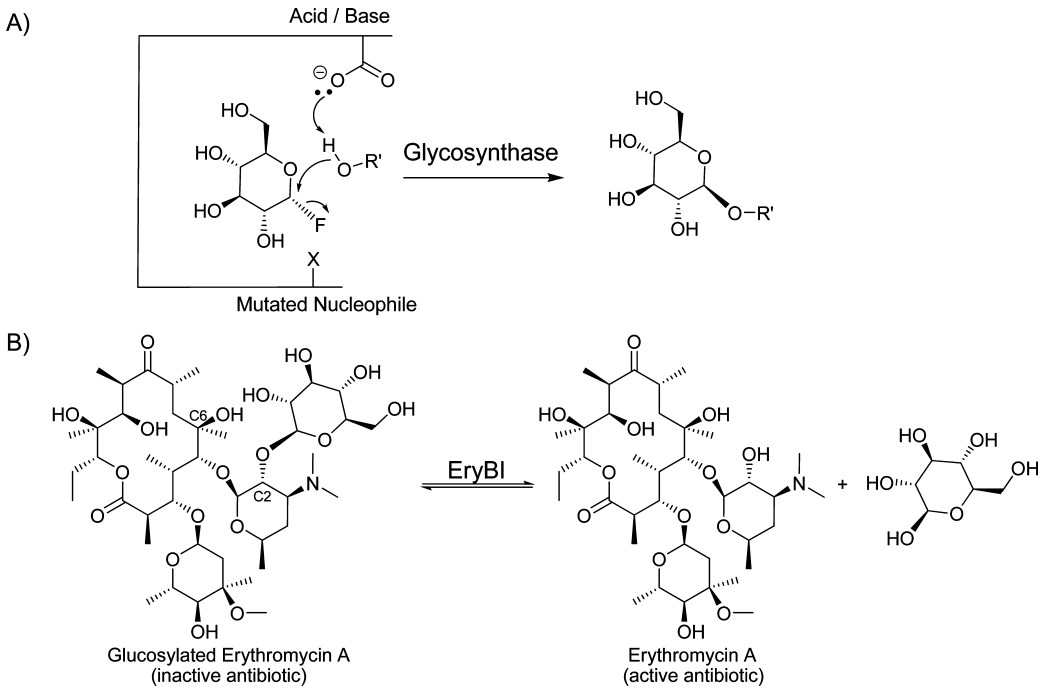


Figure 1. (A) Glycosynthase mechanism, where commonly X is Ala, Ser, or Gly and R′ is the acceptor. (B) Retaining glycosidase mechanism of EryBI. For EryBI D257G, R′ is erythromycin A. The glucosylated erythromycin is inactive as an antibiotic and is reactivated extracellularly by EryBI.

incorporating residues 29−808 of the native protein into a pET28 vector. Expression was performed in *Escherichia coli* BL21 λDE3 cells inducing protein at an OD_{600} of 0.6 and growing at 20 °C for 20 h postinduction according to published procedures for DesR, a homologous glycosidase.[21]

Purification of EryBI and Its Mutants. *E. coli* cells were collected by centrifugation and lysed with Bugbuster reagent (Novagen) according to the manufacturer's instructions. The cell debris was removed by centrifugation, and the supernatant was applied to a His trap column (Amersham Biosciences). The column was coupled to an AKTA purifier 10 instrument (Amersham Biosciences), and the protein eluted via a stepwise gradient of imidazole in PBS (pH 7.6) at 4 °C. Homogeneous samples as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis analysis were washed and concentrated using 10,000 molecular weight cutoff membranes. Protein concentrations were determined via spectrophotometric analysis (ε_{280} = 94435 M^{-1} cm^{-1}, determined using ProtParam using the protein sequence).

Construction of Site-Directed Mutants. Fragments of *eryBI* surrounding either the nucleophile or acid/base residue were subcloned into a TOPO blunt vector, and mutants were generated using a QuikChange mutagenesis strategy (Stratagene). The mutated fragments were determined by restriction analysis, and mutated DNA was recloned back into the appropriately digested pET28 vector containing the wild-type enzyme. Mutants were sequenced. Circular dichroism spectra of the wild-type and mutant proteins were essentially identical (data not shown), providing evidence that the mutants were correctly folded.

Enzyme Kinetics. The activity of EryBI was monitored at 400 nm using a Molecular Devices Spectromax 384 UV spectrometer, monitoring the release of *p*-nitrophenolate (ε_{400} = 5350 M^{-1} cm^{-1}) in PBS (pH 7.6) at 20 °C using 0.25 μM enzyme. Data were fitted using Grafit 5.0 (Erithacus Software). EryBI activity on glucosylated erythromycin was monitored by TLC using normal phase silica plates and an *n*-propanol/ammonia/water (5:1:1) eluant (R_f = 0.8).

Synthesis of Glycosyl Fluorides. Glycosyl fluorides were synthesized using literature procedures by treating peracetylated glycosides with HF-Pyridine, and the acetylated glycosyl fluorides were deprotected using catalytic sodium methoxide in methanol.[22,23] The spectroscopic data and scheme are reported in the Supporting Information.

Glycosynthase Reactions. Glycosynthase reaction mixtures were comprised of mutant glucosidase (4 μM) incubated with acceptor (saturated ∼2 mM solution) and glycosyl fluoride (20−80 mM) in PBS (pH 7.6) at 37 °C. Reactions were monitored by TLC analysis.

Production of Glucosylated Erythromycin. A 20 mL reaction was run with 40 mM αGlcF, 5 mM erythromycin, and 20 μM EryBI D257G in 225 mM sodium phosphate buffer (pH 8). The reaction mixture was rotated (60 rpm) in a 37 °C incubator for 5 h, and then 0.4 mmol of αGlcF was added and incubation continued for an additional 12 h. The pH of the mixture was adjusted to 9, and the reaction mixture was extracted with chloroform (3 × 30 mL). The combined chloroform layers were dried with anhydrous sodium sulfate. To the residual material (∼100 mg) was added a limited quantity of chloroform and the solution applied to a silica column (15 cm × 1 cm) preneutralized with a 100:1 $CHCl_3$/NH_4OH mixture. A gradient elution starting from $CHCl_3$ toward a 100:1:10 $CHCl_3$/NH_4OH/MeOH eluant was applied. Relevant fractions were combined and subjected to a second chromatographic separation using the same conditions. Glucosylated erythromycin was purified (13 mg, 14%) as a single blue spot by TLC (R_f = 0.7; 5:1:1 *n*-propanol/NH_4OH/water, visualized by spraying an acidic vanillin solution in EtOH). Erythromycin (63%) was also recovered from the reaction mixture. Spectroscopic data were consistent with the literature.[24]

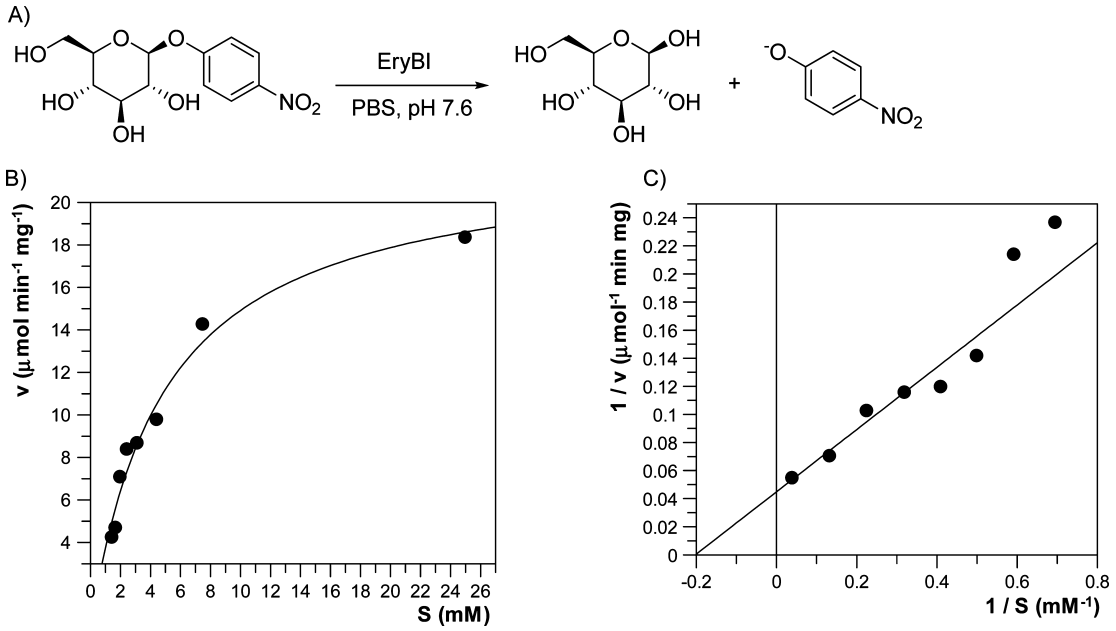


Figure 2. (A) Representative kinetic data for the hydrolysis of pNP-β-D-Glc by EryBI (K_m = 4.9 ± 0.5 mM; V_{max} = 22 µmol min^{-1} mg^{-1}). (B) Michaelis–Menten plot. (C) Lineweaver–Burk plot. Best fit data determined using GraFit 5.0. Experiments were performed in duplicate.

Determination of the Minimum Inhibitory Concentration (MIC). MIC values in liquid broth were determined using 96-well microtiter plate techniques according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).

Mass Spectrometric Analysis. Reactions were monitored using an Applied Biosystems 2000Qtrap instrument in the enhanced product ion mode with direct infusion of a methanol-diluted sample running at 10 µL/min. An electrospray source was used in the positive mode.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR and saturation transfer difference (STD) NMR spectra were recorded on a Bruker Avance 500 spectrometer as described previously.[21]

RESULTS AND DISCUSSION

Liu and co-workers predicted an EryBI homologue, DesR, as a likely secretory protein.[25] Analysis of the first 70 residues of EryBI for lipoprotein signal peptides using the LipoP server[26] indicated a likely cleavage site between residues 28 and 29. Thus, in an effort to ensure a soluble recombinant protein, a truncated version of *eryBI* DNA encoding residues 29–808 was amplified by polymerase chain reaction (PCR) from *Saccharopolyspora erythraea* ATCC 11635[27] using a high-fidelity polymerase (hereafter described as the wild type). Eight high fidelity polymerases were evaluated and screened using a variety of PCR conditions. Only the AccuPrime (Invitrogen) polymerase was able to amplify the complete sequence, presumably because of its high GC content. The gene was subcloned and ligated into a pET28 vector and sequenced. On the basis of a ClustalW sequence alignment of three functionally related family 3 glycosidases (EryBI, DesR, and OleR), residue Asp257 was identified as the putative catalytic nucleophile. On the basis of a three-dimensional model of EryBI generated using a protein homology recognition engine [PHYRE (see the Supporting Information)][28,29] and three structurally related glycosyl hydrolases,[30−32] we predicted residue D83 to be the acid/base catalyst, on the basis of its location within the modeled active site and being within 8.6 Å of D257, the postulated catalytic nucleophile. To facilitate construction of a series of nucleophile mutants, which were necessary to confirm these hypotheses and evaluate glycosynthase activity, a 650 bp portion of the gene encompassing Asp257 was subcloned. This subcloned DNA was mutated using a QuikChange mutagenesis protocol, and sequenced mutants were cloned back into the expression vector. Two mutants, D257G and D257S, were prepared. Attempts to construct the D257A mutant were unsuccessful. We also constructed the D83G mutant using a similar subcloning strategy. The wild-type and mutant proteins were expressed with an encoded His_6 N-terminal affinity tag and purified using nickel affinity chromatography using standard procedures.[21]

The wild-type EryBI enzyme was evaluated as a glycosidase with a series of nine commercially available aryl glycosides. Activity was observed with the following glycosides: pNP-β-D-Glc, pNP-β-D-Xyl, pNP-α-D-Glc, and pNP-β-D-Gal. The rates for the latter three compounds were too low to permit determination of Michaelis–Menten parameters. The activity was quantified with pNP-β-D-Glc as the substrate (Figure 2). The K_m value was determined to be 4.9 mM with a V_{max} of 22 µmol min^{-1} mg^{-1} and a V_{max}/K_m of 4.5 × 10^{-3} min^{-1} mg^{-1}. This data are highly consistent with the DesR enzyme responsible for self-resistance in the biosynthesis of methymycin.[21] Wild-type EryBI also hydrolyzed methylumbelliferyl-, bromochloroindolyl-, and 2,4-dinitrophenyl-β-D-Glc. The D257G and D257S mutants were evaluated for hydrolytic activity of pNP-β-D-Glc. No significant activity was observed with either mutant upon prolonged incubation. Greater activity was observed on pNP-β-D-Gal with both mutants, but we were not able to quantify the activity. The putative acid/base mutant D83G was evaluated over a range of aryl glycosides; however, no significant activity was observed, potentially implying this residue functions as the acid/base catalyst. These results with a series of chromogenic substrates indicate that EryBI has a broader substrate specificity than DesR, a 53% identical family 3 glucosidase responsible for self-resistance to methymycin, for

dx.doi.org/10.1021/bi201438q | *Biochemistry* 2011, 50, 10359–10366

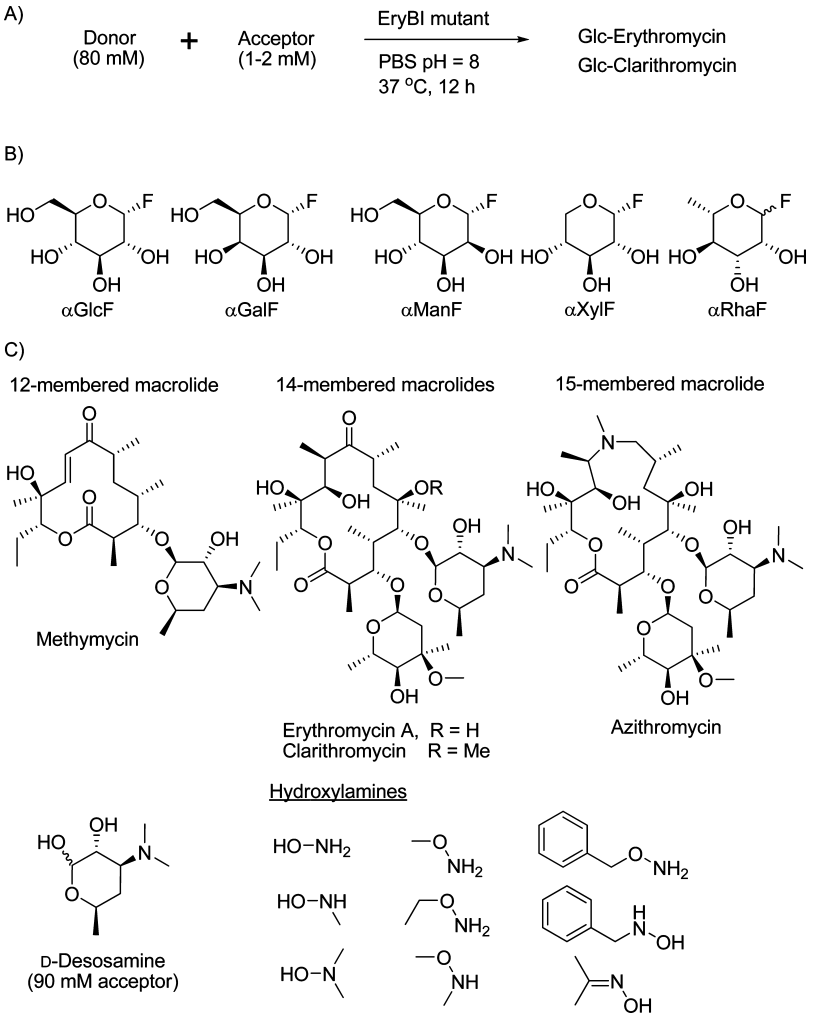


Figure 3. Structures of compounds evaluated as glycosynthase substrates with EryBI D257G: (A) screening conditions, (B) structures of donors, and (C) structures of acceptors.

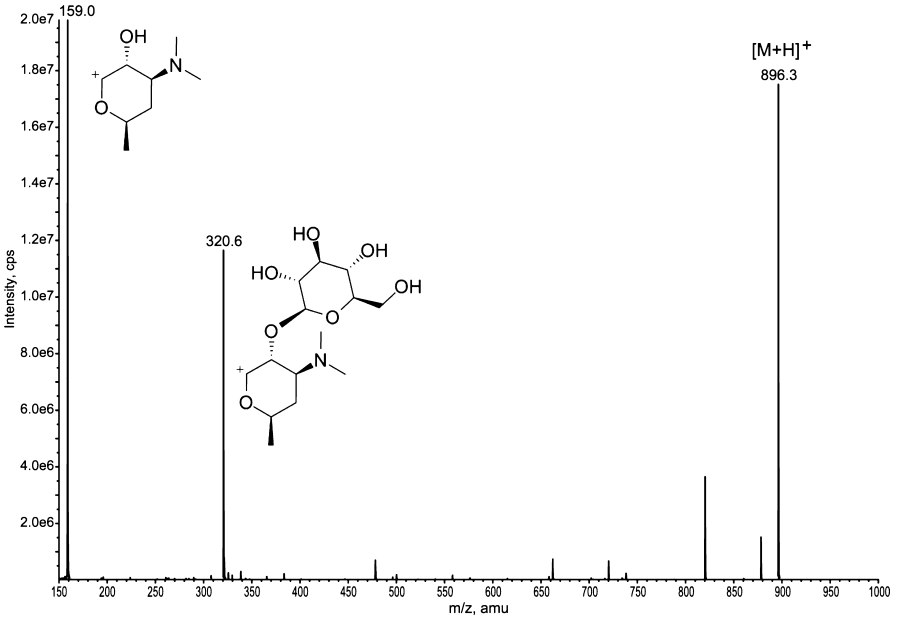


Figure 4. Electrospray tandem MS/MS spectrum of glucosylated erythromycin and fragmentation demonstrating the regioselective nature of glucosylation. The spectrum was recorded on an Applied Biosystems 2000Qtrap instrument in positive mode. The configuration of the glycosidic linkage was determined by ^{1}H NMR analysis.

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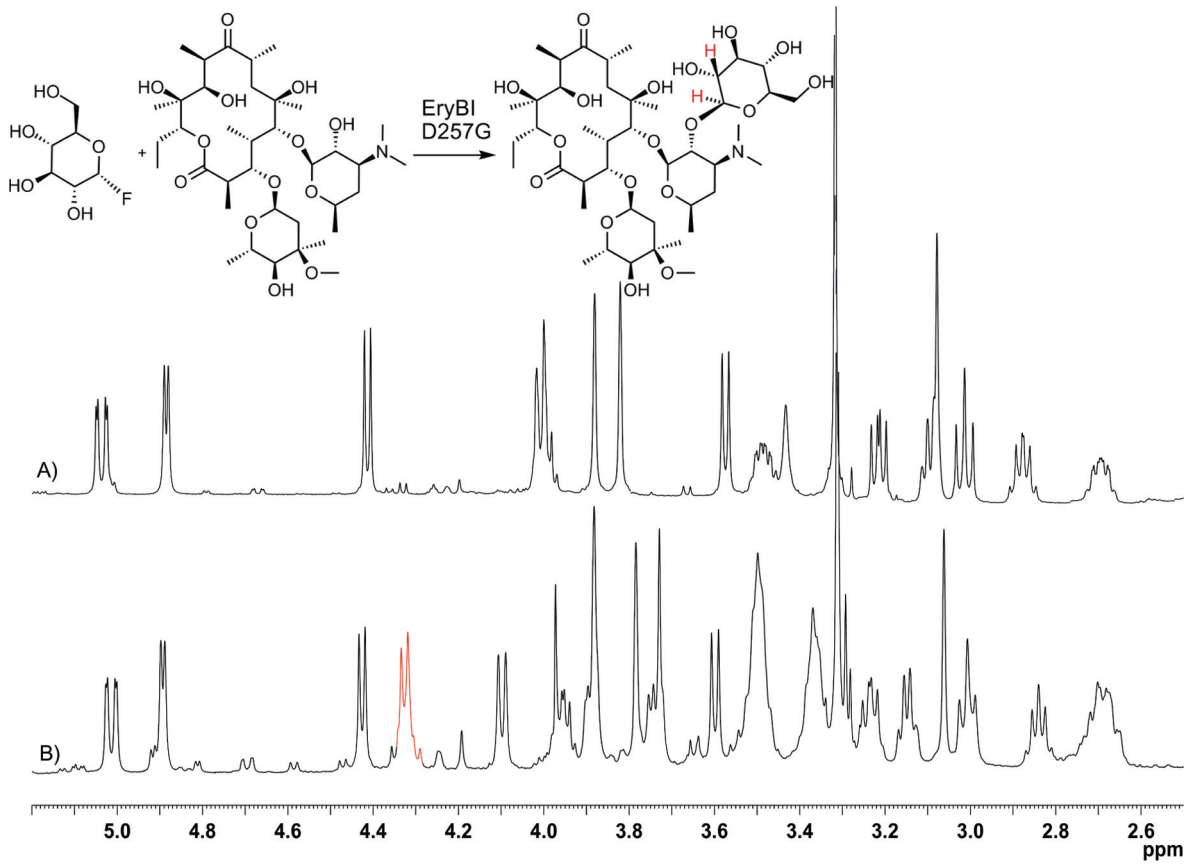

Figure 5. ^{1}H NMR spectrum (500 MHz) ($CDCl_3$) of (A) commercial erythromycin and (B) the glycosynthase product (glucosylated erythromycin). The glucosyl anomeric proton is highlighted at 4.32 ppm. The $^{3}J_{1,2}$ = 8 Hz coupling indicates a β-glycosidic linkage.

which no significant activity on the chromogenic substrates was observed.[21,25] Finally, we confirmed that the recombinantly expressed wild-type EryBI was able to hydrolyze glucosylated erythromycin to erythromycin. Reactions were monitored by TLC. This confirms the activity of EryBI to be consistent with the activities of OleR and DesR, homologous enzymes responsible for oleandomycin and methymycin self-resistance and extracellular reactivation.[18,25]

The nucleophile mutants D257G and D257S were evaluated as glycosynthases using a panel of five donor glycosyl fluorides (αGlcF, αGalF, αManF, αXylF, and αRhaF) and acceptors comprising three different macrolide antibiotics (erythromycin, clarithromycin, and azithromycin), an amino sugar (desosamine), and nine hydroxylamines (Figure 3). We anticipated that one or both of the nucleophile mutants would be able to catalyze the transfer of a glycosyl fluoride onto an acceptor. Glycosynthase reaction analysis was challenging because of the solubility limitations of the macrolide antibiotics at ~4 mM. Acceptors in glycosynthase reactions are frequently screened at concentrations of 50 mM to account for high K_m values required for enzyme activity and the hydrophilic nature of the acceptor.[33] Nevertheless, in a reaction comprised of glucosyl fluoride, erythromycin, and D257G as the catalyst, upon overnight incubation, we were pleased to observe a new spot by TLC (Supporting Information). Analysis by mass spectrometry identified a molecular ion corresponding to a glucosylated erythromycin. Subsequent tandem mass spectrometry (Figure 4) identified the site of attachment of the glucosyl unit to be the desosamine sugar. Scale-up, isolation, and characterization of the reaction product allowed NMR studies to be conducted. Glucosylated erythromycin was isolated in 14% yield, or 38% when accounting for recovered erythromycin from the reaction mixture. The ^{1}H NMR studies confirmed that the glucosyl unit was appended to the C2 hydroxyl substituent in desosamine, with a β-configured glucosidic linkage based on the $^{3}J_{H1,H2}$ = 8 Hz coupling (Figure 5). This was consistent with the literature data for the compound.[24] A similar analysis of a reaction mixture in which erythromycin was replaced with clarithromycin clearly demonstrated the formation of a similarly glucosylated macrolide antibiotic, again in a similar yield. The low yields were potentially due to the poor solubility of erythromycin or the glucosylated product, particularly in comparison to other glycosynthase reactions in which acceptor concentrations are typically 1 order of magnitude higher.[33] This demonstrates that the active site of the D257G glycosynthase is able to accommodate a methyl ether substituent attached at C6 of the macrolide ring. TLC and mass spectral analysis of reaction mixtures in which desosamine replaced erythromycin failed to show the formation of a new product, indicating that the D257G glycosynthase recognizes more than the desosamine sugar. Azithromycin, a ring-expanded derivative of erythromycin, was recalcitrant as a substrate for the glycosynthase. This indicates that the expanded macrolide ring comprising the tertiary amine present in the azithromycin macrolactone is a sufficient structural change to prohibit glycosynthase activity. No other combination of glycosyl fluorides and acceptors was transformed by EryBI D257G. This observation of glycosynthase activity with an EryBI nucleophile mutant was in contrast to our analysis of DesR, a homologous enzyme, responsible for

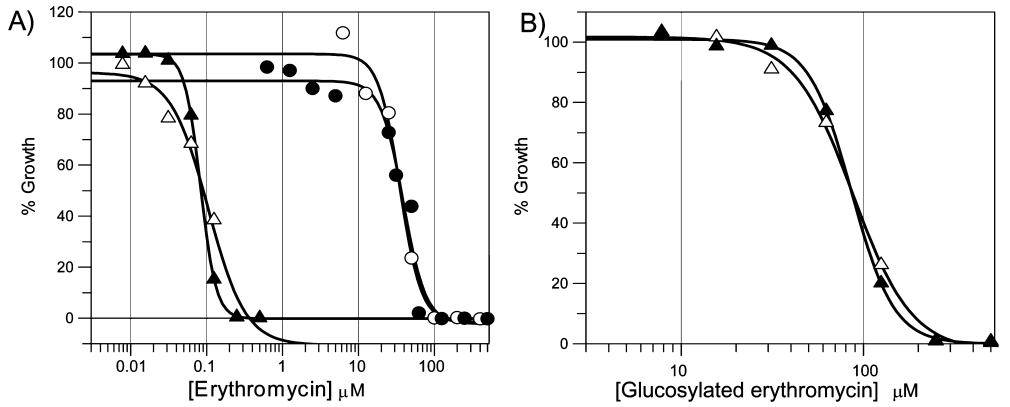

Figure 6. Antibacterial susceptibility test for *E. coli* strains vs (A) erythromycin and (B) glucosylated erythromycin. Glucosylated erythromycin is significantly less active than erythromycin: (○) BL21 λDE3, (●) BL21 λDE3 with EryBI D257G, (△) NR698 λDE3, and (▲) NR698 λDE3 with EryBI D257G.

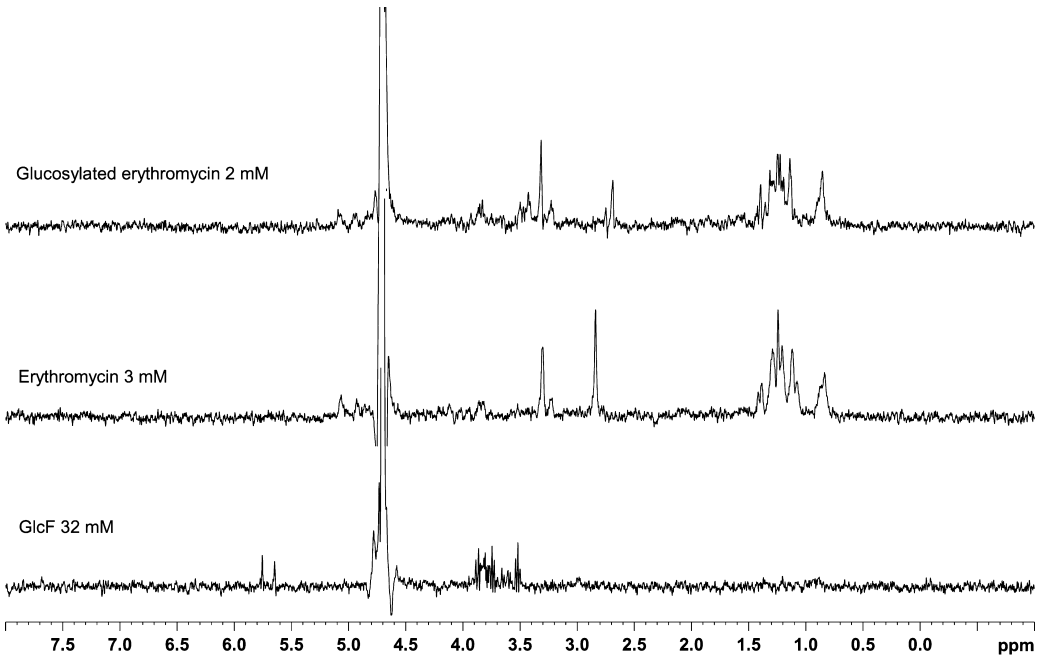

Figure 7. ^{1}H STD-NMR spectra (500 MHz) of EryBI D257G (0.1 mM) and substrates and product in deuterated phosphate-buffered saline (50 mM, pD 7.6).

the self-resistance of methymycin that was not catalytically active as a glycosynthase.[21]

We next turned our attention to the development of a cellular selection method for facilitating the directed evolution of EryBI glycosynthase to broaden its substrate specificity and enhance its catalytic rate. The basis of the selection strategy was the hypothetical lack of antibacterial activity observed with glucosylated erythromycin in contrast to the activity observed with erythromycin, particularly in view of the extracellular reactivation of macrolide antibiotics by glucosidases (Figure 1B).[18] Thus, for a bacterial cell to survive and prosper in the presence of erythromycin and glucosyl fluoride, a selective advantage was thought to exist if the bacterium harbored a plasmid containing an active EryBI glycosynthase. Application of this technology would facilitate the straightforward evaluation of large libraries of mutant glycosynthases because only those that prospered on growth media would contain active glycosynthase-encoding plasmids. This approach could also be used to facilitate modification of the glycosyl fluoride donor sugar involved in a glycosynthase reaction.

Cognizant of the fact that erythromycin is not effective against Gram-negative bacteria, we first obtained *E. coli* strain NR698, known for its permeability toward erythromycin,[34] and integrated the λDE3 prophage to facilitate expression of the T7 RNA polymerase expression vectors. Next we evaluated this strain together with strain BL21 λDE3 to determine MIC values in liquid broth for erythromycin and glucosylated erythromycin using Clinical and Laboratory Standards Institute methods [CLSI (Figure 6)]. NR698 λDE3 was 1000 times more susceptible to erythromycin than BL21 λDE3, with a minimum inhibitory concentration (MIC) of ∼100 nM. In contrast, glucosylated erythromycin was significantly less effective against NR698 λDE3, with a MIC of ∼200 μM. BL21 λDE3 cells were not susceptible to the antibacterial effects of glucosylated erythromycin up to 500 μM. We determined that strain NR698 λDE3 functioned as a suitable

expression host using plasmids expressing EryBI and EryBI D257G (data not shown).

A proof of concept study was devised to demonstrate the selection method. First, we grew two individual cultures of NR698 λDE3. The first contained the plasmid harboring EryBI glycosidase and the second the plasmid harboring EryBI D257G glycosynthase. When the cell growth was in midlog phase (OD_{600} ~ 0.7), protein induction was initiated, and 2 h later, equal quantities of cultures (based on OD) were mixed together and diluted to an OD_{600} of ~0.1. The mixture was divided into two, with one aliquot being incubated with erythromycin and glucosyl fluoride and the other being incubated with erythromycin alone. The concentrations and times for these events were varied significantly in subsequent experiments. Equal quantities of cultures were then plated and evaluated for activity on methylumbelliferyl-β-D-Glc. We anticipated that we would observe significantly more colonies on the erythromycin and glucosyl fluoride plate and that significantly more glycosynthase activity would be detected. However, despite varying reagent concentrations and timing steps throughout this method, we were unable to consistently observe results that demonstrated an enrichment of glycosynthase activity within the cultures. Nevertheless, significant numbers of colonies were picked from plates and plasmids analyzed for the presence of the glycosynthase mutant by digestion with restriction enzyme BspEI, a unique restriction site introduced into the mutagenic primer. The anticipated change in the proportion of glucosidase versus glycosynthase plasmid was not observed, again indicating that the selection procedure had been unsuccessful. One potential reason explaining the lack of selection may be the particularly susceptible nature of *E. coli* strain NR698 to erythromycin and the affinity of the drug for its cellular target, the bacterial ribosome. The affinity with which erythromycin binds the *E. coli* ribosome has been reported as 2.2×10^{-9} M.[35] As a method of gauging binding, we probed interactions of erythromycin and glucosyl fluoride with EryBI D257G glycosynthase using STD-NMR spectroscopy (Figure 7). Analysis of the ^{1}H NMR spectra indicated that substrates, glucosyl fluoride and erythromycin, in addition to the glycosynthase product, all bound to the glycosynthase. Because we observed signals in the STD-NMR spectra, this is highly indicative of binding interactions in the micromolar range.[36−41] Therefore, the interactions between erythromycin and the glycosynthase are substantially weaker than the interactions between erythromycin and the ribosome. As a consequence, the ability to select improved glycosynthase variants using glycosylation of erythromycin as an in vivo mechanism of selection requires an enzyme catalyst with significantly greater catalytic prowess than the D257G point mutant, or substrates with substantially weaker affinity for the ribosome.

In conclusion, we have demonstrated, for the first time, the in vitro glucosidase activity of EryBI and identified putative acid/base and nucleophile catalytic residues essential for hydrolytic activity. These data support the role of eryBI as a self-resistance gene in erythromycin biosynthesis, as originally proposed by Salas and co-workers for homologous genes involved in oleandomycin biosynthesis.[18] We have constructed the first glycosynthase capable of glycosylating amino sugar acceptor substrates with a β-(1,2) glycosidic linkage. Use of this mutant enzyme should allow the development of glycosylated amino sugars with novel properties, including enhanced solubility, selective targeting, or prodrug properties. The development of an in vivo selection method using a glycosynthase was compromised by the high affinity of the glycosynthase substrate (erythromycin) for its ribosomal binding site.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Four schemes, four tables, five figures, and detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We thank the Mizutani Glycoscience Foundation of Japan, the Canadian Institutes of Health Research (CIHR), and the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding.

■ ABBREVIATIONS

pNP, *p*-nitrophenyl; Glc, glucose; PBS, phosphate buffered (50 mm) saline (145 mM) pH 7.6; TLC, thin layer chromatography; αGlcF, α-D-glucosyl fluoride; αGalF, α-D-galactosyl fluoride; αManF, α-D-mannosyl fluoride; αXylF, α-D-xylosyl fluoride; αRhaF, α-L-rhamnosyl fluoride; STD-NMR, saturation transfer difference nuclear magnetic resonance; MIC, minimum inhibitory concentration.

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