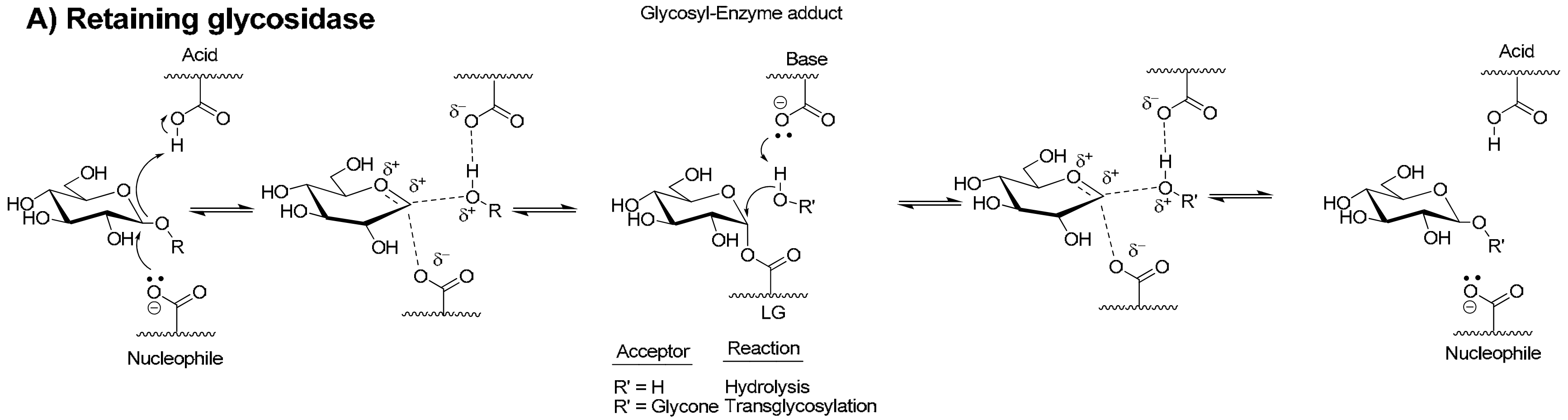
Electronic supporting information

A β-(1,2)-glycosynthase, and an attempted selection method for the directed evolution of glycosynthases

David L. Jakeman,*[1,2] and Ali Sadeghi-Khomami[1,2]
[1] *College of Pharmacy Dalhousie University, 5968 College St., Halifax, Nova Scotia, B3H 3J5 Canada. Fax: +1 902 494 1396; Tel:+1 902 494 7159; E-mail: david.jakeman@dal.ca*
[2] *Department of Chemistry, Dalhousie University, 1459 Oxford St., Halifax, Nova Scotia, B3H 4R2.*

Scheme S1. A) Retaining glycosidase mechanism and B) glycosynthase mechanism

A) Retaining glycosidase



B) Glycosynthase

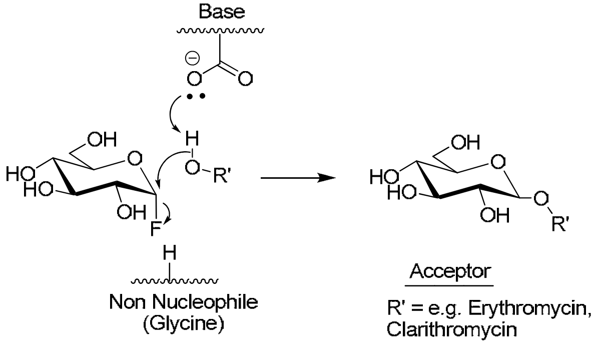


Table S1. Homology between EryBI, DesR and OleR (Family 3 glycosidases from macrolide producers)

Glycosidase	Producer		Product	Protein ID	Length	Mw (Dalton)	Identity%	Similarity%
	Name	Strain						
EryBI	*Saccharopolyspora erythraea (Streptomyces erythraeus)*	NRRL2338	Erythromycin	CAA74702	808	86678	100	100
DesR	*Streptomyces venezuelae*	ATCC15439	Methymycin	AAC68679	809	84687	53	67
OleR	*Streptomyces antibioticus*	ATCC 11891	Oleandomycin	AAC12650	769	82258	60	70

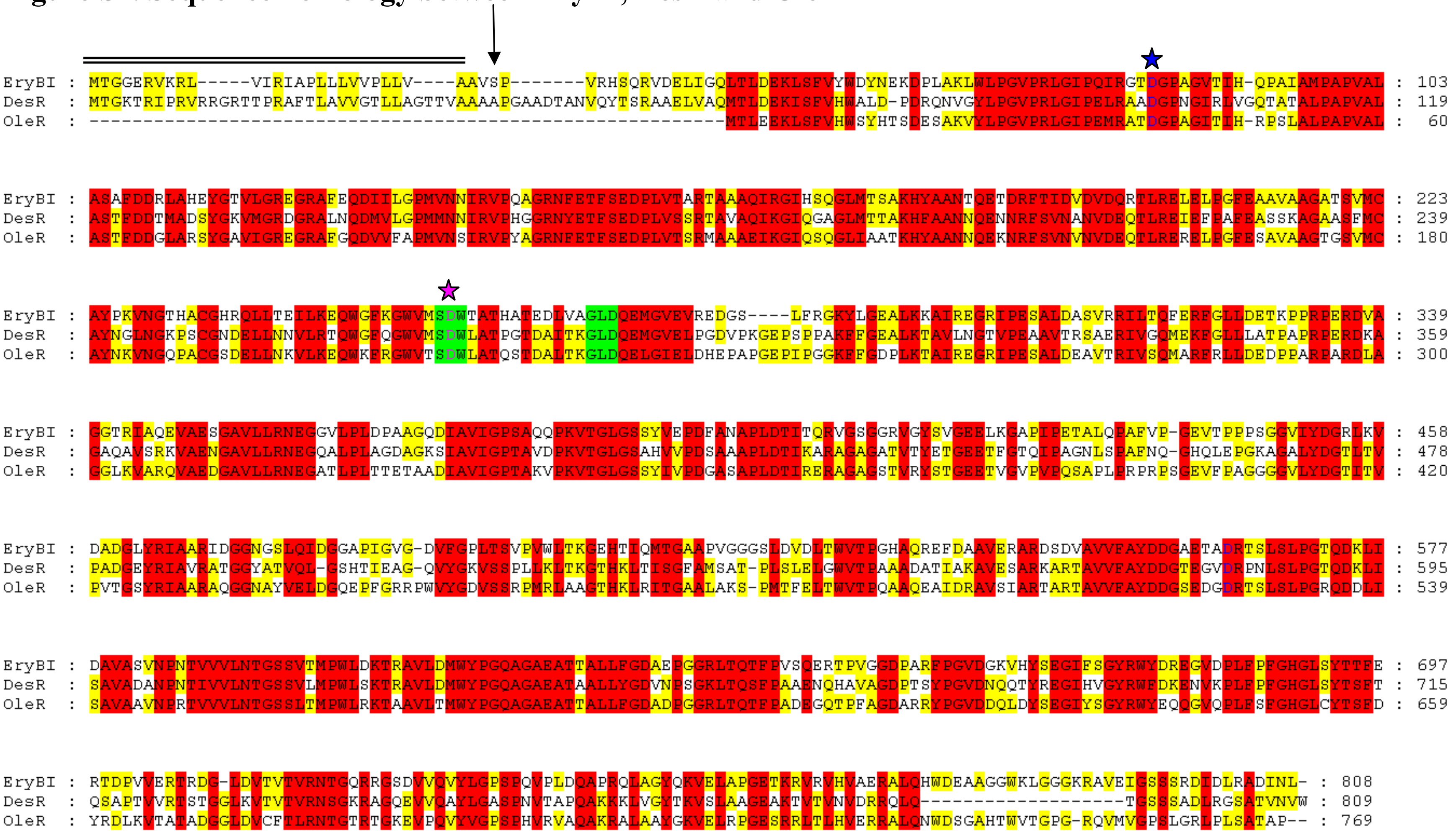
PDB structure of family 3 glycosidases, namely β-N-acetylhexosaminidase (NagZ, 3.2.1.52, 330 AA) from *Vibrio cholerae*, β-N-acetylglucosaminidase (ORF1, 3.2.1.52, 642 AA) from *Bacillus subtillis* and exo-1,3-1,4-glucanase (ExoI, 3.2.1.58, 630 AA) from *Hordeum vulgare* are available from Protein Data Bank.

Table S2. Catalytic amino acids in selected family-3 glycosidases

Glycosidase	Catalytic amino acid (AA)			PDB
	Nucleophile	Acid/Base	Carboxylate distance (C- - -C Å) between catalytic AAs	
EryBI	D257	D83	8.59*	--
DesR	D273	D98	8.51*	--
OleR	D214	D40	8.59*	--
ExoI	D285	D95 or E491	8.48 or 6.91	1IEW (Resol. 2.55 Å)
ORF1	D318	D123	8.28	3BMX (Resol. 1.4 Å)
NagZ	D242	D62	12-13	2OxN (Resol. 1.7 Å)

* 3D structures were predicted with estimated precision of 100% using Protein Homology/analogY Recognition Engine (PHYRE V.2)(Bennett-Lovsey et al., 2008).

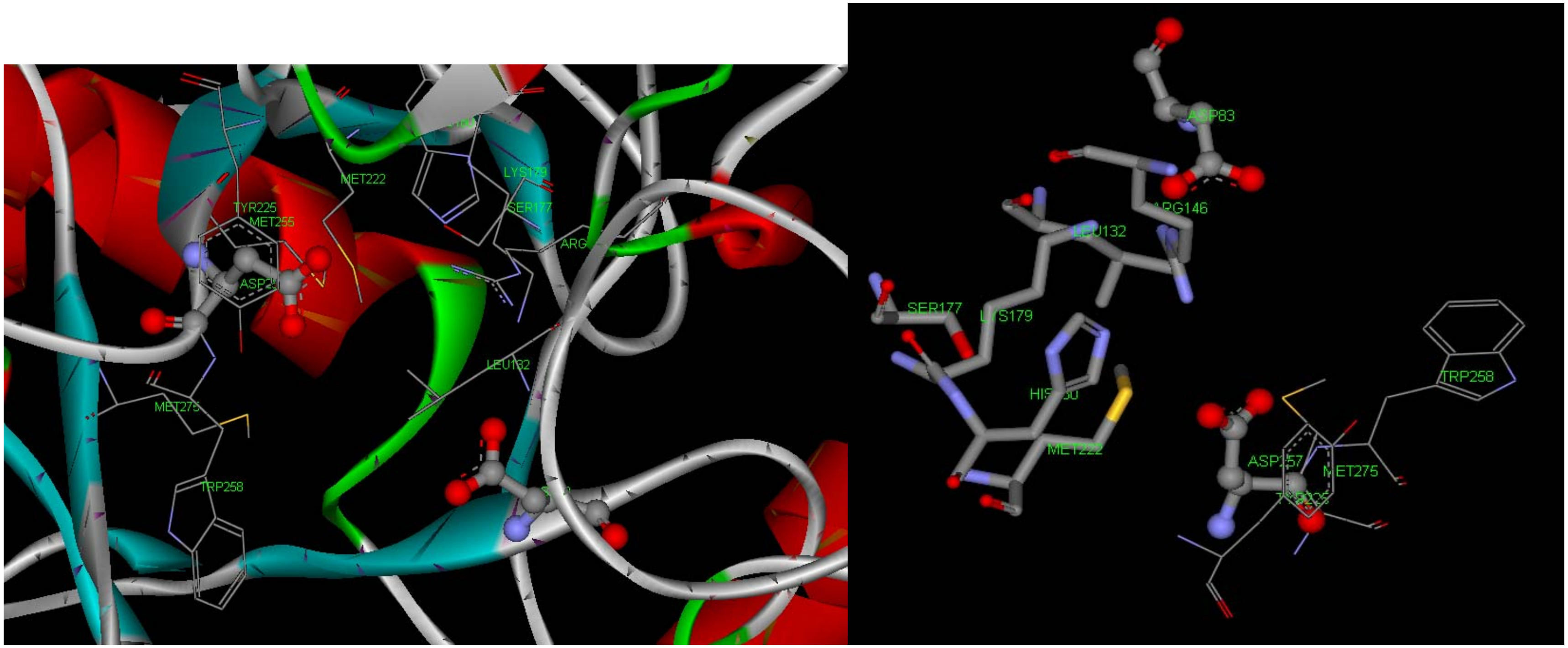
Figure S1. Sequence homology between EryBI, DesR and OleR



```
EryBI : MTGGERVKRL-----VIRIAPLLLVVPLLV----AAVSP-------VRHSQRVDELIGQLTLDEKLSFVYWDYNEKDPLAKLWLPGVPRLGIPQIRGTDGPAGVTIH-QPAIAMPAPVAL : 103
DesR  : MTGKTRIPRVRRGRTTPRAFTLAVVGTLLAGTTVAAAAPGAADTANVQYTSRAAELVAQMTLDEKISFVHWALD-PDRQNVGYLPGVPRLGIPELRAADGPNGIRLVGQTATALPAPVAL : 119
OleR  : -----------------------------------------------------------MTLEEKLSFVHWSYHTSDESAKVYLPGVPRLGIPEMRATDGPAGITIH-RPSLALPAPVAL :  60

EryBI : ASAFDDRLAHEYGTVLGREGRAFEQDIILGPMVNNIRVPQAGRNFETFSEDPLVTARTAAAQIRGIHSQGLMTSAKHYAANTQETDRFTIDVDVDQRTLRELELPGFEAAVAAGATSVMC : 223
DesR  : ASTFDDTMADSYGKVMGRDGRALNQDMVLGPMMNNIRVPHGGRNYETFSEDPLVSSRTAVAQIKGIQGAGLMTTAKHFAANNQENNRFSVNANVDEQTLREIEFPAFEASSKAGAASFMC : 239
OleR  : ASTFDDGLARSYGAVIGREGRAFGQDVVFAPMVNSIRVPYAGRNFETFSEDPLVTSRMAAAEIKGIQSQGLIAATKHYAANNQEKNRFSVNVNVDEQTLRERELPGFESAVAAGTGSVMC : 180

EryBI : AYPKVNGTHACGHRQLLTEILKEQWGFKGWVMSDWTATHATEDLVAGLDQEMGVEVREDGS----LFRGKYLGEALKKAIREGRIPESALDASVRRILTQFERFGLLDETKPPRPERDVA : 339
DesR  : AYNGLNGKPSCGNDELLNNVLRTQWGFQGWVMSDWLATPGTDAITKGLDQEMGVELPGDVPKGEPSPPAKFFGEALKTAVLNGTVPEAAVTRSAERIVGQMEKFGLLLATPAPRPERDKA : 359
OleR  : AYNKVNGQPACGSDELLNKVLKEQWKFRGWVTSDWLATQSTDALTKGLDQELGIELDHEPAPGEPIPGGKFFGDPLKTAIREGRIPESALDEAVTRIVSQMARFRLLDEDPPARPARDLA : 300

EryBI : GGTRIAQEVAESGAVLLRNEGGVLPLDPAAGQDIAVIGPSAQQPKVTGLGSSYVEPDFANAPLDTITQRVGSGGRVGYSVGEELKGAPIPETALQPAFVP-GEVTPPPSGGVIYDGRLKV : 458
DesR  : GAQAVSRKVAENGAVLLRNEGQALPLAGDAGKSIAVIGPTAVDPKVTGLGSAHVVPDSAAAPLDTIKARAGAGATVTYETGEETFGTQIPAGNLSPAFNQ-GHQLEPGKAGALYDGTLTV : 478
OleR  : GGLKVARQVAEDGAVLLRNEGATLPLTTETAADIAVIGPTAKVPKVTGLGSSYIVPDGASAPLDTIRERAGAGSTVRYSTGEETVGVPVPQSAPLPRPRPSGEVFPAGGGGVLYDGTITV : 420

EryBI : DADGLYRIAARIDGGNGSLQIDGGAPIGVG-DVFGPLTSVPVWLTKGEHTIQMTGAAPVGGGSLDVDLTWVTPGHAQREFDAAVERARDSDVAVVFAYDDGAETADRTSLSLPGTQDKLI : 577
DesR  : PADGEYRIAVRATGGYATVQL-GSHTIEAG-QVYGKVSSPLLKLTKGTHKLTISGFAMSAT-PLSLELGWVTPAAADATIAKAVESARKARTAVVFAYDDGTEGVDRPNLSLPGTQDKLI : 595
OleR  : PVTGSYRIAARAQGGNAYVELDGQEPFGRRPWVYGDVSSRPMRLAAGTHKLRITGAALAKS-PMTFELTWVTPQAAQEAIDRAVSIARTARTAVVFAYDDGSEDGDRTSLSLPGRQDDLI : 539

EryBI : DAVASVNPNTVVVLNTGSSVTMPWLDKTRAVLDMWYPGQAGAEATTALLFGDAEPGGRLTQTFPVSQERTPVGGDPARFPGVDGKVHYSEGIFSGYRWYDREGVDPLFPFGHGLSYTTFE : 697
DesR  : SAVADANPNTIVVLNTGSSVLMPWLSKTRAVLDMWYPGQAGAEATAALLYGDVNPSGKLTQSFPAAENQHAVAGDPTSYPGVDNQQTYREGIHVGYRWFDKENVKPLFPFGHGLSYTSFT : 715
OleR  : SAVAAVNPRTVVVLNTGSSLTMPWLRKTAAVLTMWYPGQAGAEATTALLFGDADPGGRLTQTFPADEGQTPFAGDARRYPGVDDQLDYSEGIYSGYRWYEQQGVQPLFSFGHGLCYTSFD : 659

EryBI : RTDPVVERTRDG-LDVTVTVRNTGQRRGSDVVQVYLGPSPQVPLDQAPRQLAGYQKVELAPGETKRVRVHVAERALQHWDEAAGGWKLGGGKRAVEIGSSSRDIDLRADINL- : 808
DesR  : QSAPTVVRTSTGGLKVTVTVRNSGKRAGQEVVQAYLGASPNVTAPQAKKKLVGYTKVSLAAGEAKTVTVNVDRRQLQ-------------------TGSSSADLRGSATVNVW : 809
OleR  : YRDLKVTATADGGLDVCFTLRNTGTRTGKEVPQVYVGPSPHVRVAQAKRALAAYGKVELRPGESRRLTLHVERRALQNWDSGAHTWVTGPG-RQVMVGPSLGRLPLSATAP-- : 769
```

Clustal W (v1.83) data presented in GeneDoc v2.6. Background of amino acids which were identical in three enzymes coloured in red, conserved amino acids between two enzymes coloured in yellow and β-glycosidase motif amino acids coloured in green. The Asp shown in pink and blue colour are the putative catalytic nucleophile (EryBI-D257) and catalytic acid/base, respectively. Double line indicates transmembrane/signal peptide predicted by Phobius and arrow shows (S29) the start point of amplification for truncated EryBI used in these studies.

Figure S2. PHYRE modeled structure of EryBI active site



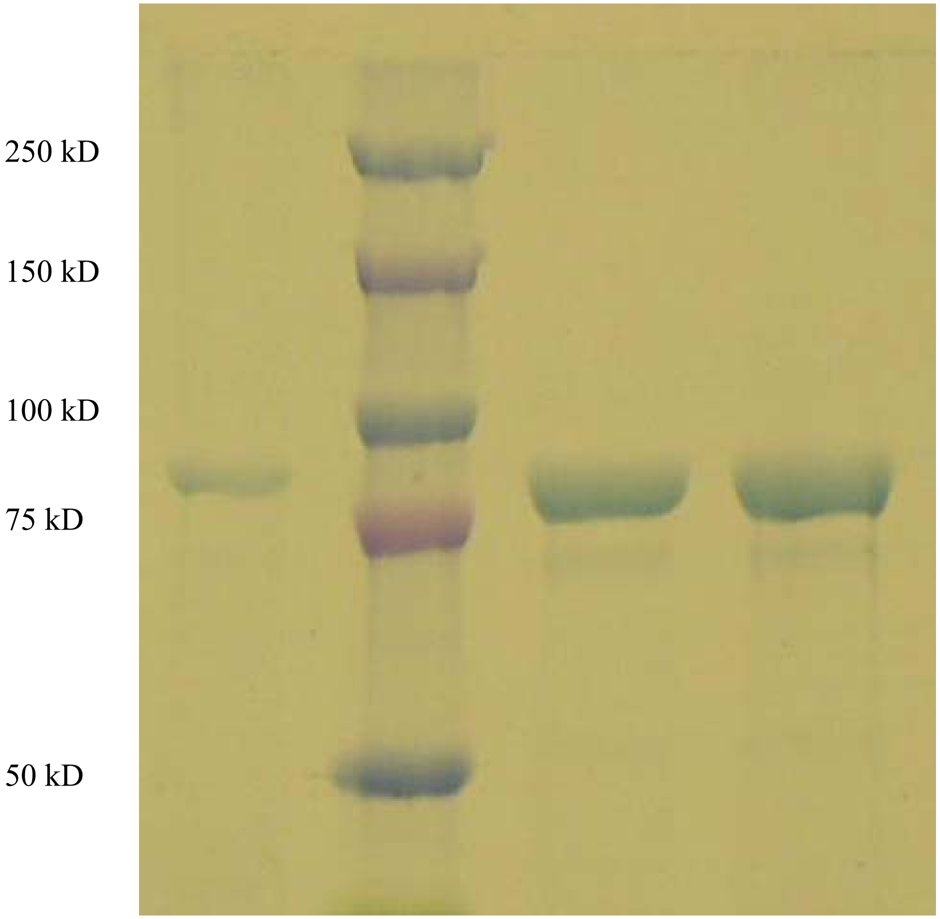
A) B)

Non-groove binding-site of glucose in 3D-model of EryBI structure predicted with estimated precision of 100% using Protein Homology/analogY Recognition Engine (PHYRE V.2)(Bennett-Lovsey et al., 2008).

A) Looking through the entrance of the glucose binding-pocket

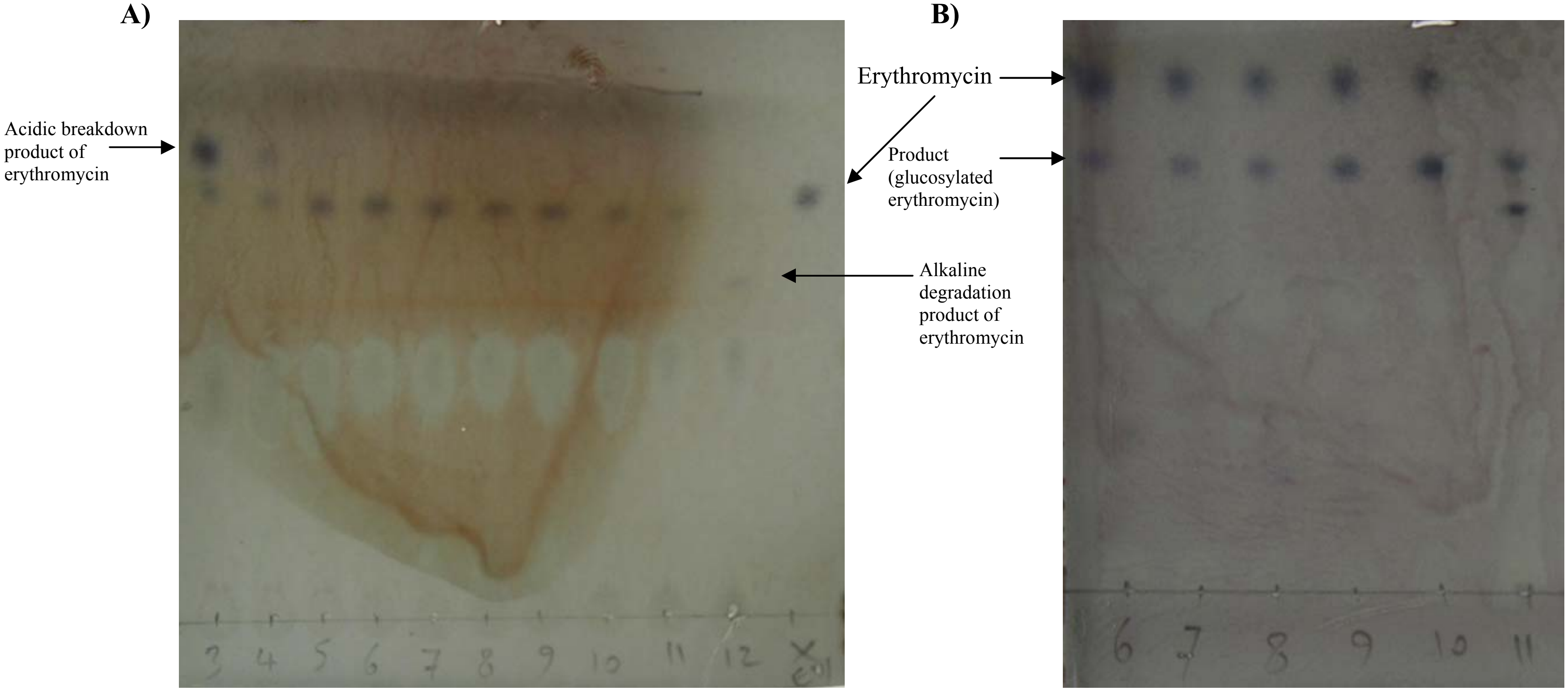
B) a simplified presentation of glucose recognition-site after *ca.* 180 ° anti-clockwise rotation, amino acids involved in the recognition of glucose are displayed as *caped-stick*, catalytic aspartic acid amino acids D257 (nucleophile) and D83 (acid / base) are displayed as *ball-and-stick* residues. W258 which is likely involved in hydrophobic interactions with erythromycin is displayed as wire-frame.

Figure S3. SDS-PAGE gel showing recombinant EryBI and mutant enzymes



Lane 1 D257S mutant, lane 2 molecular weight markers, lane 3 and 4 purified wild-type EryBI and D257G mutant, respectively.

Figure S4. TLC Evaluation of erythromycin stability at extremes of pH(A) and glycosynthase activity of EryBI D257G (B)



(A) pH effect on the decomposition of erythromycin after 2h @ 37 °C, sample pH as indicated by column numbers (3 – 12)

(B) Glycosynthase activity of EryBI-D257G after 12h @ 37 °C in reaction between erythromycin and 1F-Glc. Sample pH indicated by column number. The glycosynthase was active between pH 6 – 11. Note alkaline degradation product at pH 11.

Determination of optimum pH for glycoynthase activity of D257G mutant:
Judged by TLC, erythromycin (acceptor) at 37 °C was stable for 2 days between pH 7-9. Decomposition of α-1F-Glc was more significant at acidic pH and portionwise addition of α-1F-Glc was used to maintain a high concentration of donor fluoride.

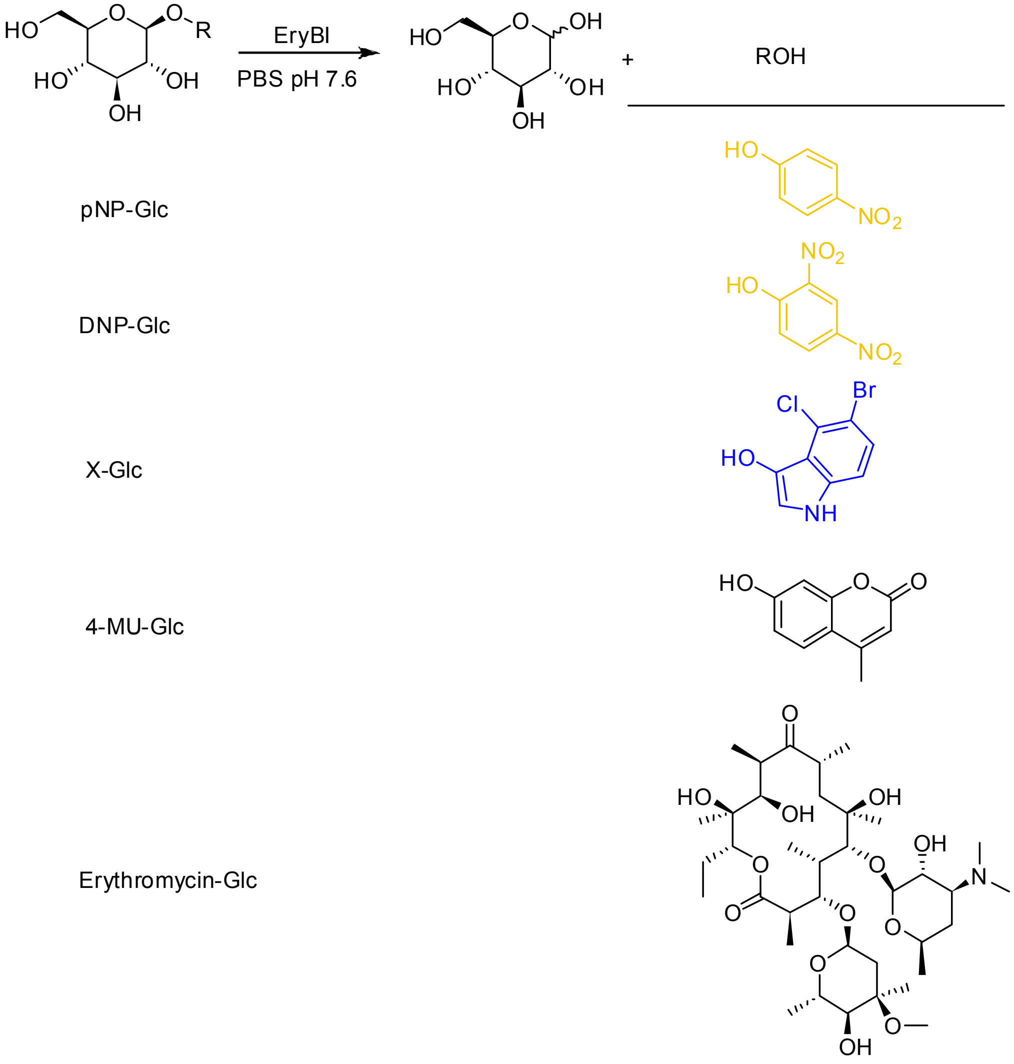
Table S3. Kinetic analysis of chromogenic substrates with EryBI

Entry	Compound	**R¹**	**R²**	**R³**	**R⁴**	**R⁵**	K_m µM	V_{max} µmol min^{-1} mg^{-1}	V_{max} / K_m x10^3
1	*p*NP-β-D-Glc	H	OH	OH	H	CH_2OH	4900	22	4.4
2	*p*NP-α-D-Glc	H	OH	OH	H	CH_2OH	-	-*	-
3	*p*NP-β-D-Xyl	H	OH	OH	H	H	+	+	+
4	*p*NP-β-D-Glu	H	OH	OH	H	CO_2H	-	-	-
5	*p*NP-β-D-GlcNAc	H	$NHCOCH_3$	OH	H	CH_2OH	-	-	-
6	*p*NP-β-D-Gal	H	OH	H	OH	CH_2OH	-	-*	-
7	*p*NP-α-D-Gal	H	OH	H	OH	CH_2OH	-	-	-
8	*p*NP-β-D-Fuc	H	OH	H	OH	CH_3	-	-	-
9	*p*NP-β-D-Man	OH	H	OH	H	CH_2OH	-	-	-
10	Erythromycin-β-D-Glc	H	OH	OH	H	CH_2OH	+	+	+

+active but not quantified, * very weak unquantifiable activity was observed, - no activity

No significant activity was observed for EryBI-D257G or S mutants for the hydrolysis of *p*NP-β-D-Glc, however they were more active in hydrolysis of *p*NP-β-D-Gal

Scheme S2. Aglycon substrate specificity of EryBI



Scheme S3. Synthesis of glycosyl fluoride donors

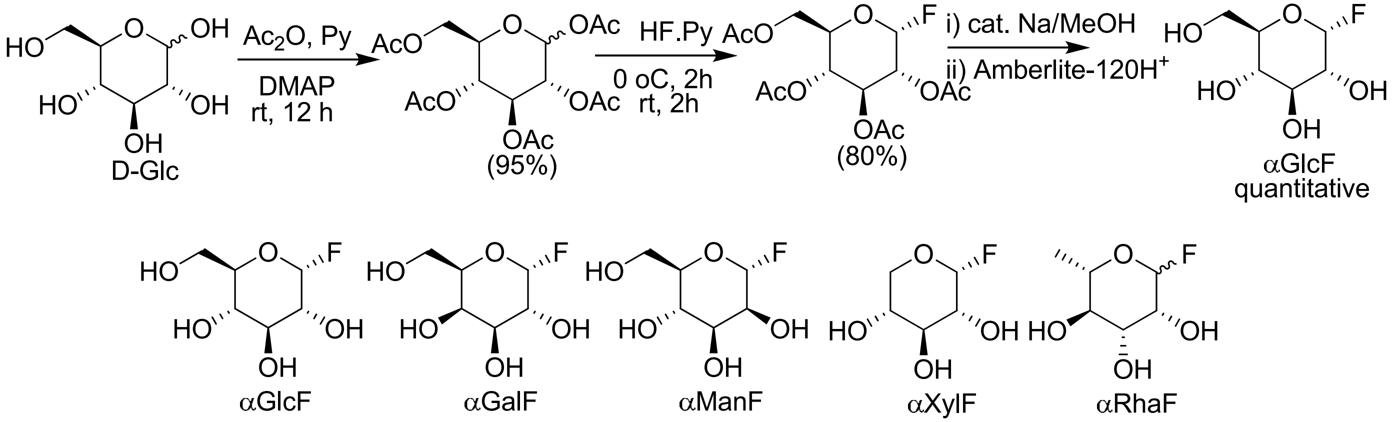


Table S4 ^{19}F, ^{13}C, ^{1}H NMR data for synthesized glycosyl fluorides

235 MHz ^{19}F-NMR of glycosyl fluorides

Ref $CF_3COO^-Na^+$ in D_2O at -74.180 ppm

	{^{1}H}δ_F
α-1F-D-Glc*p*	-148.9
α-1F-D-Gal*p*	-150.96
α-1F-D-Man*p*	-120.9
α-1F-D-Xyl*p*	-151.1
α–1F-L-Rhm*p*	-121.1

125 MHz ^{13}C-NMR of glycosyl fluorides

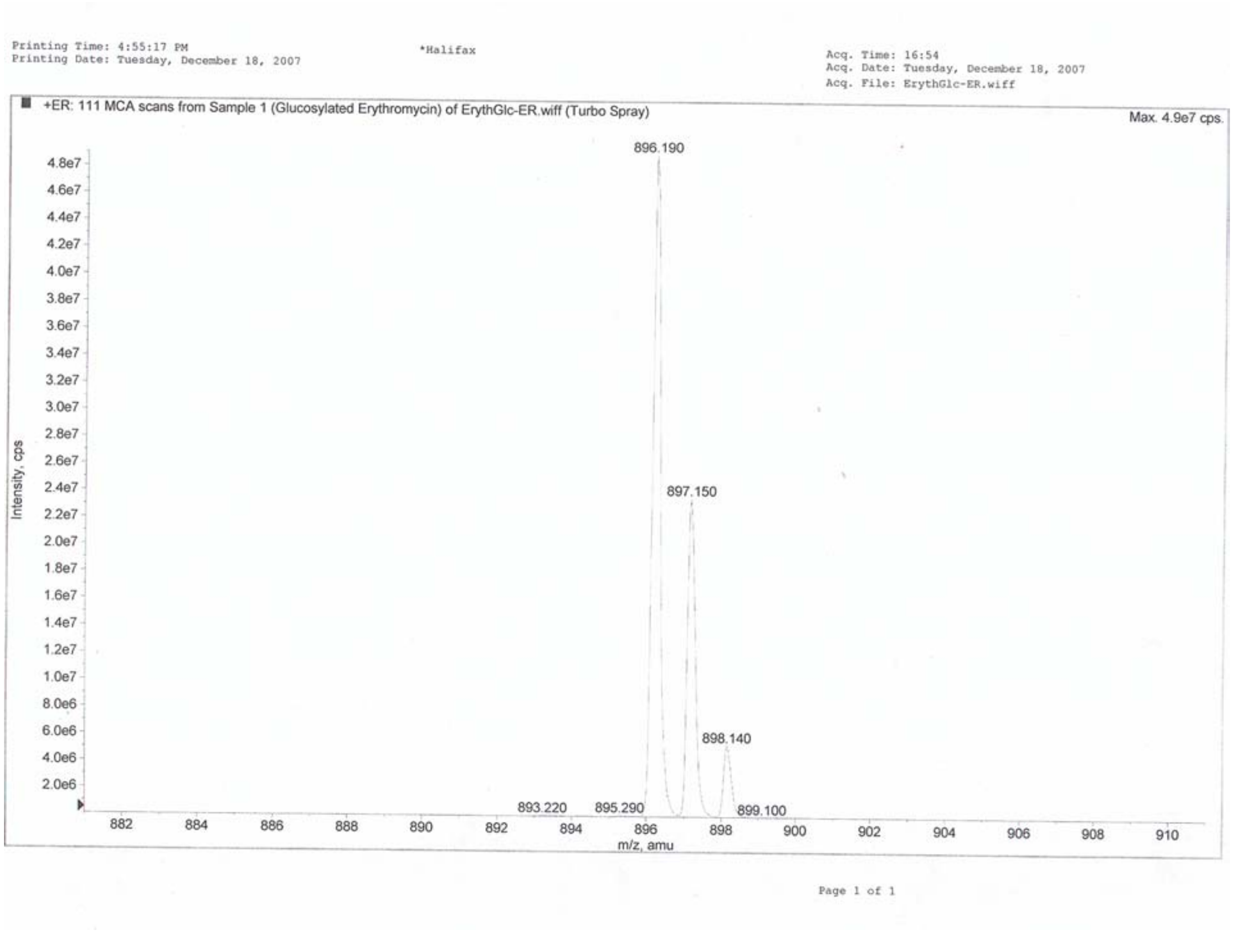
Chemical	C-1	C-2	C-3	C-4	C-5	C-6
α-1F-D-Glc*p*	107.4 d ^1J 2□3	71.1 d ^2J 2□.9	72.4	68.6	74.2 d ^3J 2.8	60.2
α-1F-D-Gal*p*	107.6 d ^1J 223	68 d ^2J 24.9	68.8	69	73.5 d ^3J 2.8	61
α-1F-D-Man*p*						
α-1F-L-Rhm*p*	107.8 d ^1J 220.5	68.4 d ^2J □3	69.5	71.3 □	71.2 d ^3J 2.5	?
α-1F-D-Xyl*p*	107.6	71.16	72.7	68.4	63.27	

	d ^1J 223	d ^2J 24.7			d ^3J 4	

500 MHz ^{1}H-NMR of glycosyl fluorides

Chemical	**1-H**	**2-H**	**3-H**	**4-H**	**5-H**	**6a-H**	**6b-H**
α-1F-D-Glc*p*	5.75 dd $J_{1,2}$ 2.6 $^2J_{1,F}$ 53.4	3.69 ddd $J_{2,3}$ 9.4 $^3J_{2,F}$ 26.4	3.8 dd $J_{3,4}$ 9.4	3.57 dd $J_{4,5}$ 9.4	3.86-3.9 m $J_{5,6a}$ 5	3.84 dd $J_{6a,6b}$ 12	3.93 dd $J_{6b,5}$ 1.9
α-1F-D-Gal*p*	5.77 br dd $^2J_{1,F}$ 53.3	3.8-3.9 m	3.94-3.96 m	4.1 br s	4.15 app t J ~ 5	3.78-3.82 m	
α-1F-D-Man*p*							
α-1F-L-Rhm*p*	5.62 br d $^2J_{1,F}$ ~49	4.14 br s	3.83 m $J_{3,4}$ 10	3.53 dd $J_{4,5}$ 10	3.88 m	?	
					5a-H	**5b-H**	
α-1F-D-Xyl*p*	5.67 br d $^2J_{1,F}$ 53.2	3.60-3.74				3.9 app dd $J_{5a,5b}$ 10 J ~ 3.8	

Figure S5. HRMS spectra for glucosylated-erythromycin, product of glycosynthase reaction using EryBI-D257G



Printing Time: 4:55:17 PM
Printing Date: Tuesday, December 18, 2007

*Halifax

Acq. Time: 16:54
Acq. Date: Tuesday, December 18, 2007
Acq. File: ErythGlc-ER.wiff

+ER: 111 MCA scans from Sample 1 (Glucosylated Erythromycin) of ErythGlc-ER.wiff (Turbo Spray)

Max. 4.9e7 cps.

Page 1 of 1

Materials and methods

Preparation of samples for STD-NMR

Samples for STD-NMR were prepared according to previously used conditions, see *Chemistry & Biology* 2008, **15**, 739-749, Glycosidase inhibition by macrolide antibiotics elucidated by STD-NMR spectroscopy, Sadeghi-Khomami,A.; Lumsden,M.D.; Jakeman,D.L.

Cloning and site directed mutagenesis

The *eryBI* DNA encoding residues 29 to 808 was amplified by PCR from *Saccharopolyspora erythraea* ATCC No. 11635[12] using a high fidelity polymerase. 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 due to its high GC %. The gene was sub-cloned and ligated into a pET28 vector and sequenced. To facilitate construction of a series of nucleophile mutants, which were necessary to confirm these hypotheses and evaluate glycosynthase activity, a 650 b.p. portion of the gene encompassing Asp257 was sub-cloned. This sub-cloned DNA was mutated using a quik-change mutagenesis protocol and sequenced mutants 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 sub-cloning strategy. The wild-type and mutant proteins were expressed with an encoded affinity tag and purified using nickel affinity chromatography using standard procedures.

Preparation of EryBI-template for PCR:
Saccharopolyspora erythraea (ATCC No: 11635) was purchased from the ATCC and was plated on yeast malt extract agar (ISP medium 2):

For 125 ml media:

Yeast Extract	0.5 g
Malt Extract	1.25 g
Dextrose	0.5 g
Agar	2.5 g

pH adjusted to 7.2 with KOH

After 4 days incubation at 27 °C, yellow-crusty colonies were formed. Further 5 days incubation caused formation of arial-hyphens which were white and powdery. Finally liquid cultures were prepared by incubation in shakers (100 rpm, 37 °C) for 2 days in the following medium:

MYM

Maltose	4g
Yeast Extract	4g
Malt Extract	10g
Distilled water	up to 1 Lit

pH adjusted to 7.0 with 5M NaOH

After incubation, production of erythromycin by *S. erythraea* was indicated by generation of clear zones of *Micrococcus luteus* on agar plate around paper-disks which were soaked into the inoculated liquid culture

Liquid culture (2 mL) was spun down and the resulting pellet was frozen at -70 °C. Addition of sterile water (100 μL) to the frozen pellet provided a suspension which was used as template for amplification of *eryBI* by PCR.

Amplification of truncated *eryBI* (hereafter *eryBI*):
S. erythraea has a high G+C content genome, thus it was difficult to amplify *eryBI* by PCR.
Following conditions were examined: screening different polymerases (Vent polymerase, pfU polymerase, Expand high fidelity (EHF-ROCHE), Platinum Taq, Platinum supermix, Pfx50, GC rich accuprime, AccuPrime pfx (invitrogen); different buffers and additives (Formamide, DMSO, β-Caroten, Mg concn); various template preparations (Sonication, genomic isolation); temperatures cycles, different primers and different ratio/concentration of template and primers. Successful amplification was achieved as follows:

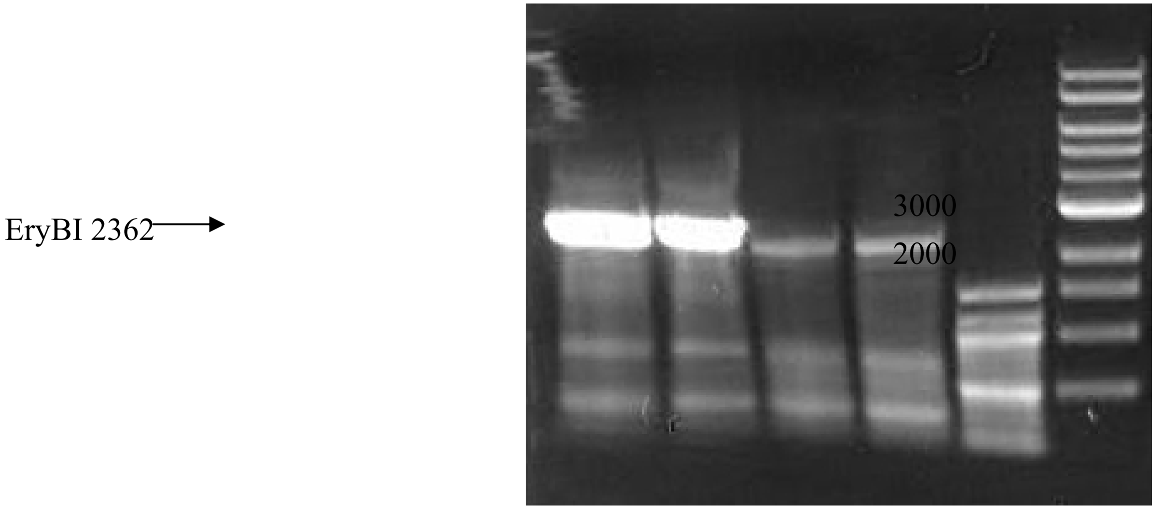
2) AccuPrime Reaction Mix	2.5 μL	
3) F-Primer EryBI-F (50 μM)	1.5 μL	5'-GCG (CAT ATG) TCG CCG GTA CGC CAT-3'
4) R-Primer EryBI-R (50 μM)	1.5 μL	5'-GCG C(GA ATT C)TC AGA GGT TGA TGT CGG-3'
5) Template (see above)	1 μL	
1) Water	18 μL	

6) AccuPrime pfx polymerase 0.5 μL was added at 95 °C.

Cycling parameters which were used for all PCR unless otherwise specified:

Step	Cycles	Temp (°C)	Time (S)
1	1	95	120
2	3*	95	30
3		60	30
4		70	120
5	30*	95	30
6		65	30
7		70	120
8	1	70	300
9	1	4	pause

Figure S6. Amplification of EryBI gene by PCR



PCR products were cloned using a Topo blunt PCR cloning kit. Upon restriction digest with EcoRI and NdeI samples were submitted for DNA-sequencing.

Ligation of *eryBI* into pET28b

After trimming DNA sequences, the desired fragments were purified and extracted from an agarose gel. Ligation steps were followed according to the manufacturer instructions of the Bio Basic rapid ligation kit. The ligation mixture was transformed into NovaBlue Giga-Singles® competent cells (Novagen, San Diego, CA). Plasmid preparations were confirmed by agarose gel analysis of digested products using restriction enzymes (HindIII , PstI, or EcoRI/NdeI) followed by DNA sequencing. Finally, *E. coli BL21 (λDE3)* Singles were transformed with *eryBI* + pET28b plasmid constructs.

Preparation of *eryBI*-D257G mutant via megaprime PCR and sub-cloning:

a) Preparation of Megaprimer-F_{D257G} :

2) AccuPrime Reaction Mix	2.5 μL	
3) F-Primer EryBI-F (50 μM)	1.5 μL	
4) R-Primer EryBI-D273G-r (50 μM)	1.5 μL	5’-GTGGCGGTCCA(TCC GGA)CATCACCC-3’
5) Template (*eryBI* + pET28b, 35 ng/μL)	1 μL	
1) Water	18 μL	

6) AccuPrime polymerase 0.5 μL was added at 95 °C.

b) Preparation of Megaprimer-R_{D257G}:

2) AccuPrime Reaction Mix	2.5 μL	
3) F-Primer EryBI-R (50 μM)	1.5 μL	
4) R-Primer EryBI-D273G-f (50 μM)	1.5 μL	5’-GGGTGATGTCCGGATGGACCGCCAC-3’
5) Template (*eryBI* + pET28b, 35 ng/μL)	1 μL	
1) Water	18 μL	

6) AccuPrime polymerase 0.5 μL was added at 95 °C.

c) Asymetric PCR:

AccuPrime Reaction Mix	2.5 μL
Megaprimer-F_{D257G} (0.1 ng/μL)	1 μL
Megaprimer-R_{D257G} (0.2 ng/μL)	1 μL
Water	20 μL

Accuprime polymerase 0.5 µL (was added at 95 °C)

*Cycling parameters:

Step	Cycles	Temp (°C)	Time (S)
1	1	95	120
2	3*	95	30
3		60	30
4		70	120
5	7*	95	30
6		65	30
7		70	120
8	1	70	300
9	1	4	pause

Followed by the second PCR:

F-Primer EryBI-F (50 µM)	1.5 µL
R-Primer EryBI-R (50 µM)	1.5 µL

AccuPrime polymerase 0.5 µL was added at 95 °C.

The PCR product was cloned into the TOPO-Blunt zero cloning kit and transformed into Top 10 competent cells. Incorporation of the mutation in the PCR product was initially confirmed by BspEI digestion and then by DNA-sequencing. Subcloning of a fragment containing D257G mutation into *eryBI*+pET28b was performed by double digestion using MreI and BamHI. The DNA fragment was ligated with appropriately digested vector and transformed into One Shot Top 10 cells. Plasmid preparations and transformation of *E. coli BL21 (λDE3)* was accomplished in preparation for protein production. DNA-sequencing confirmed the formation of the *eryBI*-D257G +pET28b plasmid construct.

Preparation of *eryBI*-D257S mutant via SDM and sub-cloning:

<u>a) PCR of mini-*eryBI* (650 bp):</u>

2) AccuPrime Reaction Mix 2.5 µL

3) F-Primer EryBI-F (5 µM)	1.5 µL	
4) R-Primer EryBI-Rmid (5 µM)	1.5 µL	5’-GGCTCGACGTAGGAGCTG-3’
5) Template (*eryBI*+pET28b)	1 µL	
1) Water	18 µL	

6) AccuPrime polymerase 0.5 µL was added at 95 °C.

The PCR product was cloned into a TOPO blunt vector to generate a PCR template for site directed mutagenesis containing 650 bp of the *eryBI* gene: Plasmid preparations were confirmed after enzymatic digestion on agarose gel.

b) Site directed mutagenesis via a modified Quikchange technique

Cycling parameters:

Segment	Cycles	Temp (°C)	Time (S)
1	1	95	60
2	**16**	95	30
		60	30
		68	**300**

5 µL of 10X reaction buffer
1 µL of dsDNA template (**NT**, at least 2.5 ng/µL)
5 µL D257S-F (5 µM) 5’-GCTGGGTGATGTCCTCGTGGACCGCCACCC-3’
5 µL D257S-R (5 µM) 5’-GGGTGGCGGTCCACGAGGACATCACCCAGC-3’
1 µL of dNTP mix (2.5 mM)
3 µL of DMSO
29 µL ddH2O (to adjust final volume to 50 µL)
Finally, 1 µL pfU polymerase (5 U/µL) was added at 95 °C.

The mixture was digested with DpnI (1.5h at 37 °C) and transformed into Nova Blue cells. Plasmid preparations were double digested using BamHI and MreI and mutated fragment sub-cloned into similarly digested *eryBI*+pET28b to generate *eryBI*-D257S+pET28b construct. Mutants were identified by enzymatic digestion (BssSI) and agarose gel analysis. They were finally confirmed by DNA sequencing and then used to transform *E. coli BL21 (λDE3)*.

Preparation of *eryBI*-D83G mutant via mini-primer and sub-cloning:

a) Preparation of Miniprimer-F_{D83G} (183 bp):

2) High G+C buffer/reaction Mix (A)	5 µL	
3) F-Primer EryBI-F (50 µM)	1.5 µL	
4) R-Primer EryBI-D83G-r (50 µM)	1.5 µL	5'-GGCCGGCCCGCCGGTGCCGCG-3'
5) Template (13 ng/µL, semi-*eryBI*)	1 µL	
1) Water	15.5 µL	

6) High G+C Accuprime polymerase 0.5 µL was added at 95 °C.

b) Preparation of semi-*eryBI*$_{D83G}$ (1361 bp):

2) High G+C buffer/reaction Mix (A)	5 µL
3) Template (*eryBI*+TOPO, 20 ng/µL)	1 µL
4) R-Primer EryBI-Rmid (5 µM)	2 µL
5) F-Primer Miniprimer-F_{D83G} (65 ng/µL, 1 µM)	1 µL
1) Water	15.5 µL

6) High G+C Accuprime polymerase 0.5 µL was added at 95 °C.

c) Sub-cloning of semi-*eryBI*$_{D83G}$

Incorporation of the mutation was confirmed by FseI digestion. A DNA fragment containing the D83G mutation was sub-cloned into *eryBI*+pET28b. After sequencing, plasmid was transformed into *E. coli BL21 (λDE3)*.

EryBI-D83G mutant was inactive in hydrolysis of β-*p*NP-Glc, β-DNP-Glc and glucosylated erythromycin. CD analysis (data not shown) of EryBI-D83G indicated a folded protein.

Thus, the predicted structure in combination with kinetic studies of active-site variants, allowed us to identify the essential catalytic residues (the nucleophile D257 and the acid/base D83) which by mutagenesis are shown to be critical for hydrolytic activity of EryBI.

Lysogenization of *E. coli*-NR698 was accomplished using the instructions as directed by the supplier Novagen.

Protein expression and purification

Standard conditions were used as described for the purification of DesR. See *Chemistry & Biology* 2008, **15**, 739-749, Glycosidase inhibition by macrolide antibiotics elucidated by STD-NMR spectroscopy, Sadeghi-Khomami,A.; Lumsden,M.D.; Jakeman,D.L.