## **Supporting Information**

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Fig. S1. SEC-MALS elution profiles of WT TarM and its trimerization interface mutant, G117R. Both proteins were run at a concentration of 25 µM. Horizontal lines correspond to calculated molecular weights as listed.



**Fig. 52.** TarM monomer catalytic site in complex with donor-substrate. (*A*) Ligand mFo-DFc OMIT maps for UDP (*Top*) and UDP-GlcNAc (*Bottom*) contoured at  $3 \sigma$ . (*B*) Interaction of active site residues with cleaved UDP remnant (*Left*) and intact UDP-GlcNAc (*Right*). Substrates and relevant residues are displayed in stick form and colored according to heteroatom type, with carbons of enzyme residues displayed in green and those of UDP/UDP-GlcNAc in yellow. Polar interactions between atoms are displayed by dotted lines. Backbone carbonyls and amides are displayed as red and blue spheres, respectively, where applicable.



Fig. S3. The products of TarM reaction mixtures containing 10 mM UDP-GlcNAc  $\pm 1$  mM fondaparinux (Fonda) and  $\pm 40\%$  glycerol where indicated were diluted 1:40 (vol/vol) with MeOH and analyzed via flow injection analysis and detected by electrospray ionization MS (positive mode).



**Fig. S4.** NMR analysis of glyceryl-GlcNAc per-O-acetate. (*A*) Heteronuclear single quantum coherence of glyceryl-GlcNAc; (*B*) heteronuclear multiple bond correlation ( $^{1}$ H,  $^{13}$ C): The only C-1 correlation detected was with H-7a and H-7b; (C)  $^{1}$ H spectrum of the anomeric proton showing a typical axial ( $\alpha$ ) glycoside coupling of  $J_{1,2} = 3.6$  Hz.



Fig. S5. SEC-MALS analysis of WTA polymer size. The horizontal line corresponds to a molecular weight distribution of ~9-11 kDa.



Fig. S6. Biolayer inferometry analysis of TarM-WTA association/dissociation constants. Association/dissociation curves were obtained with biotinylated WT/ G117R TarM loaded on a streptavidin sensor and titrated with various WTA concentrations (0.31 mM, 0.62 mM, 1.25 mM, 2.5 mM, and 5 mM).



**Fig. 57.** Effect of various ligands on TarM stability and study of enzyme kinetics and active site binding of fondaparinux. (A) Difference in thermostability ( $\Delta T_{agg}$ ) of TarM with increasing concentrations of various ligands, analyzed by differential static light scattering (StarGazer) as a measure of  $T_{agg}$  upon thermodenaturation. (B) The effect of fondaparinux on TarM catalysis, studied by fluorescence detection of the cleaved UDP product in the presence of 1 mM UDP-GlcNAc. Increasing concentrations of fondaparinux led to increased turnover of UDP-GlcNAc. The derived  $K_m$  value (compared with 540 ± 50 µM for WTA) indicates the higher affinity of fondaparinux for the acceptor substrate binding domain. (C) Overall view of the TarM active site with occupying fondaparinux, UDP, and  $\alpha$ -glyceryl-GlcNAc. Substrates and relevant residues are displayed in stick form and colored according to heteroatom type, with carbons of enzyme residues displayed in green, fondaparinux in yellow, UDP in orange, GlcNAc in blue, and the attached glyceryl moiety in purple. Polar interactions between atoms are displayed by dotted lines.



Fig. S8. Schematic of the proposed TarM reaction mechanism. (A) GT reaction between UDP-GlcNAc and glycerol resulting in UDP and  $\alpha$ -glyceryl-GlcNAc products. (B) Proposed front-face oxocarbenium ion-like transition state preceding product formation. (C) TarM ternary complex with bound UDP and  $\alpha$ -glyceryl-GlcNAc. Polar interactions between atoms are displayed by dotted lines.

S_aureus_TarM	1VIKI MWYELDINKGGITSSMFNRSHEFY ADIPADIVTEDYKGN-YDDIIKALKKQ
S_epidermidis	1VIKI MWYELDINKGGITSMINRSHYEL NGIG NIVTEDYKIN-YSDIINCHAS
T_halophilus	1VNNI MWETIG NKGGITSMILNSHYEKNSYPADIVTEDYKYN-SSKW KELKKS
L_grayi	1VTQVVILGS KENNEGIANAMYYRSTEFYNEGIRADIVTIDYNN-YATIIKN KAA
B_subtilis_TarM	1YIRI SLIE DIEKGGITSSMMSRSFTEA RGHKID VILDYNEN-YDQIEKELKNA
Kurthia_sp_TarM	1 MTK-KV MLVTVD DIKGGINSWFRRTHENNDLYTSDIITIDKPYGYADIEATHAD
S_aureus_TarM	58 57GKMLERIKMYN BYFROISNN HFK NKLLYKH SERIKN ETE SKGISRYF
S_epidermidis	58 57NKMD RICMFNESIYFR SNE HENYNOVLNEK LNI K T.EIK SKNISR F
T_halophilus	58 57GKMYSETK FNORDFESS LVTHSEKNOFLY YYDHIMN S CTVOLEKISRYF
L_grayi	58 57NKMDFRICMIN FT SDESL YLNF DSS-YKLA RLAAD TFKKTOKKEGTE YI
B_subtilis_TarM	58 57G HADVN IN DD -KNOH IKVGNAQQSYE NRNF FE PYA FHNEEKLETE
Kurthia_sp_TarM	61 60G C SSH IN DY KMT DKKV DEDREHYE HKSEKKGYKTOKPHATK
S_aureus_TarM	113 DI 115 GTV AYI K-SKSEKV DEEK NKR ERFSEIDNKY MKE ENVDNKY
S_epidermidis	113 NEN 115 GEV AYKRD-TENELHEDIEENKRYRRIYFYNNK HK DIENHNNE
T_halophilus	113 SKI 115 GEV IYKKGNEETGNYV DREENORKERVYYRKYVKE KE DYNR
L_grayi	114 SP 116DNNIW TYKINADNN NY DIEO GERKERAI ROGKIK COR ERTDGSK
B_subtilis_TarM	114 YFK 116NGLY KKK DESNN IY DHENENOQ TOR-E SGAY NYTYFDT SGK
Kurthia_sp_TarM	116 YFL 118NGO YKHKK DAGK AS DY HLTIR IAYTEEYHPAGY I KKY HP NGG
S_aureus_TarM	164 CYQ FYD 170 KG- PY SE INANNEAVG TY EVN KEEKNNLALGYYLLEKL
S_epidermidis	164 TAEQFYD 170 FG- LY YR INPLKSVGNTYLLCDKKQFKNNVEFCYFLEQL
T_halophilus	166 ISERF D 172 LG- FFLRN INRETGKVGK YLLTDNKQFENSELCYFLSGL
L_grayi	167 KSETIN 173SEE-EPYTEFIFDEE-KPTTHLLGV KEFSTD LGIHDEQL
B_subtilis_TarM	168 TNQ HYT 174APDG CYLTEWHHFNTGASQG MLFERDEKEAVFEK KHTFH HILEKL
Kurthia_sp_TarM	172 NQ LHY 178TADGHCYLARWFNSGTGKQQ VVLFSPDTKQAIGENNNEFHCYFLEL
S_aureus_TarM S_epidermidis T_halophilus L_grayi B_subtilis_TarM Kurthia_sp_TarM	215       IKUSKE-SIMI       224 CDGPGSFPK       FIGNHKNPCKY VIHVNHH       - DDT AFKKSEKY         215       ITTSKD-NIMI       224 CDGPGSFPK       FIGNHKNPCKY VIHVNHK       - DDS AKKKENY         217       IELRS-NIII       226 CDGPGSFPK       FIGNKY       FITNHINV-RNKEKNKETY         217       IELRS-NV       226 CDGPGSFPK       FIGNHKKYA       FITNHINV-RNKEKNKETY         217       IELKS-NV       226 CDGPGKVFPK       FIGNHKYA       FITNHINV-RNKEKNKETY         224       CBREKE-FIT       233 CDGVGSASK       NEMEGLAK       YC       HNHL       YPTTISK       KNHQY         228       ARQOTUKPYMI       238 CDGPGSAYK       OLDALAHH       IYA HHYO       APYTRGSV       KDNHKR         H249       H249       H249       H249       H24       H24       H24
S_aureus_TarM S_epidermidis T_halophilus L_grayi B_subtilis_TarM Kurthia_sp_TarM	269       I EN-NK NGVIVIT       283BAQ IDI NQPD ENI TISNE KIHNAPKHFQTEKIVG         269       ILKNSKN DGVI II       283BAQKDI TID ENAWISNE TNEKEECSTTNSNIVG         268       ILKN DQ DGVIVI       283BAQKDI TID ENAWISNE TNEKEECSTTNSNIVG         268       ILKN DQ DGVIVI       282BSQKNDI ID EN NN SALSNE EIPDVVNKPNNDSKKIVG         272       IFKH DA DGTIVNT       286GKQCHI D EN KN WIFNEN V DKK ISHRKTKD V         279       A EHIKDYDG IVIT       293NEQKEDI DE SNNIF IPAPRRLE LQTYEKKKNEFV         284       FINDAPHVPVVVI       298BHQKKD EADFGDNFENY ISHAMNV EQMIKKQPYTAS         N304       N304
S_aureus_TarM S_epidermidis T_halophilus L_grayi B_subtilis_TarM Kurthia_sp_TarM	<ul> <li>323 H SENVPTKRILLILEVAE 341 VV K NAWKFHLYGEGSV D. AK DE KNLERNVF</li> <li>323 H SRLVPCKG SYLLEVAE 341KVVEK EK EFH YGTGEE O. DEKOTONKNLTENVK</li> <li>324 VSRLV NKGFVYLLEVAK 342DTSIYSNYSFNIYGEGEY. S. VEELIO NGLENNFK</li> <li>326 FSRLMKKGIONAHKAFA 344LVCENPAARLILYGKG YEABURTILS KNLSNNVF</li> <li>335 VVFH EKGINAHKAFA 355VV DEPAVLNIYG GEOEAATRKLTKLKLTKHVF</li> <li>338 VSRLA EKR DLAHRAFK 356VV DEPAVLNIYG GEOEAATRKLTKLKLTKHVF</li> <li>336 K331</li> </ul>
S_aureus_TarM S_epidermidis T_halophilus L_grayi B_subtilis_TarM Kurthia_sp_TarM	379       LKGYTTTEOKC EDEKLVVSTSC       402YEGOGLS IEAM SKIPVVAFDIKYGPSDFIED         379       ILGYTNAIEK KNEGCVVSTSC       402 EGOGLS IEAM IK PVVAFDIKYGPSDFIED         380       IMGYTN ENKA SEDCVVSTSC       403 EGOGLSIEAM OC PVVFM KSCPSDFIKH         382       MGYTN IKV KNKIAS STSE       405 EGOSLSIEAM OC PVVFM KSCPSDFIKH         390       LKCYIANEAPFYQQALAT LTSK       413 EGFSIAICSSTCAPV SPDINGPSDTISD         394       LKGYTTADAVFAESOFT ITSR       417 EGGSLIA PANKTAVSFDINYLVE MYDN         394       LKGYTTADAVFAESOFT ITSR       417 EGGSLIA PANKTAVSFDINYLVE MYDN
S_aureus_TarM	435 NKNGYLTEN NIND A KI OL NNDV 461LBAEFGSKARENTI KYSTES EKWLNL
S_epidermidis	435 GKNGYLTEN NIEAPSYL KI KDQN 461LBAEFGSKARENTI KYSTES EKWLNL
T_halophilus	436 EQNGILTEN DTEK PNDT KLYNEE 46274 KMGK AREDIIKOYRFEL QOBEK
L_grayi	438 GETGLIESGN EA PNKI KAL HKK 464EM KMGKRAQAS Q PPE RICKDL
B_subtilis_TarM	446 HETGLIESGN EA PNKI YAL HKK 464EM KMGKRAQAS Q PPE RICKDL
Kurthia_sp_TarM	450 PAM-ST PDGD EA PA AT EL KDPA 476RE AMGEV YV KQHYSFESQYEKWHAL
S_aureus_TarM S_epidermidis T_halophilus L_grayi B_subtilis_TarM Kurthia_sp_TarM	491       NS       493         491       K       492         492       LDLQ       495         494       FATA       497         502       EMTR       506         506       GQQLDEK       512

Fig. S9. Sequence alignment of *Staphylococcus aureus* TarM with other bacterial species and genera. *S. aureus* TarM (National Center for Biotechnology Information reference sequence YP\_185908.1) was aligned with sequences from *Staphylococcus epidermidis* (WP\_002502944.1), *Tetragenococcus halophilus* (YP\_004887116.1), *Listeria grayi* (WP\_003756742.1), *Bacillus subtilis* (WP\_19259626.1), and *Kurthia* sp. (WP\_110286982.1). Similar sequences were identified with BLAST by using the nonredundant protein sequence database. Sequence alignment was performed with ClustalW. Letters blocked in black indicate complete identity, and those blocked in gray indicate similarity to the column consensus.

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Fig. S10. HPLC chromatogram of UDP-GlcNAc hydrolysis with increasing concentrations of glycerol. UDP-GlcNAc was incubated with WT TarM in the presence of increasing glycerol and 1 mM fondaparinux (+ Fonda) as indicated for 1 h. UDP-GlcNAc and the hydrolyzed UDP were separated on a DEAE weak anion exchange column and the peak signals detected at 254 nm. UDP-GlcNAc in the absence of TarM is shown as a control.



**Movie S1.** Structural morph between TarM open and closed states. Open state from TarM:UDP complex and closed state from TarM:UDP-GlcNAc complex (found in asymmetric unit of TarM G117R structure). Fondaparinux (yellow), glyceryl-GlcNAc (blue), and UDP (orange) substrates shown as sticks to illustrate donor/acceptor binding sites.

Movie S1