Supporting Information:

An antagonism screen for inhibitors of bacterial cell wall biogenesis uncovers a novel UppS inhibitor

Authors: Maya A. Farha[a,b], Tomasz L. Czarny[a,b], Cullen Myers[a,b], Liam J. Worrall[c], Shawn French[a,b], Deborah G. Conrady[c], Yang Wang[d], Eric Oldfield[d], Natalie C. Strynadka[c], Eric D. Brown[a,b*]

Affiliations:

[a] Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada.

[b] Michael G. DeGroote Institute of Infectious Disease Research, McMaster University, Hamilton, Ontario L8N 3Z5, Canada.

[c] Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada.

[d] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana IL 61801, USA

[*] To whom correspondence should be addressed (ebrown@mcmaster.ca)

SI Methods

Screen for antagonism with targocil in *S. aureus*.
An overnight of *S. aureus* (strain Newman) was grown in 5 mL of Mueller Hinton Broth (MHB) at 37 °C shaking at 250 rpm Following 16 h of growth the overnight was diluted 100-fold into fresh MHB broth. Once cells have reached an OD600 between 0.3 and 0.5 the cells were diluted 1,000 fold into fresh MHB broth. The culture was screened against the Pharmakon library in the presence of targocil. Screening was carried out at a 50 μL assay volume in 384-well plates (Corning MA) with a library compound concentration of 20 μM and a targocil concentration of 16 μg ml^{-1} (8xMIC value obtained under the same conditions). The final DMSO content of the screen was 2%. Thirty-two low controls per assay plate contained media with Erythromycin at 16 μg ml^{-1} and 32 high controls contained media with 2 % DMSO. Plates were incubated at 37°C shaking at 250 rpm for 12 hours and optical density was read at 600 nm using an EnVision plate reader (Perkin Elmer).

Analysis of screening data
The percent of rescued growth for each test well was calculated as (OD – mean IQ) / (mean high control – mean IQ) * 100; where mean IQ is the mean of the middle 50 % of the ordered raw data on the corresponding plate (1). A value of 0% represents no growth on the plate and 100% represents full rescue of targocil activity. A hit cut-off was determined by calculating the mean and standard deviation of the full dataset. A statistical cutoff equal to the mean + 4 standard deviations was established.

Suppression of targocil in dose and growth inhibition against *S. aureus*
Tests for targocil suppression in dose and inhibition of *S. aureus* were setup with the same conditions as the screen, except in 100 μL volumes in 96-well plates (Corning MA). Compounds were tested at concentrations between 50 μM and 0.4 μM with half-fold dilutions between and, only in the case of tests of targocil suppression, targocil was present in all wells at a concentration of 16 μg/ml. Plates were incubated for 16 hours at 37 °C shaking at 250 r.p.m.; following which optical density was read at 600nm using an EnVision plate reader (Perkin Elmer).

Checkerboard analysis
FICs were determined by setting up standard checkerboard broth microdilution assays with serially diluted 8 (or 9) concentrations of each drug, with conditions based on CLSI protocol. At least 3 replicates were done for each combination and the means used for calculation. The MIC for each drug was the lowest [drug] showing <10% growth. The FIC for each drug was calculated as the [drug in the presence of co-drug] for a well showing <10% growth, divided by the MIC for that drug. The FIC index is the sum of the two FICs. Interactions with FIC Index of less than 0.5 were deemed synergistic, and above 2, antagonistic.

Antisense interference
Since all strains displayed variable sensitivity to xylose, on average, strains were grown using their IC_{30} values to test for sensitivity to clomiphene. At this level, we observed a

modest growth defect that would allow us to capture further sensitivity. The various strains were exposed to a range of concentrations of clomiphene (0-32 μg/mL) in 96-well plates that included both ± xylose. Plates were incubated at 37°C for 16 hours and absorbance at 600nm was read. A ≥4 shift in the inhibitory concentration of clomiphene upon xylose induction was the upper limit of sensitivity. Fosfomycin was used as a positive control with the antisense strain *murA*.

Quantification of staphyloxanthin levels

Cultures of *S. aureus* (strain Newman) were grown in the presence of varying concentrations of clomiphene for 24 hours. Cells were pelleted and washed in PBS. The final pellet was resuspended in MeOH and placed 40°C water bath for 25 mins. Samples were centrifuged and the supernatant was read at 450nm. Values were normalized to initial optical density (600 nm).

High-copy suppression

E. coli MC1061 harboring pCA24N-uppS was grown overnight in LB supplemented with 50 μg/ml kanamycin, subcultured the following day (1:100 dilution in the same media) and grown to mid log (OD_{600} = 0.50) at 37°C, 250 rpm. Culture was split; one uninduced and the other induced with 1 mM IPTG, both grown for 4 hours at 30°C. Cells were then diluted 1:200 and 1μL spotted on solid agar containing 2-fold serial dilutions of clomiphene. Plated were incubated at 37°C with 80% humidity for 18hr.

Chemical supplementation

S. aureus or *B. subtilis* cultures were prepared according to the CLSI protocol and plated in 96W plates in the presence of various concentrations of inhibitors or inducer in the case of conditional deletion strains and in the presence of 150μM Und-P (Larodan, Sweden). Plates were incubated at 37°C for 18hr.

***In vitro* UppS assay**

The inhibition of UPPS by clomiphene was assessed with a coupled, spectrophotometric assay that monitored diphosphate production. Assays (100 mL) were performed at 37°C in 96-well microplates (Costar) and contained 50 mM Tris-HCl (pH 7.3), 1 mM $MgCl_2$, 50 mM KCl, 0.005% triton X-100 (w/v), 8 mM farnesyl diphosphate (Sigma), 80 mM isopentenyl diphosphate (Sigma), 0.5 units of purine nucleotide phosphorylase (Sigma), 0.2 units of *E. coli* inorganic pyrophosphatase (Sigma), 400 mM of 7-methyl-6-thioguanosine (Berry and Associates), 0.15 ng of UPPS and 0.122 – 250 mM of clomiphene citrate (Sigma). DMSO was present at a final concentration of 2% (v/v). Reactions were initiated by the simultaneous addition of farnesyl diphosphate and isopentenyl diphosphate after preincubation of UPPS with clomiphene for 30 minutes, and the absorbance at 355 nm was then continuously monitored for 10 minutes on a SpectraMax Plus microplate reader (Molecular Devices). For each concentration of clomiphene, phosphate production was expressed relative to the DMSO control to generate a dose-response curve that was fit to the non-linear regression model

$$\%\ \text{activity} = \min + \frac{\max - \min}{1 + \left(\frac{[I]}{IC50}\right)^{-h}}$$

using GraphPad Prisim 6.0, where "min" is the minimum response in the assay, "max" is the maximum response in the absence of inhibitor, [I] is the clomiphene concentration (mM), "h" is the Hill slope and IC_{50} is the concentration of clomiphene that inhibits enzyme activity by 50%.

Purification of UppS

Full-length *E. Coli* UppS was cloned into pET-28a. Expression was carried out using BL21(DE3) grown in LB media for 18 hours at 20 deg C following induction with 0.5 mM IPTG at a cell density of OD600 1-1.4. Cells were resuspended in 20 mM Hepes pH 7.5, 500 mM NaCl, EDTA free protease inhibitor tablets (Roche) and DNAse and lysed with an Avestin Emulsiflex-C5. Clarified lysate was manually loaded onto a 5 mL HisTrap HP column (GE Healthcare) equilibrated with 20 mM Hepes pH 7.5 and 500 mM NaCl. The column was washed with 6 cv equilibration buffer supplemented with 50 mM imidazole and 4 cv 100 mM imidazole. Protein was eluted with 2 cv each equilibration buffer supplemented with 200, 300 400, 500 and 1000 mM imidazole and fractions containing pure protein pooled. The eluate was dialyzed against 20 mM Hepes pH 7.5, 150 mM NaCl in the presence of thrombin to remove the N-terminal His-tag. The protein was concentrated and applied to a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in tris pH 7.4, 150 mM NaCl with fractions containing ecUppS pooled and concentrated to 5-10 mg/ml.

Crystallization and refinement

EcUppS (5-10 mg/mL) was crystallized by sitting drop vapor diffusion in the presence and absence of 1 mM clomiphene using a reservoir solution of 100 mM MES pH 6.5, 0.16 M calcium acetate, 20 % PEG 8000, 20 % glycerol (+ clomiphene) or 0.1 M lithium sulfate, 0.1 M ADA 6.5, 12 % w/v PEG 4K, 20 % glycerol (- clomiphene). Prior to data collection crystals were flash cooled directly from the drop. Crystals belonged to space group P212121 with unit cell dimensions a ~ 64 Å, b ~ 68 Å, c ~ 111 Å and two monomers in the asymmetric unit. All data were collected on beam line 08ID-1 at the Canadian Light Source at 100 K and a wavelength of 0.978 Å. Data were processed with Xia2 (2) using XDS (3) to integrate and scale the data and Aimless (4) to merge. The structures were solved using Phaser (5) using PDB 1X06 as a molecular replacement search model. Refinement and model building was carried out with Phenix (6) and Coot (7) respectively. The refined models have good stereochemistry with 97.1 % Ramachandran favoured and 0.5 % disallowed (+clomiphene; disallowed residue Phe204 in both chains supported by density)) or 0.7 % disallowed (- clomiphene; in addition to Phe204, Asp94 (chain B), which is poorly resolved). Density consistent with clomiphene was resolved in one monomer in the asymmetric unit. Weak but uninterpretable density was observed in the other monomer of the asymmetric unit, consistent with preferential binding to one monomer in previous EcUppS structures e.g. PDB ID 4H38, 2E9A and 3SGX. Data collection and refinement statistics can be found in Table S2. Data and coordinates have been deposited to the protein data bank with accession codes 5CQB (-clomiphene) and 5CQJ (+clomiphene).

Quantitative microscopy

B. subtilis 168 was cultured as previously described, in the presence of an 8 μg/mL sub-MIC concentration of clomiphene. Cells were imaged using the method of Czarny *et al.* (8), using a Nikon Eclipse TE200. About 1000 cells we imaged per treatment, and cell widths were quantified in batch using ImageJ (9). Briefly, images were converted to 8-bit greyscale, then background subtracted with a 25 pixel rolling ball radius. Background subtracted images were then thresholded (Otsu algorithm), and binary masks were subjected to a watershed transformation to separate adjacent cells. Cell widths were calculated using the minimum caliper diameter. Results from quantitative imaging were assessed for normality, then statistically analyzed using a Mann-Whitney non-parametric test.

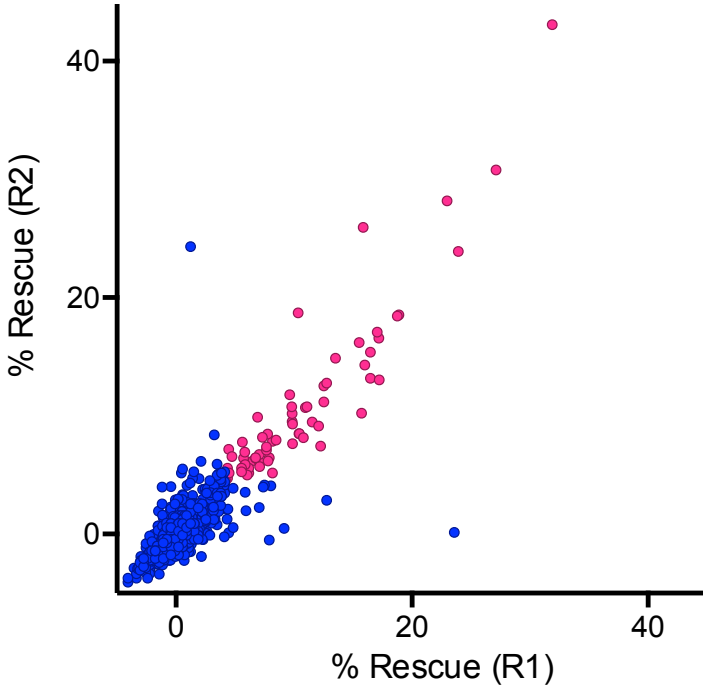

Fig. S1. Replicate plot and hit selection for the primary targocil antagonism screen of 1,600 previously approved drugs (PADs). PAD compounds above the 4.15 % cutoff were deemed hits and are depicted as red circles. Compounds, which did not register as hits, are depicted as blue circles.

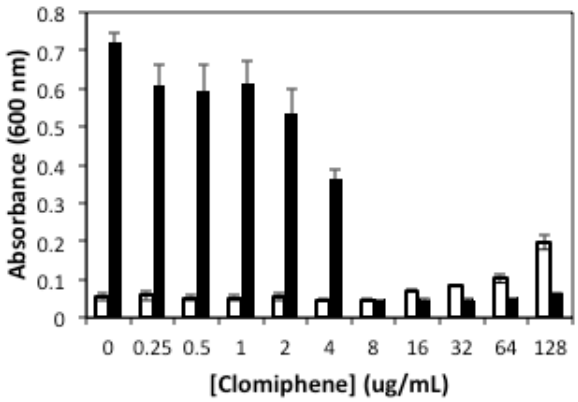

Fig. S2. Increasing concentrations of clomiphene can suppress the lethality associated with late WTA stage deletion. Shown is the growth of a *tagB* conditional deletion strain (as measured by absorbance at 600 nm) in the absence of the inducer xylose (white bars) and upon induction with 2% xylose (black bars). At concentrations starting at 32 μg/mL, clomiphene begins to recover the growth of the *tagB* conditional deletion in the absence of xylose, as shown in white bars.

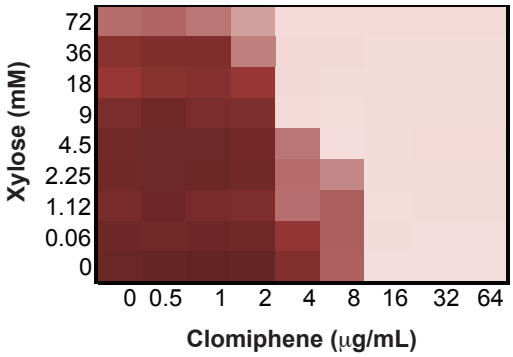

Fig. S3. Dose matrix assay showing increasing concentrations of the inducer, xylose, and clomiphene against the *uppS* antisense strain. The extent of inhibition is shown as a heat plot, such that the darkest red color represents full bacterial growth.

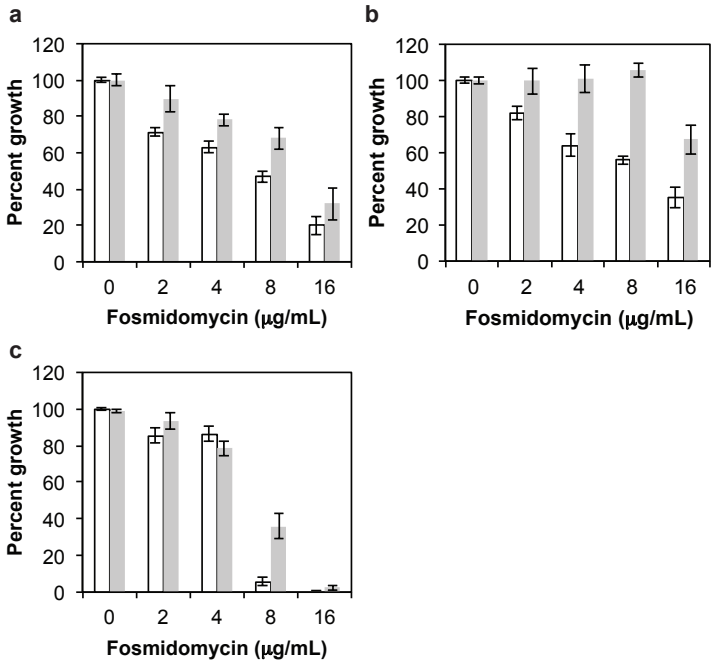

Fig. S4. The activity of fosmidomycin is suppressed upon chemical supplementation of substrates downstream of its cellular target in *B. subtilis*. (*A*) Supplementation by exogenous isopentenyl pyrophosphate (IPP) (100 μM) (*B*) Supplementation by exogenous farnesyl pyrophosphate (FPP) (100 μM) and (*C*) Supplementation by exogenous Und-P (150 μM).

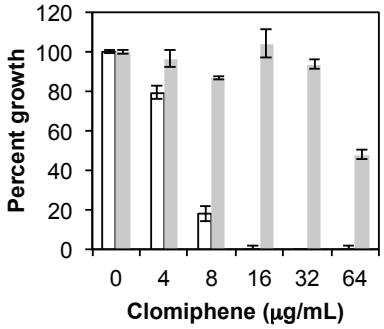

Fig. S5. Addition of exogenous Und-P (150 μM) (grey bars) suppresses the inhibitory activity of clomiphene against *B. subtilis* (white bars).

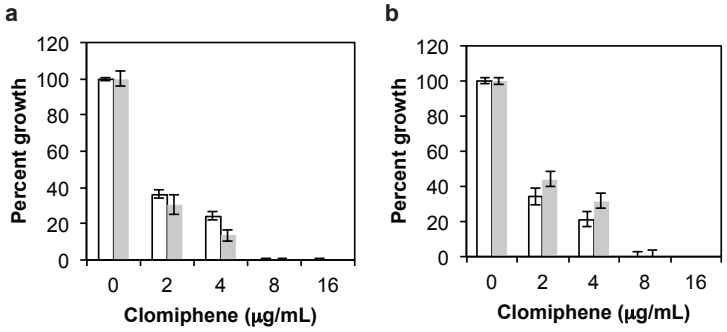

Fig. S6. Activity of clomiphene is not suppressed upon addition of exogenous (*A*) IPP and (*B*) FPP (grey bars) against *S. aureus*.

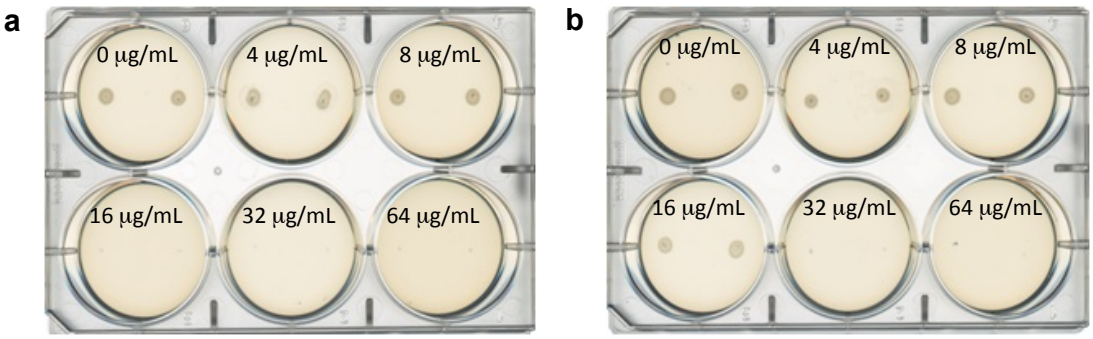

Fig. S7. Overexpression of UppS can suppress the inhibitory activity of clomiphene in *E. coli* MC1061. (*A*) Uninduced conditions where clomiphene has a MIC of 16 μg/mL. (*B*) Upon induction with 1mM IPTG to increase the expression of UppS, a two-fold suppression of activity, with clomiphene having an MIC of 32 μg/mL is observed. Duplicate samples are shown.

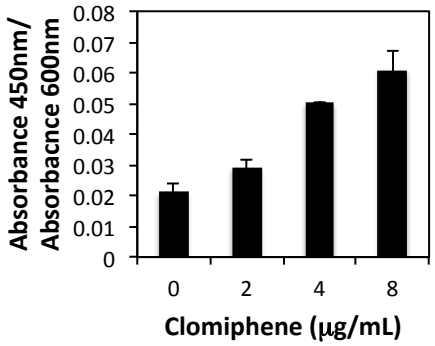

Fig. S8. Staphyloxanthin levels upon treatment with clomiphene. Staphyloxanthin was extracted by methanol and measured at 450 nm by spectrophotometry and normalized to bacterial growth (absorbance at 600nm). Data represents the means ± standard errors of the means (n=3) of one representative experiment.

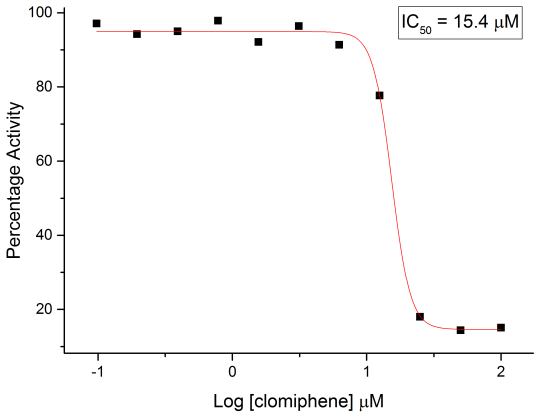

Fig. S9. Dose-response curve of clomiphene against recombinant *E. coli* UppS, yielding an IC_{50} value of 15 μM. Inhibition assay was carried out as described previously (10).

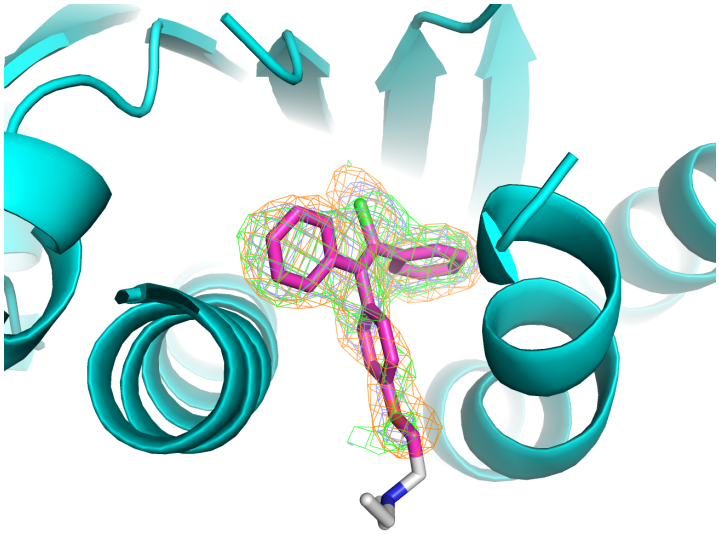

Fig. S10. Electron density maps for clomiphene. 2mFo-DFc (blue), SA-OMIT m2Fo-DFc (green) and PHENIX feature enhance map (orange) (all contoured at 1 sigma). Grey region of ligand not resolved and excluded from refinement.

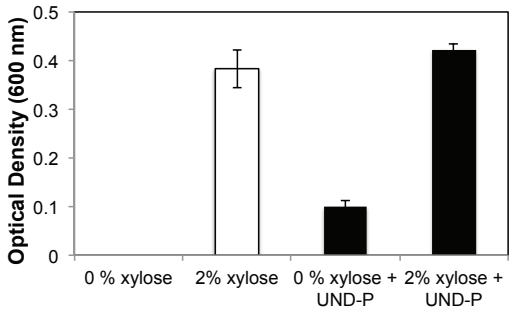

Fig. S11. Addition of exogenous Und-P can suppress the lethality associated with late-step deletion. Shown is the growth (optical density at 600 nm) of the conditional *tagF* deletion strain in the absence (white bars) and presence (black bars) of exogenous Und-P (150 μM) for both uninduced and induced (2% xylose) conditions.

Table S1. FIC indices of clomiphene in combination with various antibiotics against *B. subtilis*

Antibiotic	FIC Index[1]
Ampicillin	0.375
Cloxacillin	0.375
Nafcillin	0.375
Piperacillin	0.5
Cefmandole	0.5
Cefaclor	0.313
Cephalexin	0.375
Cefadroxil	0.5
Cefuroxime	0.313
Vancomycin	1
Bacitracin	0.375
Chloramphenicol	2
Cycloserine	0.5
Erythromycin	2
Fosfomycin	0.625
Fosmidomycin	0.38
Kanamycin	2
Norfloxacin	2
Novobiocin	2
Spectinomycin	2
Tetracycline	2

[1] Fractional Inhibitory Concentration Index: FIC index = $FIC_{clomiphene} + FIC_{antibiotic}$, where (FIC) = $[X]/MIC_X$, where [X] is the lowest inhibitory concentration of drug in the presence of the co-drug.

Table S2. Antisense strains and their respective relative hypersensitivity in the presence of clomiphene.

Pathway	Gene	Hypersensitivity
Isoprenoid	mvaD	-
	fni	-
Und-P synthesis	uppS	+++
Intracellular PG	glmM	-
	fmhB	-
	glmS	-
Extracellular PG	murA	++
	murB	++
	murC	++
	murD	++
	murE	++
	murF	++
	murZ	++
	mraY	++
	pbpA	++
	pbp2	++
Cell division	ftsA	+
	ftsZ	++
Miscellaneous	nrdF	-
	rplM	-
	hu	-
	tarL	-

Table S3. Data collection and refinement statistics for ecUppS

	ecUppS -clomiphene	ecUppS +Clomiphene
Data collection		
Space group	P 21 21 21	P 21 21 21
Cell dimensions		
a, *b*, *c* (Å)	62.98, 68.23, 111.41	63.31, 68.49, 111.63
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	46.28 - 2.2 (2.279 - 2.2)	26.51 - 2.15 (2.227 - 2.15)
R_{sym} or R_{merge}	0.129 (0.998)	0.083 (0.449)
I / σI	13.11 (1.83)	14.08 (2.03)
Completeness (%)	99.91 (100.00)	93.56 (69.43)
Redundancy	5.4 (5.4)	3.4 (2.1)
Refinement		
Resolution (Å)	46.28 – 2.2	26.51 – 2.15
No. reflections	25021 (2460)	25330 (1733)
R_{work} / R_{free}	0.185/0.249	0.183/0.244
No. atoms		
Protein	3309	3304
Ligand/ion	10	23
Water	74	96
B-factors (Å^2)		
Protein	42.10	33.70
Ligand/ion	60.30	53.10
Water	38.70	31.50
R.m.s. deviations		
Bond lengths (Å)	0.008	0.008
Bond angles (°)	1.03	1.06

*Values in parentheses are for highest-resolution shell.

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