JB Accepted Manuscript Posted Online 15 August 2016
J. Bacteriol. doi:10.1128/JB.00507-16

Depletion of undecaprenyl pyrophosphate phosphatases (UPP-Pases) disrupts cell envelope biogenesis in *Bacillus subtilis*

Running Title: Functionally redundant UPP-Pases in *Bacillus subtilis*

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Abstract

The integrity of the bacterial cell envelope is essential to sustain life by countering the high turgor pressure of the cell and providing a barrier against chemical insults. In *Bacillus subtilis*, synthesis of both peptidoglycan and wall teichoic acids requires a common C_{55} lipid carrier, undecaprenyl-pyrophosphate (UPP), to ferry precursors across the cytoplasmic membrane. The synthesis and recycling of UPP requires a phosphatase to generate the monophosphate form Und-P, which is the substrate for peptidoglycan and wall teichoic acid synthases. Using an optimized CRISPR-dCas9 based transcriptional repression system (CRISPRi), we demonstrate that *B. subtilis* requires either of two UPP phosphatases, UppP or BcrC, for viability. We show that a third predicted lipid phosphatase (YodM), with homology to diacylglycerol pyrophosphatases, can also support growth when overexpressed. Depletion of UPP phosphatase activity leads to morphological defects consistent with a failure of cell envelope synthesis and strongly activates the σ^M-dependent cell envelope stress response, including *bcrC* which encodes one of the two UPP phosphatases. These results highlight the utility of an optimized CRISPRi system for investigation of synthetic lethal gene pairs, clarify the nature of the *B. subtilis* UPP-Pase enzymes, and provide further evidence linking the σ^M regulon to cell envelope homeostasis pathways.

Importance

The emergence of antibiotic resistance amongst bacterial pathogens is of critical concern and motivates efforts to develop new therapeutics and increase the utility of those already in use. The lipid II cycle is one of the most frequently targeted processes for antibiotics and has been intensively studied. Despite these efforts, some steps have remained poorly defined, partly due to

genetic redundancy. CRISPRi provides a powerful tool to investigate the functions of essential genes and sets of genes. Here, we used an optimized CRISPRi system to demonstrate functional redundancy of two UPP-Pases required for conversion of the initially synthesized UPP lipid carrier to Und-P, the substrate for the synthesis of the initial lipid-linked precursors in peptidoglycan and wall teichoic acid synthesis.

Introduction

In bacterial peptidoglycan synthesis, a 55 carbon polyisoprenoid lipid carrier called undecaprenyl-pyrophosphate (UPP) is required to transport peptidoglycan precursor across the cell membrane (1). UPP is synthesized by UppS and then dephosphorylated by a UPP phosphatase (UPP-Pase) to Und-P (2). The MraY enzyme uses Und-P as a substrate together with UDP-MurNAc-pentapeptide to synthesize lipid I, the first membrane-bound precursor of peptidoglycan synthesis (3). Addition of N-acetylglucosamine (GlcNAc) by MurG completes the synthesis of the critical intermediate, lipid II. Lipid II is a single GlcNAc-MurNAc-pentapeptide unit linked to UPP as the lipid carrier and serves as a substrate for penicillin-binding proteins (PBPs) which incorporate the disaccharide unit into the growing glycan strands.

Our understanding of this critical lipid II cycle is compromised by a lack of mechanistic understanding for key steps. The identity of the lipid II flippase required for the export of this essential precursor from the cytoplasmic to the external face of the membrane has been controversial. Recent results provide strong support for a role of MurJ as the lipid II flippase (4) and have further shown that this enzyme is redundant in function with a stress-regulated alternate to MurJ (Amj) protein (5). The recycling of the UPP carrier to Und-P also involves redundant enzymes, perhaps to allow the conversion to occur on either the inner or outer face of the

membrane. UppS synthesizes UPP on the cytosolic face of the membrane. Subsequent dephosphorylation of UPP, also presumed to occur on the cytosolic face of the inner membrane, generates Und-P as substrate for MraY and TagO (6). After transporting cell wall precursors to the outside of membrane, the lipid carrier is released as UPP, which must again be recycled back to Und-P by the action of a UPP-Pase (7). This may occur by dephosphorylation on the outer face of the membrane, or by flipping of the UPP across the membrane to serve as a substrate for a UPP-Pase active on the inner face of the membrane. It is assumed that flipping of UPP (or Und-P) from the outer to the inner face of the membrane is facilitated by proteins, but the flippase has not been identified (6). It may be advantageous, in general, for cells to have UPP-Pase activity localized to both faces of the membrane to facilitate *de novo* Und-P synthesis (on the inner face) and UPP recycling (on the outer face), and this may account in part for the redundancy commonly observed in UPP-Pases (6-8).

In Gram-positive bacteria, the same UPP carrier is shared between the peptidoglycan and the wall teichoic acid (WTA) biosynthesis pathways. For WTA synthesis, Und-P serves as a substrate for TagO (9). As a result, mutations in later steps in WTA synthesis are lethal due to the sequestration of the limiting UPP carrier in dead-end products (10), and this observation has motivated the search for antibiotics active on late stages of WTA synthesis (11). A similar sequestration effect has been reported in *Escherichia coli,* when synthesis of the enterobacterial common antigen was impaired (1), and in *Streptococcus pneumoniae* mutants defective in synthesis of serotype 2 capsule (12).

As expected for a critical lipid carrier, the synthesis and recycling of UPP is essential and is therefore an excellent target for antibacterials. Recent *in silico*, *in vitro*, and *in vivo* approaches have identified inhibitors of UppS (13-15), including a method that used CRISPR (clustered

94 regularly interspersed short palindromic repeats) interference (CRISPRi) to identify drug targets
95 (16). We demonstrated previously that a ribosome-binding site mutation that decreased
96 expression of UppS led to vancomycin resistance and activation of the σ^M-dependent cell
97 envelope stress response (17). Compounds that inhibit the recycling of UPP may also serve as
98 effective antibiotics. The most widely used antibiotic of this class is bacitracin, which binds
99 tightly to the pyrophosphate group on surface-exposed UPP to inhibit its dephosphorylation (18).
100 Bacitracin also activates the σ^M stress response and contributes to bacitracin resistance by
101 increasing synthesis of BcrC (19-21), a predicted UPP-Pase presumed to act on the outer face of
102 the membrane to convert UPP (the target of bacitracin) into Und-P (22). Finally, a variety of
103 structurally diverse antibiotics, including glycopeptides and lantibiotics, bind to lipid II which
104 serves to both inhibit cell wall synthesis and sequester the UPP carrier lipid (23).

105 The identity of the UPP-Pases has been clearly established in *E. coli* where there are three
106 UPP-pase enzymes (BacA, YbjG, and PgpB) and a fourth enzyme (YeiU, later renamed LpxT)
107 exhibiting UPP-Pase activity *in vitro* (7). The BacA family includes the eponymous BacA
108 protein, while YbjG, PgpB and LpxT all belong to type 2 phosphatidic acid phosphatase (PAP2)
109 superfamily. BacA provides 75% of the cell's UPP-Pase activity, and overexpression of BacA
110 makes cells bacitracin resistant (7). PgpB was originally identified in mutant cells lacking
111 phosphatidylglycerol phosphate phosphatase activity (24), and is shown to have broad substrate
112 specificity (25, 26). The BacA, YbjG, and PgpB enzymes are functionally redundant;single
113 mutants lacking any one of the three genes do not show significant growth defects. However, a
114 triple mutant missing all three genes is not viable. Although LpxT displayed *in vitro* UPP-Pase
115 activity, it could not support growth in the absence of at least one of the other three UPP-Pases
116 (7). It was later found out that LpxT transfers phosphate from UPP to lipid A to produce lipid A

1-diphosphate, and in the process generates Und-P (27). In contrast, the number and identity of the UPP-Pases in *B. subtilis* has not been resolved.

Genetic approaches to analyze the function of essential genes often rely on conditional mutants in which either gene expression or protein activity can be regulated. Optimization of conditional gene expression systems can be challenging (as reported also here), since leaky expression may suffice to support viability even in the absence of induction, or the induced level may be insufficient to support viability. Recently, the CRISPR system, used by many bacterial and archaeal species to defend against foreign DNA, has been adapted as a powerful tool for conditionally regulating bacterial gene expression (16, 28-30).

Here we report the use of an optimized CRISPR interference (CRISPRi) system to investigate the essentiality of candidate UPP-Pases in *B. subtilis*. Using CRISPRi, we have identified UppP and BcrC as two functionally redundant UPP-Pases. We have also identified a third lipid phosphatase (YodM) capable of supporting growth in the absence of these two enzymes, but only when artificially overexpressed. Depletion of essential UPP-Pases, predicted to interrupt synthesis of both peptidoglycan and WTA, results in cells that cannot maintain their rod shape and leads to induction of the σ^M-dependent cell envelope stress response.

Materials and Methods

Strains, plasmids and growth condition

All strains and plasmids used in this work are listed in Tables S4. Bacteria were grown in liquid lysogeny broth (LB) medium with shaking, or on LB plates (1.5% agar; Difco) at 37 ˚C unless otherwise stated. Plasmids were constructed using standard methods (31), and amplified in *E. coli* DH5α before transforming into *B. subtilis*. For selection of transformants, 100 μg/ml

ampicillin was used for *E. coli*. Antibiotics used for selection of *B. subtilis* transformants include: kanamycin 15 µg/ml, spectinomycin 100 µg/ml, macrolide-lincosamide-streptogramin B (MLS, contains 1 µg/ml erythromycin and 25 µg/ml lincomycin), neomycin 10 µg/ml, chloramphenicol 10 µg/ml.

Genetic techniques

Chromosomal and plasmid DNA transformation was performed as previously stated (32). Markerless in-frame deletion mutants were constructed from BKE strains as described (5). Briefly, BKE strains were acquired from the Bacillus Genetics Stock Center, chromosomal DNA was extracted, and the mutation containing an *erm* cassette was transformed into our WT 168 strain. The *erm* cassette was subsequently removed by introduction of plasmid pDR244, which was later cured by growing at the non-permissive temperature of 42 ˚C. Gene deletions were confirmed by PCR screening using flanking primers. Transduction was performed to move SPβ derivatives into the 168 strain as previously described (33). Unless otherwise described, all PCR products were generated using *B. subtilis* 168 strain chromosomal DNA as template. DNA fragments used for gene over-expression were sequence verified. Null mutant construction was PCR screen verified to have the right size band.

Construction of the CRISPRi-based transcriptional repression system

The CRISPRi system was based on plasmid vectors that replicate in *E. coli* and integrate into the *B. subtilis* chromosome. Briefly, the gene encoding dCas9 is inserted into plasmid pAX01 to form pJPM1. pJMP1 is then integrated into the *B. subtilis ganA* gene by double cross-over recombination. sgRNAs targeting *bcrC* or *uppP* were incorporated into integrative vectors by

163 inverse PCR followed by cyclization by self-ligation. Expression of sgRNA is constitutive, and
164 expression of dCas9 is induced by xylose. sgRNAs were selected based on their specificity for,
165 and location within, target genes. We identified all PAM (Protospacer Adjacent Motif) sites
166 (NGG for *S. pyogenes* dCas9) within target genes and designed all possible sgRNAs for those
167 targets. We then assigned each sgRNA a specificity score by aligning progressive truncations of
168 the sgRNA sequences to the *B. subtilis* 168 genome using Bowtie (34); sgRNAs that retained
169 specificity after several truncations received better scores. We then selected the most specific
170 sgRNAs that targeted the non-template strand and were near the 5′ end of the gene where
171 CRISPR interference is thought to be most effective in bacteria (28, 30). After choosing sgRNAs
172 sequences, the constructs were made by inverse PCR using forward primer containing sequence
173 specific to gene of interest (base paring region), and universal reverse primer which binds to the
174 complementary strand of DNA adjacent to the base paring region. For example, *bcrC*-1 was
175 made using pJMP3 as template, primer 6411 CRISPRi bcrC-1 F and 6415 CRISPRi universal R.
176 Inverse PCR product was self-ligated, and transformed into *E. coli* DH5α. Plasmids extracted
177 from successful transformations were confirmed by sequencing before being used to transform *B.*
178 *subtilis*.

179 Construction of pSPβ-dCas9 was done by ligating a fragment containing P_{xyl}-*dCas9* from
180 pJMP1 into backbone of pJPM122, a plasmid that can integrate into SPβ prophage in strain
181 ZB307A as described (35). To get the fragment containing P_{xyl}-*dCas9*, pJMP1 was first digested
182 by SacII, treated with T4 DNA polymerase (NEB) to make a blunt-end, and then digested by SalI.
183 DNA fragment containing P_{xyl}-*dCas9* was gel purified. pJPM122 was digested with SalI and
184 EcoRV, and backbone was recovered from agarose gel. The fragment containing P_{xyl}-*dCas9*
185 from pJMP1 was then ligated with pJPM122, forming plasmid pSPβ-dCas9. pSPβ-dCas9 was

186 then used to transform *B. subtilis* ZB307A. Transformants were PCR screened to confirm that a
187 double cross-over recombination event occurred into the chromosome of ZB307A and named
188 ZB307A-pSPβ-dCas9. Phage lysate from ZB307A-pSPβ-dCas9 was prepared using heat shock
189 and was used to transduce *B. subtilis* 168 and its derivatives.
190
191 **Disk diffusion assays**
192 Disk diffusion assays were performed as previously described (21). Overnight cultures of test
193 strains were re-inoculated into fresh LB liquid medium and grown to early log phase (OD_{600}
194 ~0.4), and a 100 µl aliquot of the culture was mixed briefly with 4 ml LB soft agar (containing
195 0.75% agar, kept at 50 ˚C) before poured onto LB plates (containing 15 ml LB with 1.5% agar).
196 Plates were then cooled and dried in a laminar airflow hood for 5 minutes. Filter paper disks
197 were placed on the surface of the plate, and chemicals were added to the paper disks. Plates were
198 then incubated at 37˚C for 16 h before the diameter of the zone of inhibition was measured.
199 Numbers reported are diameter minus the 6.5 mm diameter of the filter paper disk. The
200 chemicals added to the disks include: xylose 20 µl of 50% solution, bacitracin 400 µg, ampicillin
201 1 mg, penicillin 1 mg, cefuroxime 10 mg, vancomycin 100 µg, fosfomycin 1mg, D-cycloserine
202 100 µg, lysozyme 100 µg, daptomycin 100 µg, nisin 1 mg, SDS 10 µl of 10% solution, Triton X-
203 100 10 µl of 25% solution, EDTA 10 µl of 0.5 M (pH 8.0) solution, novobiocin 100 µg.
204
205 **Measurement of the fraction of suppressors**
206 Strains were grown in liquid LB medium supplemented with 1% glucose overnight, and then the
207 overnight culture was re-inoculated into fresh LB liquid medium supplemented with 1% glucose
208 and grown to early log phase (OD_{600} ~0.4). Serial dilutions were performed up to 10^{-6}. Cultures

209 with 10^{-4}, 10^{-5}, and 10^{-6} dilutions were plated on LB plate supplemented with 1% glucose, and
210 cultures with no dilution, 10^{-1}, and 10^{-2} dilutions were plated on LB plate supplemented with 2%
211 xylose. Colonies were counted from plates having between 20-200 colonies, and colony forming
212 units (CFU) were calculated. Fraction of suppressors is calculated by dividing CFU of strains
213 growing on xylose LB by CFU of the same strain growing on glucose LB. This experiment was
214 done at least three times using biological replicates.
215
216 **Phase contrast time-lapse and fluorescence microscopy**
217 Strains were grown in CH medium (36) in presence of 1% (w/v) glucose at 37 °C. Then the cells
218 were harvested when the OD of the culture reaches ~0.4. Then cells were grown in CH in
219 presence of 2%(w/v) xylose in a micro-fluidic chamber. The phase contrast time lapse images
220 were acquired on a Nikon N-STORM microscope equipped with Plan Apo lambda 100x
221 objective and a Hamamatsu ORCA CMOS camera. Images were taken every 30 s. Images of
222 FM5-95 (ThermoFisher) membrane staining were acquired on a GE DeltaVision Elite with
223 DeltaVision connected to a PCO Edge sSCOS camera. A 561-nm laser and a 60X 1.45 NA Plan
224 Apo-objective were used. The excitation filter was YFP (with center wave length at 513nm and
225 17nm bandwidth) and the emission filter was mCherry (with center wave length at 632nm and
226 60nm bandwidth). The cells were stained with 1X FM5-69 (2 μM) mounted on 1% agarose in
227 CH medium pads and immediately imaged with an exposure time of 2 s. All images were
228 analyzed using Morphometrics (37) and home built MATLAB program.
229
230 **Whole genome sequencing and sequence analysis**

231 Chromosomal DNA of suppressor strains was extracted using Qiagen DNeasy Blood & Tissue
232 Kit. DNA was then sent for sequencing using Illumina HiSeq2500 High Output Mode with
233 Single-end 100 bp reads. Sequencing results were analyzed using CLC workbench version 8.5.1.
234 Single nucleotide variants (SNVs) were detected using default settings.
235
236 **Microarray analysis**
237 The wild type and depletion strains were grown separately in liquid LB medium to OD_{600} ~0.4,
238 and then each diluted 100-fold into two 250 ml flasks each containing fresh 50 ml LB medium.
239 After 1.5 hours of initial growth, one of the two flasks of each strain was supplemented with 50%
240 xylose to a final concentration of 2%, while the other flask was left untreated as control. After
241 another 2.5 hours of growth, cells were collected and total RNA was extracted using phenol-
242 chloroform based method. Total RNA was quantified and quality of RNA was tested using Bio-
243 analyzer. RNA quality numbers for all four RNA samples are 10 out of 10. Total RNA were then
244 treated with Turbo-DNAse (Thermo Fisher Scientific) to remove any possible DNA
245 contamination, and then reverse transcribed using SuperScript™ Indirect cDNA Labeling
246 System (Thermo Fisher Scientific) into cDNA. cDNA was aliquoted and labelled with either
247 fluorescent dye 555 or dye 647, and hybridized to microarray slides containing 60 nt
248 oligonucleotides for each coding region according to laboratory protocol (31). Dye swap was
249 performed to reduce systematic error caused by difference in labelling and hybridization
250 efficiency for different dyes. For instance, in one hybridization cDNA of WT strain is labelled
251 with dye 555 and cDNA of depletion strain is labelled with dye 647. In the dye swap experiment,
252 cDNA from the WT will then be labelled with dye 647 and depletion strain dye 555.

253 Transcriptome results have been deposited in the NCBI GEO database under accession number
254 (*to be added*).

255 Comparison of transcriptome among different strains and conditions were performed by
256 comparing fluorescent signal from different pairs. Among the treated group, transcriptomes of
257 the WT and depletion strains were compared to reveal change in gene transcription, and
258 untreated WT and depletion strains were used as controls. Fold change for each gene is the
259 average of two experiments containing a dye swap. Up- and down-regulated genes after UPP-
260 Pase depletion were defined as those with an average fold change of at least 3-fold, and at least
261 2-fold in each hybridization. It is also crucial that only signals well above background are
262 considered trustworthy. Gene function and regulon information was acquired from SubtiWiki
263 (http://subtiwiki.uni-goettingen.de/;(38)) and prior publications.

264 Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and Java
265 Treeview (https://sourceforge.net/projects/jtreeview/) were used to generate heat maps. Briefly,
266 we chose genes regulated by ECF sigma factors and two component systems involved in cell
267 envelope homeostasis (e.g. WalR, BceR, LiaR, PsdR and YvrHb). Gene *bcrC* was not included
268 in the list of genes (even though it is controlled by multiple ECF sigma factors), as it was
269 artificially repressed using CRISPRi. Microarray fold change data were $\log_{10}$ transformed and
270 used for hierarchical clustering (using uncentered correlation and complete linkage functions)
271 showing genes that were up-regulated (red) or down-regulated (green).

272

273 **Results**

274

275 *UppP and BcrC are functionally redundant UPP-Pases*

We identified *B. subtilis* UppP, BcrC and YodM as homologs of known UPP-Pases in *E. coli* using BLASTp with default parameters. UppP is a homolog of BacA whereas BcrC and YodM are homologs of YbjG, PgpB and LpxT, members of the PAP2 superfamily (Table S1). BcrC was previously shown to have UPP-Pase activity and to be regulated by σ^M (19, 22). UppP (YubB) was also speculated to function as a UPP-Pase based on homology, but played a less important role than BcrC in bacitracin resistance (22). Mutations that affect UPP levels were later found to affect the sensitivity of *B. subtilis* to rare earth metals and, based on this phenotype, it was also suggested that BcrC was the major enzyme with UppP having a minor role (39). However, this assay may report specifically on the levels of extracellular UPP which is accessible for binding by rare earth metals.

To investigate functional redundancy amongst these candidate UPP-Pases, we constructed single mutants with each UPP-Pase gene replaced by an antibiotic cassette, followed by transformation to make mutants missing two or all three UPP-Pases. While we could construct *yodM bcrC* and *yodM uppP* double mutants, we were unable to obtain the *bcrC uppP* double mutant in multiple attempts, despite previous suggestions that a double mutant had been obtained (22, 39). For example, when chromosomal DNA from a *bcrC*::*mls* (macrolide-lincosamide-streptogramin B) strain was used to transform a *uppP*::*spc* (spectinomycin) strain, we recovered MLS^R transformants, but only if the cells simultaneously acquired a functional copy of *uppP* (and therefore lost Spc^R). This phenomenon, in which a cell acquires two unlinked markers by transformation, is referred to as congression. Overall, our findings indicate that *bcrC* and *uppP* are a synthetic lethal gene pair and likely encode the only functional UPP-Pases in *B. subtilis,* at least in our laboratory conditions.

299 *The YodM protein can provide UPP-Pase activity when overexpressed*

300 To characterize the physiological consequences of limiting UPP-Pase activity, we sought
301 to generate conditional depletion strains. We introduced ectopic copies of *bcrC*, *uppP*, or *yodM*
302 under the control of the IPTG inducible promoter $P_{spac(hy)}$. Next, we attempted to sequentially
303 delete *bcrC* and *uppP* under conditions where the ectopic gene was expressed. However, due to
304 either leakiness or insufficient expression, we were unable to construct a depletion strain
305 conditionally expressing *bcrC* or *uppP*, even after attempts to modify the ribosome binding site
306 to change translational efficiency (Table S2)(40).

307 One interesting result that emerged from this exercise is that YodM, if overexpressed, can
308 support growth even in the absence of BcrC and UppP. In this strain, the native *yodM* RBS was
309 replaced with a strong RBS (40) and the start codon was changed from TTG to ATG. The
310 resulting $P_{spac(hy)}$-*yodM* construct allowed generation of a *bcrC uppP* double mutant strain that
311 could grow in the presence, but not in the absence of IPTG (Figure S1). Note that this strain has
312 two copies of *yodM* with one native copy and an ectopic copy controlled by $P_{spac(hy)}$ and
313 optimized for expression. Because of the homology between YodM and *E. coli* UPP-Pases and
314 the fact that overexpression of YodM can rescue growth defect of *bcrC uppP* double mutant, we
315 infer that YodM has some UPP-Pase activity, and this activity is sufficient to support growth in
316 absence of the other two UPP-Pases when it is expressed at an artificially high level. However,
317 YodM is not able to support growth of a *bcrC uppP* double mutant at its normal expression
318 levels Overall we conclude that BcrC and UppP are the two primary UPP-Pases, at least in our
319 laboratory conditions..

320

321 *Demonstration of essentiality of UPP-Pases using CRISPRi*

We next explored the use of CRISPRi as a tool to monitor the effects of UPP-Pase depletion on cell physiology. The CRISPRi system uses a catalytically inactive form of the Cas9 endonuclease (dCas9) and a single guide RNA (sgRNA) (28, 41). The sgRNA contains a customizable 20-nt base pairing region to direct dCas9 to specific DNA sequences, and the dCas9:sgRNA complex binds DNA and serves as a road block to transcription. In the system used in this work, dCas9 gene is expressed from a xylose-inducible promoter and the sgRNAs were constitutively expressed (16). Based on previous studies in *E. coli* (28) and mycobacteria (42), we anticipated that the strength of transcriptional repression would be increased by promoter proximal targeted complexes and by the use of two sgRNAs. We therefore designed *bcrC*-1 and *uppP*-1 sgRNAs to bind close to the transcription start site (predicted to have a strong repressive effect) and *bcrC*-2 and *uppP*-2 sgRNAs to bind further downstream (predicted to have weaker repressive effects).

The conditional depletion strains did not show any growth defect in the absence of the dCas9 inducer xylose, but were growth impaired in medium with xylose. In order to illustrate the functional redundancy of BcrC and UppP, we used disk diffusion assays as a measure of xylose sensitivity. *B. subtilis* is not sensitive to xylose *per se*, and can use it as a carbon source (43). As expected based on the phenotype of the null mutant strains, depletion of either *uppP* or *bcrC* using CRISPRi did not lead to xylose sensitivity (**Figure 1A**). However, strains in which both genes were targeted for transcriptional repression showed a notable sensitivity to xylose as shown by a clear zone of inhibition around the paper disk containing xylose (**Figure 1B**). A similar effect was noted in strains where *uppP* was deleted and *bcrC* was targeted for transcriptional repression. Strains containing the weak sgRNAs *bcrC*-2 and *uppP*-2 showed a small zone of inhibition, demonstrating that these cells are less xylose sensitive, and suggesting

that *bcrC*-2 and *uppP*-2 provide less efficient in transcriptional repression (**Figure 1B**). This is consistent with the previous observation that sgRNAs farther from 5' end of transcription start site caused weaker repression (42). In the *uppP* null mutant, depleting *bcrC* led to xylose-sensitivity. The use of the promoter proximal sgRNA *bcrC*-1 led to a high level of xylose sensitivity, and this was modestly increased with the inclusion of the second sgRNA, *bcrC*-2. We were not able to construct a strain with *bcrC* deleted and containing *uppP*-1, possibly due to leaky expression of dCas9 which has been shown to have moderate repressive activity even in the absence of xylose induction (16).

Optimization of CRISPRi to reduce suppressor formation in conditional depletion strains

It is apparent that suppressors arise frequently in the zone of xylose-dependent growth inhibition (**Figure 1B**). Whole genome sequencing of ten independent suppressors revealed frameshift mutations in the dCas9 gene (nine strains), and a single base pair mutation in the *bcrC* gene which altered the PAM (Protospacer Adjacent Motif) sequence for sgRNA *bcrC*-1 and thus prevent binding of dCas9:sgRNA complex (one strain). To facilitate further physiological studies, we sought to first reduce the frequency of suppressors. Since our sequencing results indicate that inactivation of the gene encoding dCas9 is the single most frequent class of suppressor mutation, we constructed a merodiploid strain containing a second copy of dCas9 integrated into the SPβ prophage. We then monitored the frequency with which suppressors arise by plating the depletion strain on LB medium supplemented with 1% glucose or 2% xylose and comparing the colony forming units (CFU). As expected, strains with two copies of the gene encoding dCas9 displayed a reduction (approximately 10-fold) in the frequency of suppressors (**Figure 2**). We also note that suppressor formation is somewhat higher in strains with dCas9 expressed only

368 from the SPβ prophage locus rather than the *ganA* locus (**Figure 2**, Figure S2A). Since it is also
369 possible to isolate suppressors that mutate either the sgRNA or its target, we reasoned that
370 having two sgRNAs targeted to the same gene would further decrease the frequency of
371 suppressors. In general, the frequency of suppressors was reduced in the *uppP* null strain
372 carrying both *bcrC*-1 and *bcrC*-2 sgRNAs relative to the strain with only *bcrC*-1, however these
373 effects were rather modest. We hypothesized that another possible class of suppressors might
374 result from up-regulation of *yodM*. Indeed, introduction of a *yodM* null mutation into the *uppP*
375 *bcrC*-1 *bcrC*-2 strain greatly reduced the frequency of suppressors, but only if dCas9 was present
376 in two copies (**Figure 2,** Figure S2B, C). These results indicate that inactivation of dCas9 is the
377 most frequent cause of suppression (as inferred from the whole genome sequencing studies), and
378 that the use of two sgRNAs and elimination of known or predicted pathways of suppression
379 (such as upregulation of *yodM*) may be required for a significant further reduction in suppressor
380 occurrence.

381 We then isolated two independent suppressors from the optimized strain (HB17235) and
382 performed whole genome sequencing. While we might have expected to discover mutations
383 revealing novel UPP-Pase(s), each suppressor contained a frameshift mutation (base deletion) in
384 a homopolymeric stretch within both dCas9 genes (one suppressor in TTCCTTTGAAAAAAA
385 and the other in CAGTATCAAAAAAAA). The fact that both dCas9 genes in each suppressor
386 strain have the same point mutation in each copy of the gene leads us to speculate that the first
387 mutation may have resulted from a relatively frequent strand slippage event in the
388 homopolymeric sequence followed by a gene conversion event to generate the double mutant.

389 One of our goals in generating depletion strains for UPP-Pases was to monitor the
390 terminal phenotype, and this is complicated if suppressors arise at a high frequency. To

391 determine if our optimized CRISPRi system had sufficiently reduced the appearance of
392 suppressors to allow growth studies, we inoculated ~4x10^4 cells into 200 µl of xylose-containing
393 medium using an automated BioScreen growth analyzer. For each strain, we monitored 30
394 replicate cultures (3 biological replicates containing 10 technical replicates each). In this assay,
395 the majority of the cultures for each strain grew after a long lag phase (Table S3), suggesting that
396 suppressors were arising that might therefore complicate analysis of the terminal phenotype. The
397 single exception was our optimized strain (HB17235) which is a dCas9 merodiploid lacking both
398 UppP and YodM and with two sgRNAs targeting *bcrC*. With this strain, there was outgrowth in
399 less than 10% of the wells.
400
401 *Dynamics of UPP-Pase depletion*
402 To determine how long it takes for the existing BcrC protein to be depleted in the
403 optimized (HB17235) strain and thereby limit growth, we monitored growth curves as a function
404 of the initial inoculum in LB with either 1% glucose or 2% xylose. With a starting culture at
405 initial OD_{600} 0.14, the culture reached a final OD_{600} of 0.7 in the presence of 2% xylose, while it
406 could reach OD_{600} ~1.2 with glucose (**Figure 3**). With each two-fold dilution of the initial
407 inoculum, the final OD_{600} reached under depletion conditions was also decreased by nearly 2-
408 fold, consistent with a growth-limiting depletion of pre-existing BcrC after 3 to 4 doublings.
409
410 *Changes in cell morphology upon depletion of UPP-Pases*
411 Defects in cell wall synthesis often cause morphological changes as the integrity of the
412 cell wall is compromised. For example, depletion of MreB and its homologs leads to a loss of
413 rod shape and formation of bulged cells (44), and cells missing all class A PBPs form longer and

bent cells with abnormal protrusions into the cell plasma (45). Cells with UppS expression repressed by CRISPRi also show bulged cells without significant lysis (16). We therefore anticipated a similar phenotype from the UPP-Pase depletion strain. Cells were grown in a micro-fluidic chamber in CH medium supplemented with 2% xylose, and images were taken every 30 seconds. At the beginning of the experiment, cells did not show any obvious morphological defect relative to WT. After about 2 hours, cells grown with xylose began to form bulges (**Figure 4**, Movie S1). In comparison, WT cells did not exhibit any growth defect in presence of 2% xylose (Movie S2). Surprisingly, few cell lysis events were observed during the time lapse experiment. This is similar to what was observed in the UppS depletion strain (16, 46), and in a strain depleted for lipid II flippases (5). The lantibiotic NAI-107 (which targets UPP linked cell wall precursors such as lipid I, II and precursors of WTA) leads to a similar terminal phenotype (47).

To quantify the fraction of cells forming bulges when UPP-Pases are depleted, we observed more than 200 cells at each time point and used Morphometrics and an in-house MATLAB script to measure cell width across the whole length of the cell, and determined the maximum cell width for each cell (**Figure 4B**). Before depletion of UPP-Pases, the majority of cells exhibited a maximum width of between 0.95 and 1.05 μm (**Figure 4C**) and only ~10% of cells had a width >1.2 μm (**Figure 4C, D**). As the cells grew under depletion conditions, the percentage of cells with a maximal width above 1.2 μm increased significantly (**Figure 4D**). Septum formation was still present in the depletion strain as visualized with the membrane stain FM5-95, even when cells exhibited bulging (Figure S3).

The UPP-Pase depletion strain is sensitive to cell envelope stress

437 It has been reported that leaky expression of dCas9 in absence of xylose may lead to ~3-
438 fold repression of target genes (16). We therefore hypothesized that our depletion strain may
439 have reduced expression of UPP-Pases even in the absence of inducer. Indeed, the depletion
440 strain exhibited more lysis than WT when stored on plates. To test this hypothesis, we used disk
441 diffusion assays under non-inducing condition to monitor sensitivity to cell envelope stress
442 conditions (**Figure 5**). In fact, the depletion strain is significantly ($P<0.01$) more sensitive to both
443 bacitracin, which binds UPP and thereby inhibits UPP-Pase activity, and daptomycin. A more
444 modest level of sensitivity ($P<0.05$) was noted for the peptidoglycan synthesis inhibitors
445 vancomycin and fosfomycin. The depletion strain was not significantly more sensitive to several
446 other tested stress conditions including compounds that elicit membrane stress (Nisin, SDS, and
447 Triton X-100). The reason for the increased sensitivity to some, but not other, peptidoglycan
448 synthesis inhibitors is presently unclear, but interpreting such effects can be complex, as
449 previously discussed (17).
450
451 *Depletion of UPP-Pases triggers a cell envelope stress response*
452 To further characterize the effects of UPP-Pase depletion, we monitored changes in the
453 transcriptome 2.5 h after addition of xylose to deplete BcrC, a time known to coincide with the
454 onset of visible morphological changes and decreased growth (**Figure 3, 4**). In the depletion
455 strain, >150 genes were >3-fold up-regulated and nearly 300 genes were >3-fold down-regulated
456 (SI Excel Sheets). The set of up-regulated genes includes known cell envelope stress responses
457 including, most notably, the σ^M regulon. In contrast, down-regulated genes include many genes
458 and regulons consistent with catabolite repression in the xylose-treated cultures, and with entry
459 of the untreated cells into transition phase during the 2.5 h of incubation. The up-regulation of

460 the flagellar and motility systems controlled by σ^D in the control cells as they enter into
461 transition phase likely explains the apparent down-regulation of these genes in the depletion
462 strain.

463 To gain further insights into the effects of UPP-Pase depletion on cell envelope stress
464 responses we used hierarchical clustering to compare the expression of genes regulated by ECF
465 sigma factors and the WalR, BceR, PsdR, LiaR and YvrHb two component system regulons
466 (**Figure 6**). Microarray data generated in this study (UPP-Pase depletion), as well as for cells
467 treated with targocil (46), bacitracin (48), D-cycloserine (49), Triton X-100 (49), and
468 vancomycin (50) (SI Excel Sheets) were used for clustering and heat map generation as
469 previously described (50). Based on the observations above, we anticipated that the effects of
470 UPP-Pase depletion (occurring over 2.5 h) might not be directly comparable to those elicited by
471 relatively short treatments with cell envelope antibiotics. Nevertheless, we noted several striking
472 similarities. Specifically, the σ^M regulon is selectively induced by UPP-Pase depletion, with
473 comparatively weak effects noted for other ECF σ factor stress responses. Induction of the σ^M
474 regulon is consistent with the inclusion of the BcrC UPP-Pase as part of this regulon (19), as well
475 as the previously noted induction in cells treated with bacitracin (48) or containing mutations that
476 decrease UPP synthesis (17). In contrast with σ^M, the σ^W regulon, most strongly activated by
477 membrane stress conditions (50), was down-regulated in the depleted cells compared to the
478 control.

479 In addition to clustering based on genes, we also determined the clustering based on
480 treatments. Among the antibiotics used for comparison, the treatment condition most similar to
481 UPP-Pase depletion is targocil, which inhibits WTA export and therefore also causes a gradual
482 depletion of the Und-P pool. D-cycloserine, an inhibitor of early steps in PG synthesis that

converge with Und-P to allow lipid II synthesis, also leads to a similar set of stress responses. We might have expected bacitracin (which binds directly to UPP) to trigger a response similar to UPP-Pase depletion. However, despite several similarities, bacitracin additionally elicits strong (albeit transient) induction of the BceR and LiaR regulons after 5 min of treatment (51). This activation can happen at bacitracin concentrations well below the MIC and does not depend on cell wall damage (52). The least similar stress conditions, compared to UPP-Pase depletion, were those elicited by Triton-X-100 and vancomycin, which both lead to induction of the large σ^W regulon under the tested conditions.

Discussion

UPP is a crucial lipid carrier for synthesis of the cell envelope, including both peptidoglycan and WTA. In peptidoglycan synthesis, UPP serves as lipid carrier for the disaccharide-pentapeptide precursor in the central intermediate lipid II. Although initially synthesized as the pyrophosphate form (UPP), dephosphorylation is required to generate the substrate (Und-P) for MraY. Similarly, in *B. subtilis* strain 168, Und-P is required for WTA synthesis which is initiated by transfer of GlcNac-1-P from UDP-GlcNac to Und-P by TagO to generate the initial membrane-bound precursor. The peptidoglycan precursor, lipid II, is flipped across the membrane by either of two flippases, MurJ or Amj, where it serves as substrate for synthesis of the glycan polymer by PBPs. In contrast, synthesis of the WTA polymer occurs in the cytosol and the completed polymer (a glycerol-phosphate alternating copolymer in *B. subtilis*) is exported and then attached to PG. In both peptidoglycan and WTA synthesis, the UPP carrier is released on the extracytoplasmic face of the membrane (by PBPs or the TagTUV family of WTA-attachases) and recycled (9).

Disruption of cell envelope synthesis is one of the most effective strategies for inhibiting bacterial growth and numerous antibiotics target these processes. The lipid II cycle can be disrupted by compounds that inhibit UPP synthesis or that bind directly to lipid II (nisin, vancomycin, ramoplanin), and to UPP itself (bacitracin). Other PG synthesis inhibitors target the PBPs and thereby inhibit the transpeptidation (β-lactams) or transglycosylation (moenomycin) reactions. It is also possible to disrupt the lipid II cycle by blocking the synthesis of WTA at late steps, thereby leading to sequestration of this limiting lipid carrier (11).

A common feature of many different compounds that inhibit the lipid II cycle is that they invoke cell envelope stress responses including activation of the σ^M regulon (53). A major function of the σ^M regulon is to increase the expression of key enzymes involved in cell envelope synthesis and to up-regulate alternative enzymes to replace those that might be inhibited. Notable examples of σ^M-dependent compensation reactions include up-regulation of the lipid II flippase Amj (which substitutes for MurJ; (5)), the lipoteichoic acid synthase LtaSa (YfnI, which substitutes for LtaS), and BcrC (which substitutes for UppP; (19, 50)). Similarly, antibiotics that inhibit the lipid II cycle, including bacitracin (48), vancomycin (50), moenomycin (54), and the WTA-targeting targocil (46), all activate the σ^M regulon (53).

Here, we set out to explore the role of three genes encoding candidate UPP-Pases in the lipid II cycle in *B. subtilis*. The CRISPRi system has been recently adapted as a tool to repress bacterial gene expression and facilitate the analysis of essential gene functions (16). Our inability to construct a *uppP bcrC* double mutant suggested that these two genes might be a synthetic lethal pair (in contrast with a previous report; (22, 39)), although they could both be deleted when a third candidate phosphatase, YodM, was overexpressed. The ability of YodM to rescue cell growth in a *uppP bcrC* double mutant and its homology to other UPP-Pases support a

529 possible role for YodM as a UPP-Pase that is active under growth conditions not yet identified.
530 Alternatively, this may be an adventitious reaction that only occurs when this enzyme is
531 artificially overexpressed. This may be similar to the case in *E.coli,* where LpxT transfers
532 phosphate from UPP to lipid A and as a product also generates Und-P (27). The efficiency of
533 recycling UPP by this reaction is not enough to support cell growth, but this enzyme indeed has
534 UPP-Pase activity. To test the hypothesis that *uppP* and *bcrC* are a synthetic lethal pair, and to
535 explore the physiological consequences of UPP-Pase depletion, we optimized a previously
536 described CRISPRi repression system (16) to minimize the appearance of suppressor mutations.
537 We have here used two copies of dCas9 to reduce the occurrence of suppressor mutations. The
538 frequency of suppressor mutations varies significantly between loci (unpublished results), and
539 may be affected by the length of time required to deplete the cell of essential proteins. Whereas
540 one sgRNA generally suffices for gene knockdown (16), here we included two sgRNAs targeting
541 the *bcrC* gene. The presence of two sgRNAs leads to a modest increase in repression efficiency
542 as judged by xylose sensitivity (**Figure 1B**), and also slightly reduced the frequency of
543 suppressors (**Figure 2**). In this system, the greatest reduction in suppressor frequency required
544 deletion of *yodM* which eliminates the only other known protein with UPP-Pase activity (**Figure**
545 **2**). In general, optimization of CRISPRi for knockdown of essential genes (or sets of genes) may
546 benefit from use of a dCas9 merodiploid since inactivation of dCas9 is one frequent cause of
547 suppression. Further modifications worth exploring include the use of two sgRNAs per gene
548 (although this effect may be minor) and elimination of known or predicted pathways of
549 suppression.

550 The two independent suppressor analyzed from our optimized strain each contained
551 mutations in both copies of the dCas9 gene. The occurrence of frameshift mutations in runs of

552 adenines suggests that these may be hot spots for mutagenesis, and the fact that each strain
553 contains the same point mutation in both dCas9 genes suggests a gene conversion event after the
554 first mutation. One way to further reduce the fraction of suppressors might be to use a dCas9
555 with a different coding sequence as the second copy. For example, swapping alternate but still
556 preferred codons for *B. subtilis* (55) could be used to reduce the number of homopolymeric
557 sequences and also to reduce sequence-sequence similarity between two dCas9 genes.
558 Nevertheless, the fraction of suppressors in the optimized strain used in this study is sufficiently
559 low to carry out the physiological assays performed here.

560 Depletion of UPP-Pases leads to a cessation of growth after ~3-4 doublings. Even mild
561 depletion (due to the leaky expression of dCas9 in the depletion strain) leads to an increase in
562 sensitivity to bacitracin. As UPP is further depleted, the cells begin to display morphological
563 abnormalities including a prominent bulging of the cells. This is consistent with the
564 morphological changes induced by other genetic and chemical perturbations known to affect PG
565 synthesis. Concomitant with the onset of morphological defects resulting from depletion of UPP,
566 the cells strongly activate the σ^M cell envelope stress response, consistent with the effects of
567 other antibiotics targeting the lipid II cycle (53).

568 In conclusion, our results establish that *B. subtilis* requires either of two UPP-Pases for
569 viability, UppP and BcrC. Because of the nature of Und-P synthesis and recycling, it is
570 reasonable to speculate that one UPP-Pase has a cytosolic-facing active site and functions to
571 convert UPP produced by UppS into Und-P for use in cell wall synthesis, while the other one
572 recycles UPP produced as a product of the transglycosylase reaction catalyzed by class A PBPs.
573 We speculate that the former activity is UppP, which might account for the minor role of this
574 enzyme with respect to sensitivity to rare earth metals (39). After each addition to the growing

peptidoglycan (or WTA) polymer, UPP is released and must be dephosphorylated and flipped back to the cytoplasmic face of the membrane for reuse. This is the likely role for BcrC, which by reducing surface exposed UPP can prevent binding of bacitracin to its target. In contrast, all four of the *E. coli* UPP-Pases may have active sites on the outer face of the membrane. These include the PAP2 family UPP-Pases PgpB (26), YbjG and YeiU (56) and, based on protein modeling, BacA (57). Manat and co-workers provided supporting evidence for this model using truncated BacA-β-lactamase reporters (58).

Regardless of whether the two UPP-Pases in *B. subtilis* have their active sites on the same or opposite faces of the membrane, the fact that *B. subtilis* can survive with only one UPP-Pase suggests that UPP-Pase function needs only be localized to one face to support viability. This suggests that either *de novo* synthesized UPP is dephosphorylated inside the cell and the UPP product released by the transglycosylase reaction can be flipped from outside to inside, or that UPP synthesized in the cytoplasm is first flipped to the outside to be dephosphorylated, and then flipped back to the cytoplasmic face to support peptidoglycan (MraY) and WTA (TagO) synthesis. Flipping of Und-P or UPP is unlikely to be spontaneous, although the identity of any proteins that help catalyze this reaction is not yet clear. Ongoing efforts to define this and other poorly understood steps in the lipid II cycle will help to identify new candidates for the targeting of antibacterials and will refine our understanding of the complex mechanisms of acclimation to antibiotics in bacteria.

Acknowledgements: We thank Pete Chandrangsu and Ahmed Gaballa for help with the experimental design and interpretation.

Funding Information: Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number GM047446 (JDH).

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752 **Legends**

753 **Fig.1. Growth inhibition by depletion of BcrC and UppP.** Photos of disk diffusion assay of
754 WT and mutant strains. Cells were grown on LB plates with xylose on the filter paper disk to
755 induce dCas9 and inhibit transcription of *bcrC* and/or *uppP*. (A) WT and mutants with either
756 *bcrC* or *uppP* null mutations, or containing sgRNA targeting only one UPP-Pase are not sensitive
757 to induction of dCas9 by xylose. (B) Mutants containing sgRNA for both *bcrC* and *uppP* (*bcrC*-
758 1, *uppP*-1*; bcrC*-2*, uppP*-2), and mutants with a *uppP* null mutant and sgRNA for *bcrC* (*ΔuppP,*
759 *bcrC*-1*; ΔuppP, bcrC*-2*; ΔuppP, bcrC*-1&2) are sensitive to xylose. (C) Mutant strain shows
760 similar phenotypes in a Δ*yodM* background.

761

762 **Fig.2. Reduction of suppressor mutations in depletion strains.** Fraction of suppressors in
763 populations of various depletion strains. UPP-Pases depletion strains cannot grow on LB plates
764 containing 2% xylose unless they have a suppressor mutation. Cells were plated on LB plates
765 with or without 2% xylose, and the number of cells on each type of plate were counted and back
766 calculated to CFU/ml. Fraction of suppressor in each population was calculated by dividing CFU
767 of suppressors over CFU of total cells.
768
769 **Fig. 3. Growth of depletion strain in depletion/non-depletion condition with different initial**
770 **culture population size.** Depletion strain (*ganA*::*dCas9* SPβ::*dCas9 ΔyodM uppP bcrC*-1&2)
771 growing at logarithm phase was diluted and re-inoculate into liquid LB medium supplemented
772 with either 1% (w/v) glucose or 2% (w/v) xylose. Growth was carried at 37℃ with shaking.
773 OD_{600} was measured every 15 minutes. Starting OD_{600} of ~0.1 was designated at "Dilution 1",
774 and further two-fold dilution was performed until the smallest inoculum contained 1/8 amount of
775 the cells in "Dilution 1". Different amount of initial inoculums affects final OD of cells growing
776 in LB xylose medium, while had no significant on cells growing in LB glucose medium.
777
778 **Fig. 4. UPP-Pase depletion leads to curved and bulged cells**. Microscopy images of depletion
779 strains under depletion conditions. Cells were observed using phase contract and under 100X
780 magnification (each pixel size is 65 nm). (A) Depletion strain (HB17325) growing in CH
781 medium supplemented with 2% xylose at 37˚C. Cells showed normal morphology at the
782 beginning of the experiment (0 min) and showed curved and bulged cells as UPP-Pases were
783 depleted (200 min). (B) Example showing how cell width was measured. Lines were draw
784 across cell width, and cell width at each line was measured. The max width of each cell was used

for quantification. (C) Distribution of max cell width of depletion strain at the beginning of treatment (0 min). The majority of cells had max width of 0.95-1.05 μm, while approximately 10% of cells have max width bigger than 1.2 μm. (D) Change of fraction of cells having max cell width (Dmax, max diameter of cells) larger than 1.2 μm through time. The fraction increased significantly after 120 minutes and reached its peak after 180 minutes, indicating more and more cells began to form bulge as UPP-Pases were depleted.

Fig. 5. Sensitivity of depletion strain against stresses under partial depletion condition. Cells were grown on LB plates with chemicals added onto the filter paper disk. Sensitivity of depletion strain was measured as the clear zone of inhibition around the paper disk. Chemicals used in this assay include bacitracin 400 μg, ampicillin 1 mg, penicillin 1 mg, cefuroxime 10 mg, vancomycin 100 μg, fosfomycin 1 mg, D-cycloserine 100 μg, lysozyme 100 μg, daptomycin 100 μg, nisin 1 mg, SDS 10 μl of 10% solution, Triton X-100 10 μl of 25% solution, EDTA 10 μl of 0.5 M (pH 8.0) solution, novobiocin 100 μg. The data are expressed as mean ± standard error (n=3). Statistical assay was performed using T test with two samples assuming unequal variances. * indicates a two tail P value <0.05, ** indicates a two tail P value <0.01.

Fig. 6. UPP-Pase depletion induces the σ^M cell envelope stress response. Hierarchical clustering was used to generate a heat map of up-regulation (red) or down-regulation (green) of known cell envelope stress responsive genes (regulons indicated to the right). Cells were either depleted of UPP-Pase (compared to non-depletion control cells), or treated with targocil (TAR), D-cycloserine (CYC), bacitracin (BAC), Triton X-100 (TRI), or vancomycin (VAN). Numbers indicate the minutes of treatment with each antibiotic.