YhcB coordinates peptidoglycan and LPS biogenesis with phospholipid synthesis during *Escherichia coli* cell growth.

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Abstract

The cell envelope is essential for viability in all kingdoms of life. It retains enzymes and substrates within a confined space while providing a protective barrier to the external environment. Destabilising the envelope of bacterial pathogens is a common strategy employed by antimicrobial treatment. However, even in one of the most well studied organisms, *Escherichia coli*, there remain gaps in our understanding of how the synthesis of the successive layers of the cell envelope are coordinated during growth and cell division. Here, we used a whole genome phenotypic screen to identify mutants with a defective cell envelope. We report that loss of *yhcB*, a conserved gene of unknown function, results in loss of envelope stability, increased cell permeability and dysregulated control of cell size. Using whole genome transposon mutagenesis strategies we report the complete genetic interaction network of *yhcB,* revealing all genes with a synthetic negative and a synthetic positive relationship. These genes include those previously reported to have a role in cell envelope biogenesis. Surprisingly, we identified genes previously annotated as essential that became non-essential in a Δ*yhcB* background. Subsequent analyses suggest that YhcB sits at the junction of several envelope biosynthetic pathways coordinating the spatiotemporal growth of the cell, highlighting YhcB as an as yet unexplored antimicrobial target.

Introduction

The bacterial cell envelope plays a fundamental role in protection, host interaction, energy generation, expulsion of toxic substances and coordination of growth and cell division. As a physical barrier, it has a central role in acquired and intrinsic antimicrobial resistance. The cell envelope biogenesis systems are therefore important drug targets. In Gram-negative bacteria, the tripartite cell envelope is composed of the cytoplasmic membrane (CM), peptidoglycan (PG) layer and an outer membrane (OM) (Silhavy et al.

2010). The OM is an asymmetrical bilayer of phospholipids and lipopolysaccharide (LPS), which forms a strong permeability barrier conferring resistance to many toxic antimicrobials. Both membranes are studded with integral membrane proteins and peripheral lipoproteins that facilitate cellular functions (Lugtenberg 1981; Molloy et al. 2000; Luirink et al. 2012). Each component of the cell envelope must be synthesised and assembled in a coordinated fashion to maintain cell envelope homeostasis and viability. Thus, understanding how this complex envelope is synthesised and maintained is instrumental to understanding how to disrupt its function, to kill the bacterium, or render the organism susceptible to otherwise ineffective treatments.

Over the last 50 years a variety of sophisticated and complex multiprotein machineries have been discovered that are required for the synthesis of the Gram-negative cell envelope. The Lpt machinery facilitates insertion of LPS into the OM (Sperandeo et al. 2008; Polissi and Sperandeo 2014; May et al. 2015; Simpson et al. 2015; Okuda et al. 2016; Sherman et al. 2018), the BAM complex coordinates OM protein assembly and insertion (Ruiz et al. 2005; Wu et al. 2005; Knowles et al. 2009), the Lol system traffics lipoproteins across the periplasm for incorporation into the OM (Matsuyama et al. 1995; Matsuyama et al. 1997; Yakushi et al. 2000; Okuda and Tokuda 2011), the Mla complex enables phospholipids transport between the two membranes (Malinverni and Silhavy 2009; Thong et al. 2016; Abellón-Ruiz et al. 2017; Powers and Trent 2018; Ercan et al. 2019; Hughes et al. 2019; Shrivastava and Chng 2019; Chi et al. 2020), and the elongasome and divisome are the architects of peptidoglycan assembly during cell elongation and division (Goehring and Beckwith 2005; Den Blaauwen et al. 2008). Each pathway in isolation is broadly understood. However, the precise molecular events that govern the crosstalk between the different biosynthetic pathways, both spatially and temporally during growth and cell division, remain to be elucidated.

78 Genetic screens to identify synthetically lethal interactions and suppressor
79 mutations have played a significant role in identifying the interconnected pathways of
80 envelope biosynthesis (Ogura et al. 1999; Klein et al. 2014). Transposon insertion
81 sequencing (TIS) is one approach that enables the study of genetic interactions on a
82 whole genome scale (DeJesus *et al*. 2017). Here we used a chemical genomics approach
83 to identify genes required for cell envelope homeostasis in *Escherichia coli,* coupling
84 TraDIS, a TIS method (Langridge et al. 2009; Cain et al. 2020), with the membrane
85 targeting antibiotic polymyxin B. We demonstrate that a poorly characterised protein,
86 YhcB, is crucial for tolerance to polymyxin B and for maintenance of cell envelope
87 homeostasis in *E. coli*. Previous studies revealed that YhcB is a CM protein widely
88 conserved in Gammaproteobacteria that physically interacts with the elongasome, the
89 machine that coordinates peptidoglycan synthesis along the cylindrical part of the cell,
90 and with LapA, a protein involved in regulating LPS biosynthesis (Mogi et al. 2006; Den
91 Blaauwen et al. 2008; Maddalo et al. 2011; Li et al. 2012; Klein et al. 2014; Mahalakshmi
92 et al. 2014; Fivenson and Bernhardt 2020). We demonstrate that loss of YhcB results in
93 dysregulation of cell length and width. We created a high-density transposon mutant
94 library in an *E. coli* Δ*yhcB* strain to gain a whole genome view of genetic interactions with
95 *yhcB*. Subsequent screening of this library against chemical stresses revealed all genes
96 that suppress the envelope defects associated with the loss of *yhcB.* These data suggest
97 that YhcB is stationed at the interface between PG and LPS synthesis, and phospholipid
98 membrane biogenesis, and has a role in coordinating the spatial and temporal assembly
99 of the cell envelope.
100
101 **Results**
102 ***Identification of envelope barrier-defective mutants***

103 Previously, we described a high-density transposon-mutant library of *E. coli* K-12
104 (Goodall et al. 2018). To identify genes required for cell envelope biogenesis we applied
105 a chemical genomics approach exposing our library to sub-inhibitory concentrations of
106 the membrane-acting antibiotic polymyxin B (Fig. S1A). Polymyxins bind the lipid A
107 moiety of LPS, displacing divalent cations, disrupting the integrity of LPS-crosslinks, and
108 resulting in an increase in membrane permeability (Newton 1953; Dixon and Chopra
109 1986; Trimble et al. 2016; Li and Velkov 2019). We posited that if the transposon disrupts
110 a gene required for the maintenance of cell envelope integrity, the resulting mutant will
111 become more susceptible to polymyxin B, and the gene would be identified through our
112 TIS screen as these mutants will be outcompeted during growth; identifiable as a
113 depletion in transposon-insertions. We grew the transposon library in LB broth with or
114 without 0.2 µg/ml polymyxin B, in duplicate, and harvested cells after ~5 generations of
115 growth (Fig. 1A). The sequencing yielded a total of >4.2 M reads, estimated to sample
116 >99% of the possible unique insertion sites (Fig. S1B). We used the BioTraDIS pipeline
117 for data processing and analysis (Barquist et al. 2016), and mapped the processed reads
118 to the *E. coli* BW25113 reference genome obtained from NCBI (accession CP009273.1).
119 The insertion index scores (IISs; the number of insertions per gene, normalised for gene
120 length) between replicates were highly comparable (Fig. S1C). We identified 54 genes
121 required for growth in sub-inhibitory concentrations of polymyxin B (Fig. 1B; Table S1).
122 Comparison of the relative abundance of COG (Cluster of Orthologous Groups)
123 categories of these 54 genes showed a marked increase in genes involved in cell
124 wall/membrane/envelope biogenesis (COG category 'M'), supporting the validity of the
125 TIS screen (Fig. S1D). Many of these genes (e.g. *bamB*, *degP*, *galE*, *surA*, and
126 *waaBCFGJPR*) have been identified in a similar TIS screen using *Klebsiella pneumoniae*
127 or have previously been reported as mutants sensitive to polymyxin (Yethon et al. 2000;
128 Jana et al. 2017). The COG analysis identified only three genes (*ydbH*, *yqjA* and *yhcB*)

129 of unknown function (category 'S' in Fig. S1D) that were important for survival when
130 exposed to sub-inhibitory concentrations of polymyxin B (Fig. 1C and Fig. S1E). YdbH is
131 a putative autotransporter, while YqjA belongs to the DedA family of proteins that have
132 been suggested to be involved with membrane homeostasis (Keseler et al. 2017). Given
133 that *yhcB* is conserved in three out of the six ESKAPE pathogens, is required for tolerance
134 to polymyxin, an antibiotic of last resort, and is the target of a sRNA toxin that causes cell
135 death, we decided to investigate the role of YhcB further (Mogi et al. 2006; Choi et al.
136 2018).
137
138 ***Deletion of* yhcB *confirms an envelope defect***
139 To confirm the barrier defect of the *yhcB* mutant, we grew the parent strain, *E. coli*
140 BW25113, and an isogenic Δ*yhcB* mutant on LB agar plates supplemented with either
141 vancomycin, a high molecular weight antibiotic that is typically unable to cross the Gram-
142 negative OM, or plates supplemented with SDS and EDTA (Nikaido and Vaara 1985;
143 Nikaido 2005). The Δ*yhcB* mutant was more sensitive than the parent strain to both
144 conditions (Fig. 2A). Ectopic expression of YhcB under arabinose induction on a pBAD
145 plasmid was able to complement sensitivity defects, consistent with previous reports
146 (Sung et al. 2020). These data confirm that loss of *yhcB* results in a severe envelope
147 defect (Fig. S2A).
148
149 ***Effect of YhcB on cell size***
150 Despite normalising cultures by optical density, the number of viable colony
151 forming units (CFUs) of the Δ*yhcB* mutant were 10-fold fewer than the parent on the LB
152 control plate (Fig. 2A). Analysis of cellular morphology by DIC microscopy revealed that
153 the Δ*yhcB* mutant cells were significantly larger than the parent strain when grown in LB
154 (Fig. 2B). Expression of *yhcB* in the Δ*yhcB* mutant background substantially decreased

155 the cell dimensions relative to the Δ*yhcB*-pBAD empty vector strain but did not restore
156 cell size completely (Fig S2B). We also observed that the ectopic expression of *yhcB* in
157 the *E. coli* BW25113 parent strain resulted in longer cells than the control. Taken together,
158 these data suggest that the abundance of YhcB within the cell impacts cellular
159 dimensions, and that YhcB is important for maintaining cell size and shape.

160 Bacterial cell dimensions are influenced by a number of factors (reviewed by Cesar
161 and Huang 2017), but cell size is fundamentally determined by growth rate and nutrient
162 availability (Schaechter et al. 1958; Hill et al. 2013; Vadia and Levin 2015; Vadia et al.
163 2017). A shift from growth in nutrient-restricted to nutrient-rich medium results in an
164 increase in both cell length and cell width (Pierucci 1978). Therefore, we investigated the
165 effect of growth medium on the size of the Δ*yhcB* mutant. Overnight growth in M9 minimal
166 medium supplemented with glucose (M9-glucose) substantially decreased the cell size
167 of the Δ*yhcB* mutant compared to growth in LB, although a small subset of cells within
168 the population still exhibited a division defect and increased cell length (Fig. 2B-C).
169 Moreover, overnight growth in M9-glucose restored resistance to membrane-acting
170 compounds SDS and EDTA, but cells remained susceptible to vancomycin (Fig. 2A).
171 Changes in temperature alter growth rate but not size (Schaechter et al. 1958). To
172 understand whether the fitness advantage conferred by growth in M9-glucose was due
173 to a slower doubling time, or nutrient-dependent, we grew the Δ*yhcB* mutant in a range
174 of conditions. Overnight growth in M9-glucose and M9-glycerol at 37˚C, and LB at 16˚C,
175 restored resistance to both SDS and EDTA, but no growth condition conferred resistance
176 to vancomycin (Fig. S3). Altogether these results indicate that a slower growth rate
177 partially alleviates the defect caused by deletion of *yhcB* but does not restore the
178 envelope permeability barrier.

179 We hypothesised that there is an envelope synthesis defect in a Δ*yhcB* mutant that
180 becomes more severe over time during growth in LB. To investigate this, overnight

181 cultures (grown first in M9 medium) were sub-cultured in LB at a starting optical density
182 (OD) of 0.05. Bacteria were grown at 37˚C with aeration, and both the OD_{600} and the
183 corresponding number of CFUs were recorded hourly. The rate of increase in biomass of
184 the mutant, as assessed by optical density, was comparable to that of *E. coli* BW25113
185 (Fig. 3). In contrast, upon transition to stationary phase, the number of Δ*yhcB* CFUs
186 decreased 10-fold from ~3.5×10^8 to ~3.5×10^7. These data suggest that the fast-growing
187 mutant cells gradually expand in cell size, fail to divide, but lose viability when they enter
188 the stationary growth phase.
189
190 ***The domains of YhcB contribute to survival under different conditions***
191 To gain a better understanding of how YhcB functions, we scrutinised the primary
192 structure of the protein. A prediction of YhcB secondary structure of YhcB by PSIPRED
193 supports the model put forward by Mogi et al. (2006; Jones 1999; Buchan and Jones
194 2019). YhcB has a single transmembrane (TM) domain, with a cytoplasmic helical domain
195 and a disordered cytoplasmic C-terminal region (Fig. S4A); the topology of which has
196 been confirmed by GFP- and PhoA-fusion analysis (Maddalo et al. 2011). A
197 multisequence alignment of the amino acid sequence of YhcB from 150 different species
198 was generated using the online program ConSurf (Ashkenazy et al. 2016). This revealed
199 two highly conserved amino acid motifs within the disordered C-terminus: a 'NPF' motif
200 after the long cytoplasmic helical domain, followed by a non-conserved linker region, and
201 a highly conserved 'PRDY' motif at the extreme C-terminus (Fig S4B). A large number of
202 transposon insertions were identified within the 3′ end of *yhcB* in the initial polymyxin TIS
203 screen; the *yhcB* gene only met the stringency criteria for identification when 20% of the
204 3′ end of the coding sequence (CDS) was discarded. We mapped the coordinates of
205 these domains to the original TIS data and the data suggest that the 'PRDY' domain is
206 dispensable for survival in the presence of polymyxin B (Fig. 4A). To confirm the

207 functional importance of the 'PRDY' domain, which is highly conserved (Fig. S5), we
208 constructed complementation vectors encoding truncated variants of YhcB and repeated
209 our plate-based envelope screens (Fig. 4B). Consistent with results reported by Sung *et*
210 *al*. (2020), the ΔTM construct fully complemented both phenotypes. However, deletion of
211 the 'PRDY' domain rendered the mutant sensitive to vancomycin, but not SDS-EDTA
212 (Fig. 4C). These data suggest a dual function of the YhcB protein: the cytoplasmic helical
213 domain is needed to suppress sensitivity to SDS and EDTA, while the function of the
214 'PRDY' domain is needed for survival in the presence of vancomycin, but not polymyxin
215 B or SDS and EDTA.
216
217 ***Identification of genetic interactions with* yhcB**
218 To identify all genes with a synthetically lethal relationship with *yhcB*, a transposon
219 mutant library was constructed in a Δ*yhcB* background using a mini Tn*5* transposon
220 encoding a kanamycin resistance gene. A second library was constructed in the *E. coli*
221 BW25113 parent strain using the same transposon as a control. Approximately 800,000
222 transposon-mutants were collected and pooled for each library. Two technical replicates
223 of each library were sequenced (Fig. S6A; Table S2). There was a high correlation
224 coefficient between the gene insertion index scores of each replicate for both libraries (R^2
225 = 0.93 and R^2 = 0.96 for the WT and Δ*yhcB* library respectively). The insertion sites were
226 evenly distributed around the genome (Fig. 5A and Fig. S6B), and the respective insertion
227 density amounted to an average of one insertion every 6.28 bp in the WT library, and one
228 insertion every 6.99 bp in the Δ*yhcB* library. We quantified insertions per CDS to identify
229 genes that were sparsely disrupted by transposon insertion events i.e. essential genes in
230 each genetic background (Barquist et al. 2016). There was an overlap of 382 essential
231 genes between the two datasets and these were not considered further (Fig. 5B).

232 Previously, Li *et al.* (2012) reported that the deletion of both *rodZ* and *yhcB* in
233 combination is lethal, genetically linking the elongasome with the function of YhcB. Our
234 data confirmed a synthetic lethal interaction between *rodZ* and *yhcB*. However, we
235 observed that only the 5′ end of the *rodZ* gene, corresponding with the cytoplasmic helix-
236 turn-helix and transmembrane domains of the RodZ protein, is essential in a *yhcB*
237 background (Fig. 5C). These domains are required for maintaining cell shape (Shiomi et
238 al. 2008; Bendezú et al. 2009). This result supports the validity of our approach.
239 Surprisingly, our bimodal analysis of the data revealed 28 genes that were predicted to
240 be essential in a WT library but no longer essential when *yhcB* is deleted (Fig. 5B, Table
241 S3). This list includes *ftsH* (Fig. S6C), which encodes a metalloprotease that degrades
242 several proteins, including LpxC. Deletion of FtsH results in toxic accumulation of LPS
243 via increased LpxC stability, which is lethal to the cell (Ogura et al. 1999). The increased
244 number of transposons in *ftsH* in the Δ*yhcB* library suggest that an increase in LpxC
245 stability in this background is less toxic.

246 The bimodal analysis revealed 121 genes that were predicted to be essential in a
247 Δ*yhcB* background. However, this analysis pipeline does not identify the non-essential
248 genes that contribute to fitness: such mutants are viable but either more or less fit.
249 Consequently, when compared to the control library they are represented by higher or
250 lower numbers of sequencing reads, respectively. Therefore we further analysed our data
251 using the tradis_comparison.R script (Barquist et al. 2016). This analysis uses edgeR and
252 measures the fold-change of sequence read depth between a condition and control
253 (Robinson et al. 2009). The 382 genes reported to be essential in both libraries were
254 removed from this analysis. After filtering our data, this method identified 22 genes that,
255 when disrupted, confer a fitness advantage (Fig.5D, blue), and 163 genes that, when
256 disrupted, confer a fitness defect in a Δ*yhcB* mutant (Fig. 5D, red; Table S4; Fold change
257 > 2, Q-value < 0.01). By combining these analyses, we defined synthetically lethal genes

258 as those that are both significantly underrepresented by transposon insertion mutants
259 and that have a >2-fold decrease in reads when compared to the control with a Q-value
260 < 0.01. Altogether 87 genes met these criteria: we consider these genes to be
261 synthetically lethal with *yhcB* and they are discussed below (Fig. 5E).
262
263 ***Identification of pathways important for viability of a* yhcB *mutant***
264 As YhcB interacts directly with components of the elongasome, and is synthetically
265 lethal with *rodZ*, we inspected the list of synthetic lethal genes for additional genes
266 involved in peptidoglycan synthesis, remodelling or recycling. Five genes (*mepM*, *mepS*,
267 *dacA, dapF* and *ldcA*) were identified (Fig. 6A). The genes *mepM* and *mepS* encode two
268 of the three peptidoglycan endopeptidases that cleave the 3-4 *meso*-Dap-D-Ala crosslink
269 and are collectively essential for cell elongation in *E. coli* (Singh et al. 2012). However, a
270 third endopeptidase of this group encoded by *mepH,* was not essential in a Δ*yhcB* mutant
271 (Fig. 6A-B). DapF is an epimerase that catalyses the conversion of L,L-diaminopimelate
272 (LL-DAP) to *meso*-diaminopimelate (*meso*-DAP), which is an integral component of the
273 peptidoglycan stem peptide and the primary residue from which cross-links are formed
274 (Richaud et al. 1987). Sacculi isolated from *dapF* mutants have fewer crosslinks (Mengin-
275 Lecreulx et al. 1988). The DD-carboxypeptidase PBP5, encoded by *dacA*, removes the
276 terminal D-alanine from peptidoglycan pentapeptides (Spratt and Strominger 1976), and
277 is the primary carboxypeptidase under standard laboratory growth conditions (Nelson and
278 Young 2000; Nelson and Young 2001). Finally, the cytosolic LD-carboxypeptidase LdcA
279 participates in the recycling of peptidoglycan turnover products (Fig. 6A). Deletion of *ldcA*
280 results in the lysis of cells during entry into the stationary growth phase due to the
281 accumulation of peptidoglycan crosslinking defects (Templin et al. 1999). From these
282 data, it could be inferred that a Δ*yhcB* mutant cannot survive when additional mutations
283 weaken the integrity of the peptidoglycan layer.

284 To determine whether other cellular processes or pathways are enriched among
285 the genes identified as synthetically lethal with *yhcB,* we used the PANTHER
286 overrepresentation test (Mi et al. 2021). This analysis compares the identified proportion
287 of genes in a given functional category to the expected number of genes in a pathway
288 derived from whole genome data (Table S6). Four processes were identified as
289 functionally enriched among the list of synthetic lethal genes: the 'enterobacterial
290 common antigen (ECA) biosynthetic pathway', 'LPS core biosynthesis', 'cell division', and
291 'response to abiotic stimulus' (Fig. 7A). With the exception of the *tol-pal* system (Fig.
292 S7A), the remaining genes listed within the 'cell division' and 'response to abiotic stimulus'
293 categories did not share overlapping functions and we did not pursue these further.

294 Upon closer inspection of LPS biosynthetic pathways, both the heptosyl
295 transferases WaaC and WaaF, and the entire heptose biosynthetic pathway were
296 synthetically lethal (Fig. 7B-C, Fig. S7B). The WaaP kinase, which phosphorylates the
297 first heptose of the inner core, was also essential in a *yhcB* mutant. Enzymes
298 WaaBGQRY were not essential in a Δ*yhcB* background, but mutants were less fit. In
299 contrast, ligation of the third Kdo moiety by WaaZ was non-essential. The acylation of
300 lipid A by LpxM was also essential in a Δ*yhcB* strain (Fig. 7C). Together these data
301 suggest that LPS stability, whether mediated by Lipid-A hexa-acylation or LPS
302 crosslinking interactions, is important for viability of a Δ*yhcB* strain.

303 Within the locus required for ECA biosynthesis the genes *wecCDEF*, *rffH* and
304 *wzxE* were synthetically lethal with *yhcB*, while disruption to *wecB* and *wecG* suggests
305 these genes were not synthetically lethal but the mutants were sick (Fig. 7D, Fig S7C).
306 There were no significant differences in the insertion frequency within genes *wzzE* and
307 *wecA*. WzzE mediates the length of the ECA chains by determining the number of
308 repeating units (Barr et al. 1999), and WecA catalyses the first committed step in ECA
309 biosynthesis: the transfer of *N*-acetylglucosamine-1-phosphate onto undecaprenyl

310 phosphate (Und-P) to form ECA-lipid I. In short, an ECA-null mutant is viable and mutants
311 with variable ECA lengths are also viable; only mid-pathway blocks are lethal. Mutations
312 that introduce a mid-pathway block in ECA biogenesis are known to cause an aberrant
313 cell morphology (Jorgenson *et al*. 2016), due to accumulation of undecaprenyl-linked
314 ECA-lipid II intermediates creating limited availability of Und-P: a compound of limited
315 abundance that sits at the start of several cell envelope biosynthetic pathways (Jorgenson
316 *et al*. 2016). These observations suggest that the combined defects of *yhcB*-deletion and
317 Und-P sequestration are lethal, and indicate that the availability of Und-P might be limited
318 in a *yhcB* mutant.
319
320 ***Identification of mutations that suppress a* yhcB*-deletion defect***
321 In addition to synthetic lethal interactions, mutations that restore a phenotypic
322 defect, so-called suppressor mutations, can assist in the identification of interconnected
323 cellular pathways (Ogura et al. 1999). We applied TIS to identify, at a whole genome
324 scale, all mutations that can restore tolerance to vancomycin or SDS and EDTA, and
325 therefore suppress a Δ*yhcB* phenotype. First, the Δ*yhcB* mutant library was screened on
326 LB agar supplemented with either SDS and EDTA or vancomycin at concentrations that
327 kill the Δ*yhcB* strain. Transposon mutants of the Δ*yhcB* library able to grow under these
328 conditions were identified as before (Fig. 8A-B). We used the recently published
329 AlbaTraDIS package to analyse our data (Page et al. 2020). We identified 28 gene-
330 deletion suppressor mutations shared between both conditions, and therefore considered
331 these to be universal suppressors (Fig. 8C; Table S7). Of note among the 28 universal
332 suppressors was the Mla pathway. All genes of the pathway have insertions along the full
333 length of each CDS indicating a disruption at any stage of the pathway is restorative to
334 both vancomycin and SDS and EDTA sensitivity (Fig. 8D). This was unexpected as *mla*

335 mutants are highly sensitive to SDS and EDTA (Malinverni and Silhavy 2009; Ekiert et al.
336 2017; Isom et al. 2017).

337 Deletion of *nlpI* was also identified as a universal suppressor (Fig. S8A). NlpI is an
338 OM lipoprotein that functions as an adaptor protein for peptidoglycan endopeptidases
339 (Banzhaf *et al*., 2020) and mediates the degradation of MepS by the protease Prc
340 (Tadokoro et al. 2004; Singh et al. 2015). In a Δ*nlpI* strain, MepS activity is significantly
341 increased (Singh *et al*. 2015), which has been shown to enhance peptidoglycan synthesis
342 by stimulating PBP1B-mediated peptidoglycan synthesis and directing peptidoglycan
343 precursors away from the elongasome complex (Lai et al. 2017), presumably to facilitate
344 the repair of defects in the PG (More et al., 2019). Consistent with this hypothesis, *mrcB*
345 (PBP1B) was identified in the list of genes that confer a fitness defect on a *yhcB* mutant
346 strain when deleted (Table S4), while deletion of *mrcA* (PBP1A) and *lpoA*, which encodes
347 the PBP1A activator, were identified as universal suppressors. Suppressor analysis
348 therefore supported a functional link between YhcB and PG synthesis.

349 Another universal suppressor we identified was deletion of *fabF,* genetically linking
350 *yhcB* with fatty acid biosynthesis (Fig. S8B). FabF, together with FabB, are the two β-
351 ketoacyl-[acyl carrier protein] synthases involved in fatty acid elongation (Garwin et al.
352 1980a). FabB is the major synthase and is essential while FabF is predominantly required
353 at low temperatures to increase membrane fluidity by increasing the proportion of
354 diunsaturated phospholipids (Garwin et al. 1980b), as FabF is more efficient than FabB
355 at elongating palmitoleic acid (16:1Δ9) to cis-vaccinic acid (18:1Δ11). Therefore, loss of
356 *fabF* might result in decreased membrane fluidity or a decreased rate of phospholipid
357 synthesis, or both.

358 We plotted the total suppressor insertion data on a genome map to view relative
359 mutant abundance; the assumption here is that read depth is representative of mutant
360 abundance, which correlates with fitness and is an indicator of the strength of the

361 suppression. In addition to Δ*fabF*, disruption of the *mla* operon and Δ*nlpI* we observed a
362 substantial peak at the *uppS-cdsA* locus in both conditions (Fig. 9A). UppS, encoded by
363 *uppS* (*ispU*), is an undecaprenyl pyrophosphate synthase, which synthesises
364 undecaprenyl diphosphate (Und-PP), the only source of *de novo* synthesised Und-PP
365 and the precursor to Und-P (Apfel et al. 1999; Kato et al. 1999). CdsA sits before the
366 branchpoint in the synthesis of the major phospholipids and catalyses the synthesis of
367 cytidine diphosphate-diacylglycerol from phosphatidic acid (Kanfer and Kennedy 1964;
368 Ganong et al. 1980). Both UppS and CdsA are essential, as such, neither gene can be
369 disrupted by transposon insertion. However, we observed that disruption upstream of the
370 *uppS-cdsA* operon is significant in both suppressor conditions (Fig. 9B). It was unclear
371 from the insertion pattern the effect that disruption at this locus would have. However,
372 these data suggest that deregulation of the native level of expression of *uppS-cdsA* is
373 restorative in a *yhcB* mutant background.

374 Finally, during screening of the transposon mutant library on supplemented agar
375 plates, we also isolated six colonies of the parent Δ*yhcB* strain growing on the control
376 plates of LB supplemented with vancomycin, assumed to be natural revertant
377 suppressors of Δ*yhcB*. The revertant suppression was confirmed by plating on both
378 vancomycin and SDS-EDTA, as well as confirming gene-deletion by PCR of the *yhcB*
379 locus (Fig. S9A-B). We sequenced the genomes of these isolates and identified the
380 mutations listed in Table S8. Two isolates contained either the reported *mlaA** mutation
381 or a variation of this mutation (mut4 and mut6), while one isolate had a genome inversion
382 that resulted in separation of the *mla* operon from its promoter region (mut2). The *mla*
383 inversion mutation restored resistance to both vancomycin and SDS-EDTA, consistent
384 with the earlier TIS data, but did not fully restore cell size (Fig. S9A-C). The *mlaA**
385 mutation is well characterised and is toxic in a WT background as it results in LPS
386 overproduction as a consequence of increased LpxC stability (May and Silhavy 2018).

387 However, these mutations are not toxic in a Δ*yhcB* mutant and restore cell size and
388 resistance to vancomycin (Fig. S9A-C). We also identified a single nucleotide
389 polymorphism (SNP) in *lpxC* that restored resistance to vancomycin and SDS-EDTA but
390 did not restore cell size (mut1). These data are suggestive of either a defect in LPS
391 synthesis or export, or an imbalance in LPS to phospholipid at the outer membrane of a
392 Δ*yhcB* mutant, rendering the *mlaA** mutation non-lethal in this background.

393 Finally, two isolates each had a SNP that resulted in a single amino acid
394 substitution in CdsA. We used Phyre2 to predict the structure of CdsA (Kelley et al. 2015).
395 The predicted structure was very similar to the experimentally determined structure of
396 CdsA, derived from *Thermotaga maritama*, with an RMSD value of 0.404 Å (Fig. S9D)
397 (Liu et al. 2014). The active site of CdsA is a conserved, negatively charged pocket
398 occupied by two cation cofactors that are critical for function (Fig. S9D). Mutations within
399 this region that hinder cation binding result in decreased enzyme activity (Liu et al. 2014).
400 The mutations identified within CdsA in our revertant suppressor mutants (S231L and
401 S239G, respectively) each result in substitution of a serine residue proximal to the metal
402 ion binding site (Fig. S9D). A mutation of S223C in *T. maritama* CdsA, which corresponds
403 with residue S239 in *E. coli*, results in severely reduced CdsA function (Liu et al. 2014).
404 The two mutations within CdsA in the revertant suppressor mutants likely result in
405 decreased function of CdsA. Both mutants with a SNP in CdsA (mut3 and mut5) restored
406 resistance to both vancomycin and SDS and EDTA, in addition to cell size (Fig. S10A-C).
407

408 ***Characterisation of a* yhcB*-mutant envelope***

409 Our genetic screening revealed a connection with phospholipid biosynthesis (*fabF,*
410 *cdsA*) and trafficking (*mla*), Previous research has shown that fatty acid availability
411 determines the cell size via phospholipid biosynthesis (Vadia et al. 2017). Increased
412 phospholipid production resulted in a larger cell size that was reminiscent of a Δ*yhcB*

413 mutant (Vadia et al. 2017). As such, we hypothesised that phospholipid production might
414 be increased in a *yhcB* mutant. We first investigated whether membrane "lipid ruffles"
415 resulting from increased phospholipid synthesis, and resembling those reported by Vadia
416 et al. (2017), could be detected in a *yhcB* mutant. Following 3 hours of growth in LB, *E.*
417 *coli* BW25113 and an isogenic *yhcB* mutant were stained with MitoTracker to label
418 internal lipid structures (Vadia et al. 2017). Microscopic evaluation of these cells revealed
419 that *E. coli* BW25113 membranes stained uniformly. In contrast, intensely stained spots
420 indicative of accumulation of lipid structures were observed in the *yhcB* mutant (Fig.
421 S12A). Subsequent imaging of cells by transmission electron microscopy confirmed the
422 aberrant size of *yhcB* mutant cells and revealed the presence of internal membrane
423 structures (Fig. 10A, Fig. S10). These structures were further examined in fast frozen
424 cells after freeze substitution (Walser et al, 2012) avoiding artefacts associated with
425 chemical fixation. Internal membranes showed a range of different morphologies
426 including tubular and vacuolar structures (Fig. 10B, Fig. S11) as well as striking tightly-
427 stacked membrane arrays (yellow arrowheads Fig. S11).

428 Given the increase in phospholipid biogenesis noted above we next investigated
429 the composition of phospholipids species. We extracted the total phospholipid species
430 using the Bligh and Dyer method (Bligh and Dyer 1959), and separated these by thin
431 layer chromatography (Matsumoto et al. 1998). The phospholipid composition of *E. coli*
432 is approximately 75% phosphatidyl ethanolamine, 20% phosphatidyl glycerol and 5%
433 cardiolipin with lysophospholipids typically making up <1% of total phospholipid species
434 (Rowlett et al. 2017; Zheng et al. 2017). The relative ratios of phosphatidylethanolamine,
435 phosphatidylglycerol and cardiolipin were similar in the parent and Δ*yhcB* strains.
436 However, during growth in LB, we observed an additional phospholipid species that was
437 present in both strains, but only the Δ*yhcB* mutant strain in stationary phase (Fig.10B and
438 S10B). This was not observed when the mutant was grown in M9-glucose (Fig. S10C).

439 By using known lipid standards, the additional spot in the Δ*yhcB* mutant stationary phase
440 was identified as most likely lysophosphatidylethanolamine (LPE; Fig. 10B).
441 LPE can be derived from PE hydrolysis or lipoprotein maturation. However, in a
442 cell with a stressed envelope, the primary source of LPE accumulation occurs via PE
443 hydrolysis (Zheng et al. 2017). Two OM proteins, PldA and PagP, maintain the OM
444 asymmetry by cleaving phospholipids that accumulate in the outer leaflet of the OM. PldA
445 degrades phospholipids while PagP, a Lipid A palmitoyltransferase, cleaves an acyl chain
446 from PE and catalyses its transfer to Lipid A resulting in hepta-acylated LPS (Bishop et
447 al. 2000). Both reactions result in an increase in free LPE. As PagP is usually inactive in
448 the OM and activity is stimulated in response to migration of phospholipids into the outer
449 leaflet (Jia et al. 2004), measuring the amount of hepta-acylated LPS is an established
450 proxy for detecting loss of OM asymmetry (Chong et al. 2015). We quantified the amount
451 of hepta-acylated lipid A relative to hexa-acylated lipid A and identified a significant
452 increase in hepta-acylated lipid A in the Δ*yhcB* mutant compared to the WT; a phenotype
453 that is exacerbated by prolonged growth (Fig. 10C). Together these data suggest that
454 excess phospholipid production in a *yhcB* mutant background results in an increase in
455 cell size and loss of OM asymmetry, resulting in hepta-acylation of lipid A and
456 accumulation of LPE.
457 Cell membrane synthesis must be coordinated with peptidoglycan biogenesis to
458 maintain cell envelope integrity. As the peptidoglycan layer defines cellular morphology,
459 and we had observed a swollen morphology of *yhcB* mutants, we hypothesised that the
460 peptidoglycan layer of a *yhcB* mutant may be compromised. Indeed, our genetic evidence
461 supports the hypothesis that mutations (*dapF, dacA*) that weaken integrity of the
462 peptidoglycan are lethal. We therefore analysed the muropeptide composition of the cell
463 wall. Cells were grown in LB and collected at both exponential and stationary phase.
464 Peptidoglycan was purified and digested by the muramidase cellosyl, and the resulting

465 muropeptides were analysed by HPLC (Glauner 1988). There were no significant
466 differences in the muropeptide species identified (Fig S11; Table S5). However, we did
467 identify differences in the amount of crosslinking between strains and growth phases (Fig.
468 10D). While the degree of peptidoglycan crosslinking in the WT strain was increased in
469 stationary phase compared to exponential phase, consistent with the literature (Templin
470 et al. 1999), this same transition was not observed in the *yhcB* mutant, suggesting either
471 a limitation in the rate of peptidoglycan crosslinking or an increased rate of crosslinking
472 hydrolysis in a *yhcB* mutant. The muropeptide analysis, together with the phospholipid
473 analysis, support our finding that a *yhcB* mutant is unable to sustain rapid exponential
474 growth and indicate a defect in the ability to transition into stationary phase.

475 In addition to a synthetically lethal relationship with genes involved in
476 peptidoglycan assembly, recycling and remodelling (*mepS, mepM, ldcA, nlpI*) our TIS
477 data also indicated that mutations in the ECA biosynthetic pathway that sequester
478 undecaprenol phosphate (Und-P) are lethal in a *yhcB* mutant. We hypothesised that the
479 availability of Und-P might be limited in a Δ*yhcB* mutant, affecting peptidoglycan synthesis
480 (Danese *et al*. 1998; Ramos-Morales *et al*. 2003; Jorgenson *et al*. 2016). In addition to
481 peptidoglycan and ECA biosynthesis, Und-P is also a lipid carrier for O-antigen, however,
482 most *E. coli* K-12 strains, including BW25113, do not have O-antigen as a result of an
483 insertion element in *wbbL* (Liu and Reeves 1994). Therefore, to investigate whether
484 sequestration of Und-P is toxic to a *yhcB* mutant, we tried to restore O-antigen. The
485 transformation efficiency of the *yhcB* mutant with *wbbL* to restore O-antigen was strikingly
486 reduced, suggesting that Und-P limitation is lethal to a *yhcB* mutant (Fig. S10D). One
487 mechanism to suppress Und-P limitation is to upregulate expression of *uppS,* the gene
488 required for Und-P synthesis. Indeed, our TIS data reveal insertions that suppress the
489 *yhcB* phenotype in the promoter region to *uppS*. Furthermore, complementation of a *yhcB*

490 mutant with ectopically expressed *uppS*, but not *cdsA,* suppressed both the vancomycin
491 and SDS-EDTA sensitivity defect of a *yhcB* mutant (Fig. 10E).

492 During our analysis we noted that *uppS* and *cdsA* are highly co-conserved across
493 the bacterial kingdom (Fig. S12) and share a conserved operon structure in many
494 bacterial phyla (Fig.S13) (Saha et al. 2020). We hypothesise that the upregulation of
495 *uppS* would result in increased expression of *cdsA*, and in turn give rise to excess
496 phospholipid synthesis. This hypothesis is supported by our finding that loss of function
497 mutations in the metal-binding pocket of CdsA restore all *yhcB* defective phenotypes (Fig.
498 9C).

499

500 **Discussion**

501 Here we have applied a high-density mutagenesis screen to comprehensively map
502 the genetic interactions of a single gene, *yhcB*. With thousands of deleterious and
503 restorative genetic interactions discovered, to the best of our knowledge no other gene
504 has been scrutinised genetically at such scale and resolution. Our data reveal that a
505 Δ*yhcB* mutant has a cell envelope permeability defect, dysregulated cell size, and
506 epistatic interactions with multiple pathways of cell envelope biogenesis. The defect
507 introduced by deletion of *yhcB* is most severe during rapid growth in rich media, when the
508 demand for synthesis of cell envelope material is greatest. In addition, toxicity progresses
509 with sustained growth in rich medium, with a decrease in cell viability observed upon
510 transition to stationary phase, a period of growth when *de novo* synthesis machineries
511 are switched off. Taken together, these observations indicate that a *yhcB* mutant is either
512 limited in the ability to recycle existing components of the envelope during synthesis, or
513 limited in *de novo* synthesis of one or more cell envelope components. This hypothesis
514 is supported by the cell growth model put forward by Harris and Theriot (2016) that when
515 cell envelope synthesis is impeded, an increase in cell width is observed decreasing the

516 surface area to cell volume ratio and enabling the same amount of cell volume to be
517 encapsulated by less material.

518 The pathways for synthesis of the different components of the Gram-negative cell
519 envelope are intrinsically linked: they share several common precursors and tightly
520 interconnected feedback mechanisms (Fig. 11A). If the rate of peptidoglycan biosynthesis
521 is decreased, the rate of LPS biosynthesis needs to be decreased accordingly to avoid
522 the toxic effects of LPS accumulation (Sullivan and Donachie 1984; May and Silhavy
523 2018). Moreover, a recent paper identified that deletion of the *mla* pathway confers a
524 fitness advantage in cells exposed to fosfomcin, a MurA inhibitor, connecting decreased
525 PG synthesis with a requirement for decreased PL trafficking (Turner et al. 2020).
526 Similarly, when LptC, a component of the LPS export system, is depleted the relative
527 abundance of proteins involved in phospholipid biosynthesis and trafficking are also
528 depleted indicating a feedback mechanism that couples phospholipid synthesis and
529 export with LPS demand (Martorana et al. 2014). In a *yhcB* mutant we observed
530 weakened peptidoglycan integrity and a loss of OM asymmetry, coupled with an increase
531 in cell size and internal phospholipid membrane structures (Fig. 11B). These findings
532 connect YhcB with every layer of the cell envelope.

533 Our observations of excess phospholipid membranes and an increased cell size
534 in a *yhcB* mutant resemble the phenotype reported by Vadia *et al.* (2017) that results from
535 increased phospholipid synthesis. We hypothesise that the increase in phospholipid
536 production results in loss of OM asymmetry, with substantial amounts of phospholipid
537 integrating into the outer leaflet of the OM to compromise the barrier function of the cell
538 envelope. This hypothesis is supported by several lines of evidence. First inhibition of
539 phospholipid synthesis by mutations within the metal ion binding site of CdsA, or loss of
540 FabF, which regulates membrane fluidity, are restorative to a *yhcB* mutant. Furthermore,
541 treatment with sub-inhibitory concentrations of cerulenin, an antibiotic that targets the

542 FabB and FabF elongation step of fatty acid synthesis, is beneficial to growth of a *yhcB*
543 mutant (Nichols et al. 2011). Second, a *mlaA** mutation, which increases mislocalisation
544 of phospholipids at the OM triggering subsequent phospholipid degradation by PldA and
545 is toxic in a wild-type background, is tolerated in a *yhcB* mutant, restores cell size, and
546 re-establishes the barrier function of the OM. Third, an increase in LPS production
547 through the loss of the essential protease FtsH, the introduction of *mlaA** which
548 upregulates LpxC production, or by mutation of its target LpxC can be tolerated. Overall,
549 mutations that result in a decrease of phospholipid biosynthesis and trafficking, or an
550 increase in LPS biosynthesis are restorative, indicative of an underlying imbalance in PL
551 to LPS levels.

552 We found that mutations that weaken PG integrity are lethal in a *yhcB* mutant
553 indicating that the rate of PG synthesis is also not coordinated with cell expansion. While
554 the composition of the PG in a *yhcB* mutant resembles that of the WT, our data reveal
555 that loss of PG synthesis by the elongasome is restorative. In contrast, loss of PG
556 synthesis by the divisome had a negative effect while mutations that stimulate PG
557 synthesis via the divisome are restorative. Given the location of YhcB and the phenotypes
558 of a deletion mutant, an attractive hypothesis is that YhcB is involved in switching PG
559 synthesis between the elongasome and divisome. In support of this hypothesis a recent
560 paper reported the interaction and co-purification of YhcB with proteins of the divisome
561 (Mehla et al. 2021).

562 Another attractive hypothesis is that YhcB has a role in sensing the availability of
563 Und-P, or recycling/synthesis of Und-P. This latter hypothesis is supported by several
564 observations. First, mutations in the ECA biosynthesis pathway that sequester Und-P and
565 limit the available pool are lethal. Second, restoration of WbbL activity is toxic in a *yhcB*
566 mutant, suggesting that Und-P availability is limited. Third, a *yhcB* mutant is reported to
567 be more sensitive to bacitracin (Nichols et al. 2011), which targets BacA. BacA is

568 responsible for recycling approximately three quarters of Und-PP back to Und-P following
569 release of its substrate in the periplasm. Fourth, deletion of *mrcB* in cells with low levels
570 of Und-P results in heterogenous cell lysis, and *mrcB* was identified in the list genes in
571 which an epistatic interaction with *yhcB* causes a fitness defect. These data are
572 consistent with the finding that only a sub-population of the Und-P depleted *mrcB* mutants
573 lyse (Jorgenson et al. 2019). Lastly, upregulation of *uppS*, but not *cdsA*, was universally
574 restorative in a *yhcB* mutant.

575 The observation that the *uppS-cdsA* operon structure is conserved across bacteria
576 reveals the tight linkage between phospholipid production and the synthesis of
577 polysaccharide components of the cell envelope. The observation that cell size,
578 resistance to vancomycin, and resistance to SDS-EDTA can be decoupled in suppressor
579 screens indicate that YhcB interacts with multiple cell envelope biogenesis pathways.
580 Indeed, in addition to forming a dimer and reports of larger homo-multimer structures
581 (Maddalo et al. 2011; Li et al. 2012; Mehla et al. 2021), YhcB is known to interact with
582 several components of the elongasome (Li et al. 2012), and has also been identified as
583 an interaction partner of LapA (Hu et al. 2009; Li et al. 2012). LapA (YciS) forms a
584 complex with LapB (YciM), FtsH and YejM to regulate LpxC stability and thus LPS
585 abundance (Clairfeuille et al. 2020; Fivenson and Bernhardt 2020; Guest et al. 2020).
586 The role of LapA is unclear but deletion of *lapA* results in minor accumulation of
587 incomplete LPS precursors (Klein et al. 2014). The interaction of YhcB with the
588 elongasome and the LpxC regulation complex via LapA positions YhcB at the interface
589 between two of the major complexes that coordinate envelope synthesis (Fig. 11C). This
590 hypothesis is supported by the observation that deletion of different YhcB domains gives
591 rise to different chemical sensitivity profiles.

592 In conclusion, YhcB plays an important regulatory function at the interface between
593 all of the cell envelope biogenesis pathways, and this function is mediated by physical

interactions between members of the elongasome and LPS regulation systems. While the precise molecular events facilitating the regulation of these pathways has yet to be elucidated, as *yhcB* is conserved among Gammaproteobacteria, is required for tolerance to polymyxin B, colistin, and vancomycin, and is necessary for the colonisation of different hosts (Harvey et al. 2011; Brooks et al. 2014; Jana et al. 2017), it provides a novel antimicrobial target for exploitation against clinically important pathogens.

Materials and Methods

Strains, media and growth conditions

The parent strain for this work was *E. coli* K-12 strain BW25113. Gene deletion mutants were constructed by P1 transduction and using the Keio library strains as donors (Baba et al. 2006; Thomason et al. 2007). The kanamycin cassette was removed using the pCP20 vector (Datsenko and Wanner 2000). The mutant was confirmed by PCR and Sanger sequencing. Bacteria were grown in Luria-Bertani (LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl) medium or on LB plates (LB supplemented with 1.5% nutrient agar) and incubated at 37°C. When required, media were supplemented with 50 µg/ml kanamycin or 100 µg/ml carbenicillin. The M9-glucose recipe used was: 1x M9 salts (Sigma Aldrich), 200 µl filter sterilised 1 M $MgSO_4$, 10 µl filter sterilised 1 M $CaCl_2$, and 2 ml of either a 20% (w/v) D-glucose or 20% (v/v) glycerol solution per 100 ml. For micro-dilution spot plates, unless otherwise stated, bacteria were grown overnight in 5 ml LB medium at 37°C with aeration. Cultures were normalised by optical density to an OD_{600} of 1.00, 10-fold serially diluted in LB, and 2 µl of each dilution was inoculated onto LB agar plates.

Complementation assays

619 The BW25113 and *yhcB* strains were transformed with a pBAD-Myc-His-A plasmid with
620 and without the *yhcB* coding sequence under the control of the arabinose promoter. O-
621 antigen was restored as described previously (Browning et al. 2013). In brief, cells were
622 transformed with a pET20b plasmid DNA encoding *wbbL* Additional copies of *cdsA* and
623 *uppS* were introduced, independently, on a pASK-IBA2C plasmid under the control of a
624 tetracyclin-inducible promoter.
625
626 *Polymyxin B TIS screen*
627 An amended version of the Andrews broth microdilution protocol in 96-well plate format,
628 adjusting the starting inoculum to an initial OD_{600} of 0.05 and the growth medium to LB,
629 was used to identify an initial inhibitory concentration range or polymyxin B (Andrews
630 2001). Growth curve experiments of *E. coli* BW25113 were then repeated in 50 ml LB
631 supplemented with polymyxin B in glass flasks, reflecting the conditions of the TIS screen.
632 A concentration of 0.2 µg/ml polymyxin B was identified as sub-inhibitory for growht of
633 BW25113. The transposon library was inoculated into 50 ml LB medium with and without
634 0.2 µg/ml polymyxin B (Sigma Aldrich), in duplicate, at a starting optical density of 0.05,
635 equivalent to a copy number of ~2,500 of each mutant, and grown to OD_{600} = 1.00, at
636 37˚C with aeration. Cells were harvested, and genomic DNA prepared for sequencing.
637 As we knew the density of this library (Goodall et al. 2018), we estimated the number of
638 mapped reads, and therefore sequencing coverage, needed to ensure sufficient sampling
639 of the library using the equation $\mathrm{I} = s - s(\frac{s-1}{s})^n$, where I = insertions, and n = number of
640 mapped reads (Fig. S1B). For a theoretical library of 1 million mutants, assuming no loss
641 of mutants and an equal chance of each transposon junction being sampled, ~2.3M reads
642 are needed to ensure sampling of 90% of the library, with diminishing returns with further
643 sequencing. Obtaining >4.2 M reads should enable sampling of 99% of the possible
644 unique insertion sites. Therefore, we collected >2 M sequencing reads per replicate, and

645 a combined total of >4.2 M reads per condition, to give us confidence that any observed
646 loss of mutants is not due to insufficient sampling.
647
648 *Microscopy*
649 Samples were taken directly from overnight cultures grown at 37˚C in their respective
650 media for 16 h and diluted to an OD_{600} of 0.10. 5 µl cells were spread on a 1 mm glass
651 slide pre-treated with 5 µl poly-L-lysine (Sigma Aldrich). A Nikon 90i eclipse microscope
652 was used to capture differential interference contrast (DIC) images of cells, using a 40x
653 objective lens with a Nikon immersion oil. These images were collected at the University
654 of Birmingham. Cell dimensions were measured using ImageJ (Schindelin et al. 2012).
655 Fluorescent labelling of lipids was previously described by Vadia *et al*. (2017). These
656 experiments were done at the University of Queensland. M9-glucose overnight cultures
657 were diluted into fresh LB medium and after 2 h of growth at 37˚C, 0.2 µM MitoTracker®
658 Green FM (Invitrogen) was added to each sample to label lipids. After incubation for a
659 further 1 h, bacteria from 1 ml samples were harvested by centrifugation and
660 resuspended in 500 µL of FM™ 4-64FX (Invitrogen) at 5 µg/mL, to label *E. coli*
661 membranes, and incubated for 5 min at room temperature. Bacteria were embedded on
662 1% agarose pads and imaged using a confocal microscope Inverted LSM 880 Fast
663 Airyscan (63x/1.40 OIL). Electron microscopy was performed at the University of
664 Queensland. Conventional fixation and embedding in resin was undertaken using a
665 protocol adapted from (Fassel et al. 1997). Briefly, bacteria were applied to dishes with
666 glass coverslips coated in poly-L-lysine and then fixed in 2% PFA, 2.5% Glutaraldehyde,
667 and 0.075% Ruthenium Red. Samples were then washed 3x10min in PBS and postfixed
668 in 1% osmium, then again in 1% osmium with 3% potassium ferricyanide. Bacteria
669 underwent serial dehydration in increasing concentrations of ethanol and were then
670 infiltrated and embedded with Epon resin. Fast freezing and freeze substitution of bacteria

671 was undertaken as described previously (Ariotti et al. 2012; Walser et al. 2012). Briefly,
672 bacteria were applied to carbon-coated poly-L-lysine treated sapphire discs and frozen in
673 a Leica EMPACT 2 high pressure freezer. They were then freeze substituted in a EM
674 AFS 2 system (Leica Microsystems GmbH, Wetzlar, Germany) and embedded into Epon
675 resin. Ultra-thin sections were obtained using a Leica Ultracut UC6 Ultramicrotome and
676 micrographs acquired on a JEOL 1011 transmission electron microscope equipped with
677 a Morada CCD camera.
678
679 *Phospholipid extraction*
680 Total phospholipids were extracted using an amended version of the Bligh-Dyer method
681 (Bligh and Dyer 1959). 10 ml of culture at an OD_{600} ~ 4.00, or 100 ml of culture at an
682 OD_{600} of 0.40 was centrifuged at 4˚C to harvest cells. The supernatant was discarded,
683 and pellet resuspended in 1 ml ddH_2O and transferred to a glass tube. 1.25 ml chloroform
684 and 2.5 ml methanol were added to the sample using glass pipettes. The sample was
685 vortexed for 20 s to create a single-phase solution which was then incubated at 50°C for
686 30 min. A further 1.25 ml chloroform and 1.25 ml water was added to the sample to create
687 a 2-phase solution and incubated again at 50°C for 30 min. The sample was centrifuged
688 at 400 x *g* for 10 min at RT and the lower organic phase containing phospholipids was
689 transferred to a new glass tube using a glass Pasteur pipette. Chloroform was
690 evaporated by placing the glass tubes in a heat block at 50°C under a stream of nitrogen.
691
692 *Thin Layer Chromatography*
693 10 µl of sample was spotted onto the origin of a TLC silica gel membrane (Merck) using
694 a 5 µl glass capillary tube (Sigma Aldrich). Once dry, the membrane was transferred to
695 an equilibrated solvent system of 65:25:4 chloroform:methanol:water (Matsumoto et al.
696 1998). The samples were separated until the solvent front had migrated sufficiently from

697 the origin then the membrane was removed from the solvent tank and was air dried at
698 RT. Samples were stained with phosphomolybdic acid (PMA) 10% solution in ethanol
699 and heated with a heat gun to activate the PMA until lipid species were visible. Lipid
700 standards were purchased from Avanti Polar Lipids and handled according to
701 manufacturer instructions.
702
703 *Lipid A palmitoylation assay*
704 Lipid A labelling, extraction and analysis were done as described and demonstrated
705 previously (Chong et al. 2015). As a positive control, BW25113 was exposed to 25 mM
706 EDTA for 10 min to induce PagP mediated palmitoylation of Lipid A, before harvesting
707 cells by centrifugation.
708
709 *Peptidoglycan extraction and analysis*
710 Cell cultures were grown in LB medium at 37˚C and harvested at an OD of 0.4 and 4.00,
711 in triplicate. After pelleting the cells and resuspending in ice-cold water, the cell
712 suspension was dropped into 8% boiling SDS solution. Peptidoglycan was purified, and
713 muropeptides were released with cellosyl and analysed by HPLC as described (Glauner
714 1988).
715
716 *Construction of transposon-mutant libraries*
717 10 ml of 2x TY broth was inoculated with a single colony and grown overnight at 37°C
718 with aeration. The 10 ml overnight culture was used to inoculate 800 ml 2x TY broth in a
719 2 L flask and grown at 37°C with aeration until OD_{600} 0.6-0.9. At the desired OD, cells
720 were collected and stored on ice for 30 min before centrifugation to pellet the cells.
721 Electrocompetent cells were prepared by repeatedly centrifuging cells and resuspending
722 in decreasing amounts of ice-cold 10% (v/v) glycerol. The final resuspension was in 1 ml

723 of 10% glycerol resulting in a dense ~2 ml cell culture. Aliquots of 200 μl cells were
724 distributed between 1.5 ml microcentrifuge tubes. 0.2 μl EZ-Tn5™ transposome (Epibio)
725 was mixed with each aliquot of cells and incubated on ice for 30 min. Samples were
726 transferred to pre-chilled 2 mm gap electroporation cuvettes (Cell Projects Ltd.). Cells
727 were pulsed at 2200 V and 2 ml of pre-warmed SOC medium was immediately added to
728 the sample for recovery. Samples were transferred to a 15 ml falcon tube to allow for
729 maximum aeration and were incubated at 37°C for 2 h. 5 ml of LB broth was added to
730 each 15 ml falcon tube. ~4-5 drops of cells (equivalent to ~200 μl) were spread per LB
731 agar plate supplemented with 50 μg/ml kanamycin. Sufficient cells were inoculated per
732 plate to form non-touching single colonies. Plates were incubated overnight at 37°C for
733 18 h. Following incubation, 500 μl 30% glycerol-LB broth was added to each plate and
734 using a 'hockey-stick' spreader, colonies were scraped off the surface of the agar plate
735 and pooled. Cells were mixed thoroughly before storing at -80°C.
736
737 *Screening of the* yhcB *transposon-mutant library to identify suppressor mutations*
738 Cell cultures were resuspended in 1 ml of OD_{600} = 1.00, 200 µl of culture was plated
739 across 5x LB agar plates with or without additional chemical stresses. Assuming a library
740 of ~500,000 mutants (as judged from the unique insertions), this number of cells equates
741 to approximately 2,000 independent copies of each individual transposon mutant. We
742 chose a plate-based screening method as this minimises competition between mutants,
743 allowing for identification of slow growing mutants in addition to strong suppressor
744 mutations. Cultures were diluted to such quantities that single colonies of transposon
745 mutants could be isolated on the plates supplemented with stresses to prevent the growth
746 of satellite colonies and therefore false positive results. Plates were incubated at 37˚C for
747 24 h. Colonies were scraped and pooled for sequencing.
748

749 *TIS sequencing*

750 Genomic DNA (gDNA) was extracted and quantified using Qubit™ dsDNA HS Assay kit
751 (Invitrogen). 1 µg of gDNA was fragmented by mechanical shearing using a bioruptor
752 (Diagenode) using the shearing profile 30 s ON, 90 s OFF at low intensity, resulting in
753 DNA fragments with an average length of ~300 bp. Fragmented DNA was end-repaired
754 using the NEBNext Ultra I kit (New England Biolabs). The fragments were then ligated
755 with an adapter, and the sample was purified in a size-selection step using AMPure XP
756 SPRI beads (Beckman Coulter) before transposon-junctions were enriched by PCR with
757 primers specific for the transposon and the adapter. The Transposon-gDNA junctions
758 were prepared for sequencing by PCR addition of Illumina adapters using the NEBNext
759 Multiplex Oligos for Illumina (New England Biolabs). However, the forward Universal
760 primer was replaced with custom primers that include the Universal primer sequence
761 followed by a 6-9 nucleotide barcode (to introduce complexity and stagger the start of the
762 transposon sequence) and a 22 nt sequence with homology to the transposon at the 3′
763 end of the primer. Samples were purified after each PCR step using SPRI beads at a
764 ratio of 0.9:1 beads to sample. Finally, the sample was quantified using the KAPA Library
765 Quant Kit (Illumina) Universal qPCR Mix (Kapa Biosystems). Samples were pooled,
766 denatured and diluted to 18 pM and sequenced using an Illumina MiSeq, with 5% (v/v)
767 20 pM PhiX (Illumina), using 150 cycle v3 cartridges. Data are available at the European
768 Nucleotide Archive (accession: PRJEB43420).

769

770 *TIS analysis*

771 Data were first demultiplexed using the Fastx barcode splitter, to remove the 5′ end
772 barcode (inline index) unique to each sample (Pearson et al. 1997). The BioTraDIS
773 analysis package (version 1.4.5) was used for the remaining data processing (Barquist
774 et al. 2016). We allow for up to 4 bp mismatches in the transposon pattern matching step.

775 When successfully identified transposon sequences have been identified, the transposon
776 is trimmed and the remaining read is mapped to the BW25113 reference genome using
777 bwa (accession CP009273.1) to generate insertion plot files. These data can be viewed
778 online at our browser: http://tradis-vault.qfab.org/. The plot files are input to the
779 tradis_gene_insert_sites script to calculate the number of insertions per gene (insertion
780 index score). Unless otherwise stated, 5% trim was applied to both the 5′ and 3′ end of
781 each gene. The tradis_essentiality.R script was used to calculate the probability of
782 belonging to each mode representing essential and non-essential gene populations
783 respectively. The tradis_comparison.R script was used to compare read depth between
784 control and condition samples, per gene, using a threshold of >2-fold change and a Q-
785 value < 0.01. Plot files generated by the BioTraDIS analysis were also used as inputs for
786 AlbaTraDIS. AlbaTraDIS (version 1.0.1) was used for comparative analysis between
787 suppressor TIS datasets (Page et al. 2020). The reference genome annotation was used
788 to define gene boundaries. We set the minimum log counts per million threshold at 10,
789 for all other settings the default conditions were used.
790
791 *Whole genome sequencing*
792 Whole genome sequencing was done by MicrobesNG, University of Birmingham, UK,
793 using Illumina platforms generating short read data. Sequencing data were aligned to the
794 *E. coli* BW25113 reference genome available from the NCBI database (CP009273.1)
795 using bwa mem and then converted to bam files and sorted and indexed using SAMtools
796 (Li et al. 2009; Langmead and Salzberg 2012). Data are available at the European
797 Nucleotide Archive (accession: PRJEB43420). The programs Snippy and VarScan were
798 used to identify SNPs and indels, BreSeq was used to identify large chromosomal
799 rearrangement events (Koboldt *et al*. 2012; Deatherage & Barrick 2014; Seeman 2015).
800

801 *Phylogenetic analysis*

802 All searches were performed against the UniProt database of Reference Proteomes
803 (v2020_06, 02-Dec-2020), which includes only complete proteomes of reference
804 organisms, to confidently determine presence as well as absence. Search was performed
805 with PF06295.13 using hmmsearch (3.1b2; (Eddy 2011)) with the --max setting. Search
806 results were analysed manually as well as by all-against-all blast and subsequent
807 clustering in cytoscape, to determine the search cutoff used. Taxonomy information was
808 equally obtained from the UniProt Reference Proteomes ftp site (v2020_06, 02-Dec-
809 2020). Phylogenetic inference was performed on the sequences aligned via mafft (Katoh
810 and Standley 2013), and tree calculation using IQ-TREE (Nguyen et al. 2015) with the
811 built-in model test (Kalyaanamoorthy et al. 2017), which resulted in LG+G4. Tree
812 visualisation was performed using iTol (https://doi.org/10.1093/nar/gkz239).

813

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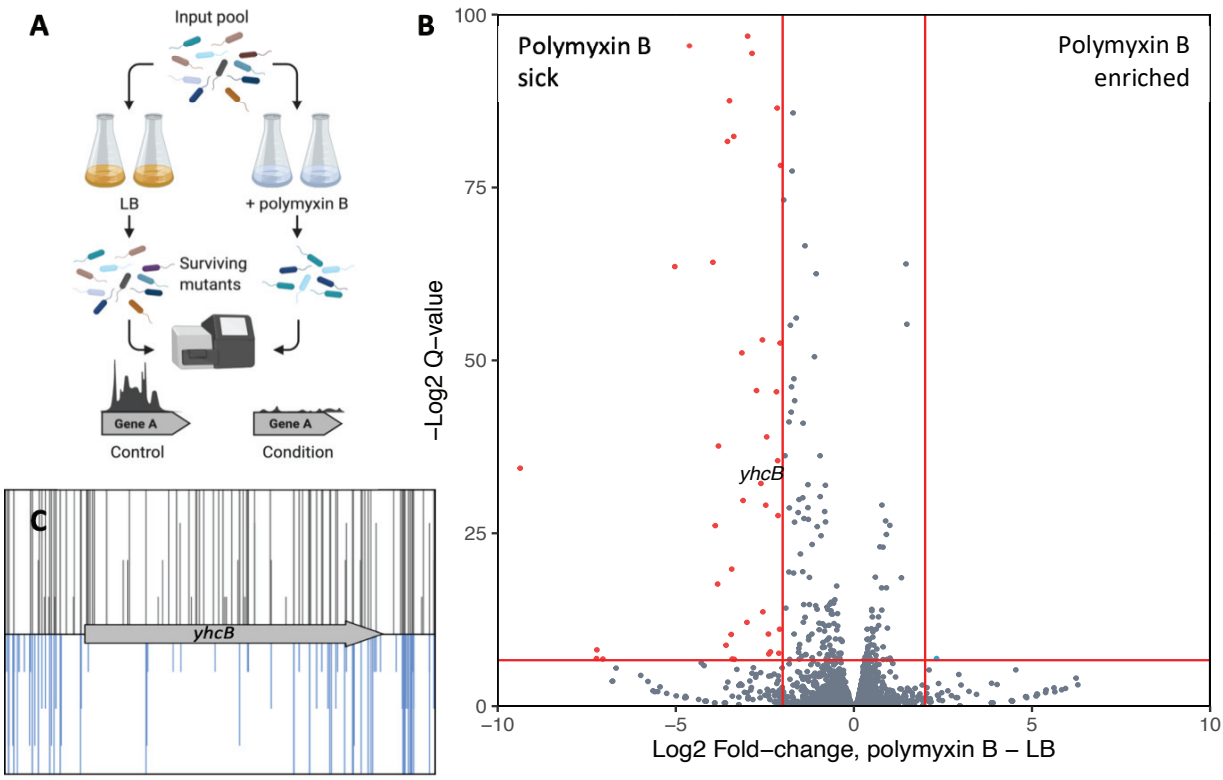


Figure 1. Screening the BW25113 transposon library in sub-inhibitory concentrations of polymyxin B

(A) Schematic showing the TIS experiment for identification of polymyxin B-sensitive mutants. (B) Volcano plot of the fold-change in mapped reads between conditions, calculated using BioTraDIS. Datapoints in red correspond with genes with >2-fold decrease in reads in the polymyxin B dataset relative to the LB outgrowth dataset, and with a Q-value > 0.01 (red horizontal line). While one datapoint in blue had a >2-fold increase in reads in polymyxin B dataset relative to the control, and a Q-value > 0.01. A label for the *yhcB* datapoint has been added above the point for clarity. (C) Image of the *yhcB* insertion data following outgrowth in LB only (grey, above) or in LB supplemented with polymyxin B (blue, below). The transposon insertion position along the gene is marked by a vertical line, with the vertical line size corresponding with read depth, with visibly fewer transposon-insertion sites identified within *yhcB* following outgrowth in LB supplemented with polymyxin B.

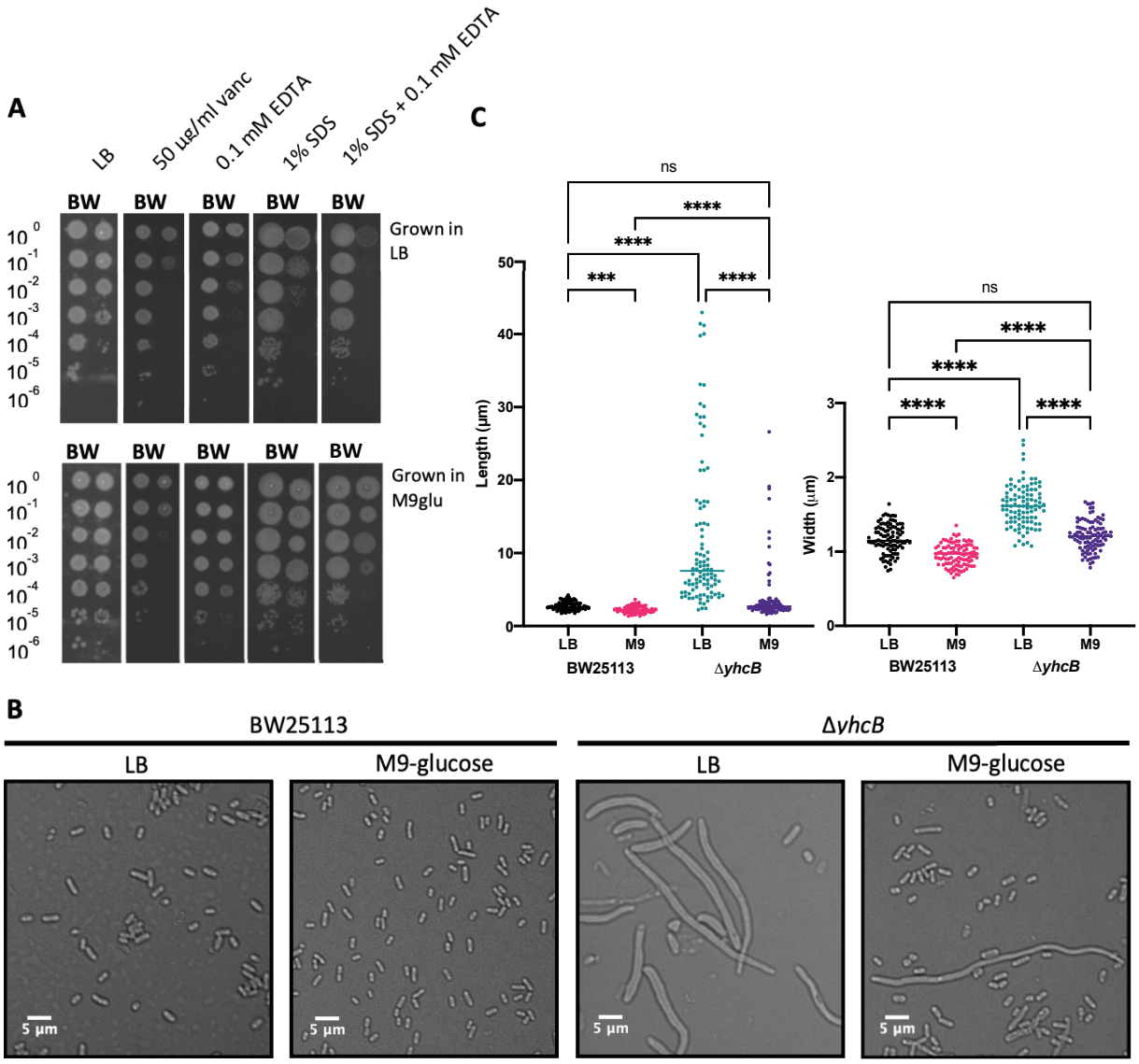
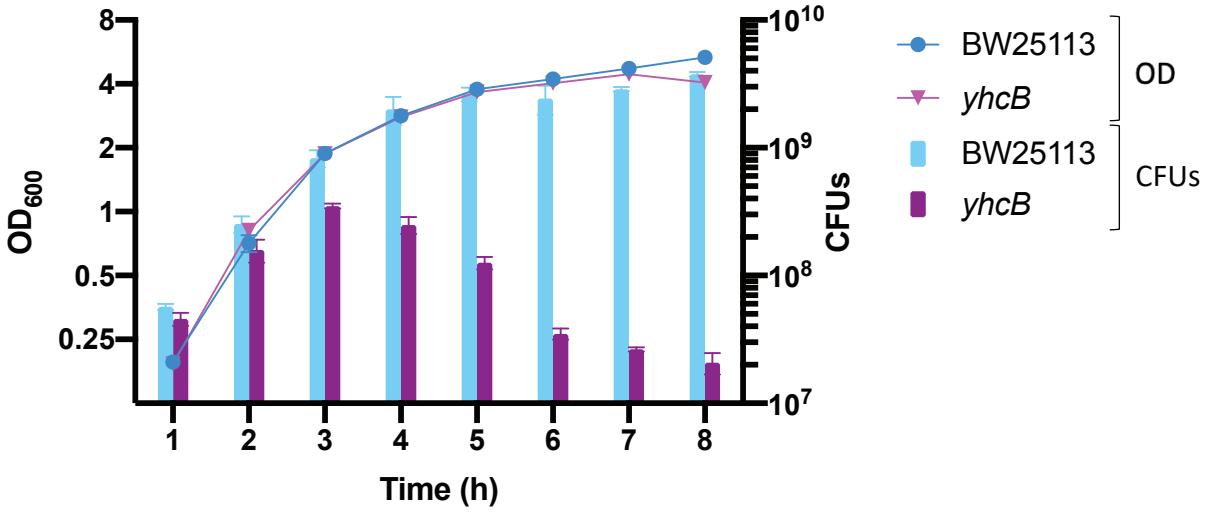


Figure 2. Validation of a *yhcB* mutant cell envelope defect and the effect of growth medium on cell size

(A) Overnight cultures of the WT strain BW25113 (BW) and Δ*yhcB* (Y) strain grown in LB or M9-glucose at 37˚C, normalised to an OD_{600} of 1.00 and 10-fold serially diluted before inoculating LB agar plates supplemented with various compounds. (B) The WT and Δ*yhcB* mutant were grown overnight in LB or M9-glucose media at 37˚C. Cells were visualised using a Nikon 90i microscope, a 5 µm bar is shown for scale. (C) Cell measurements for BW25113 and Δ*yhcB* mutant grown in LB and M9-glucose. Images were taken after overnight growth and are representative of n=4 experiments, data from 1 experiment is presented. Width and Length measurements were taken of 100 cells in each condition. A Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare between samples: $p < 0.0001$ (****); $p < 0.0002$ (***).



1321

1322 **Figure 3. The effect of *yhcB*–deletion during growth in rich media**

1323 (A) The optical density (OD) and number of colony forming units (CFUs) were recorded
1324 hourly over 8 h during growth in LB at 37˚C. Strains were grown in triplicate, the mean is
1325 plotted and the s.d. represented by error bars. BW25113 is shown in blue (•), *yhcB* is
1326 shown in pink (▾).

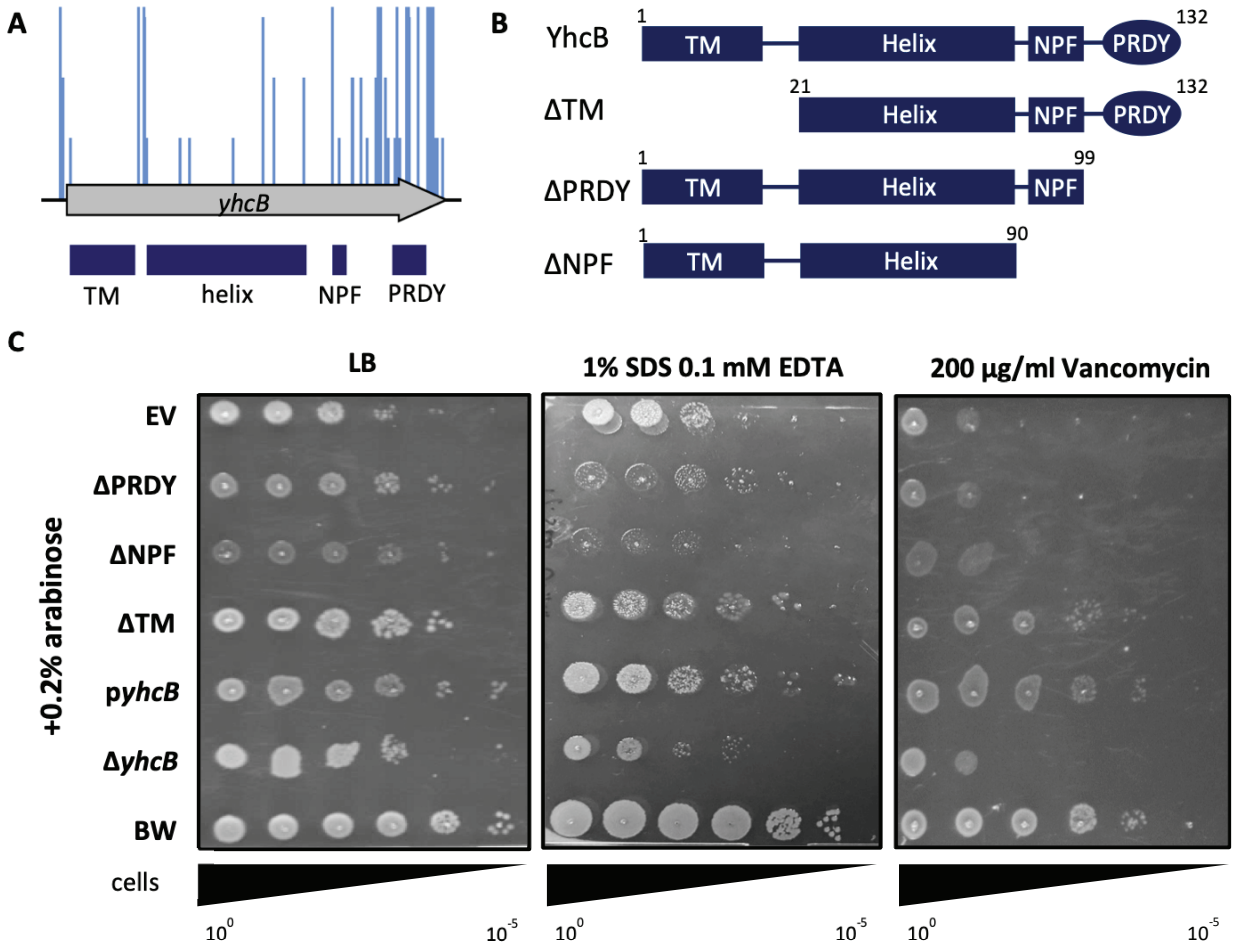


Figure 4. The structural and functional contribution of the domains of YhcB
(A) Polymyxin B TIS screen data (blue insertions) with the domains of YhcB mapped underneath (dark blue boxes). (B) Schematics of the plasmid-based YhcB complementation constructs. (C) Overnight cultures grown in LB supplemented with carbenicillin (for plasmid selection), normalised to an OD_{600} of 1.00 and 10-fold serially diluted before inoculating LB agar plates supplemented with vancomycin or SDS and EDTA, with 0.2% arabinose.

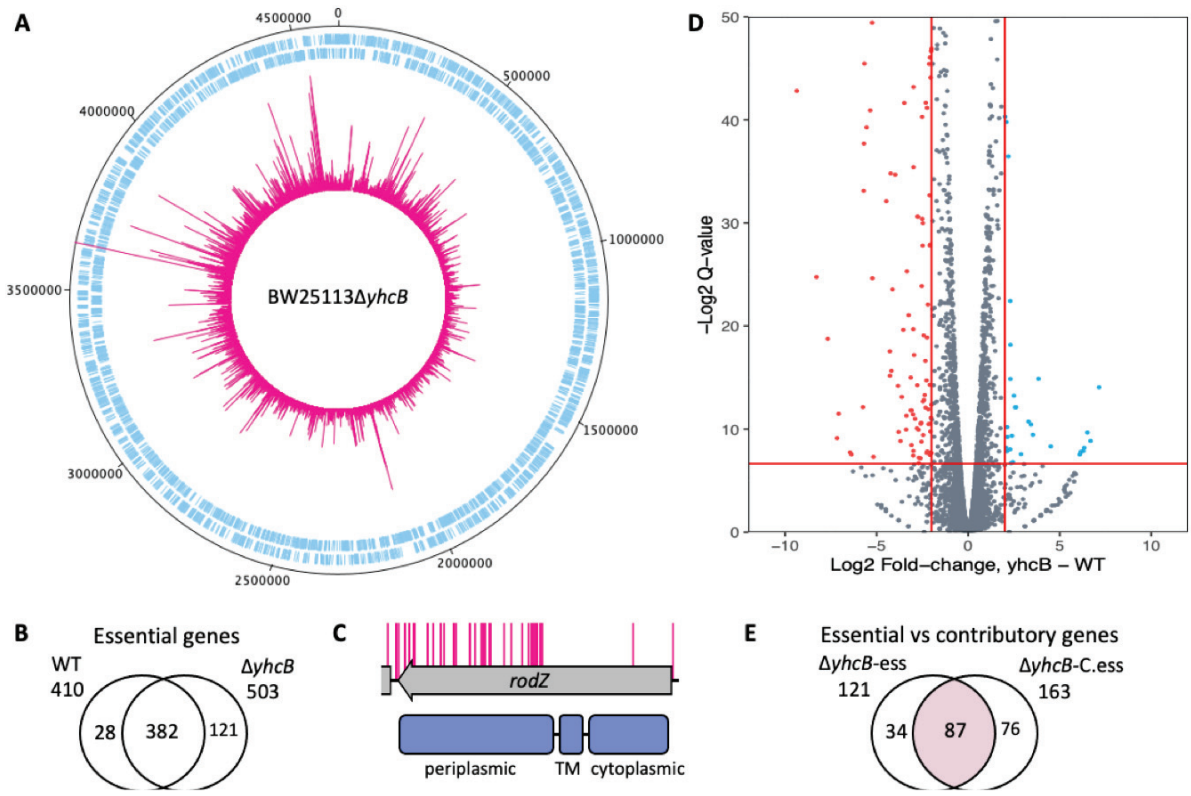


Figure 5. Construction of a transposon library in a *yhcB* mutant and identification of genes with a synthetic lethal relationship with *yhcB*

(A) The Δ*yhcB* transposon library. A genome map of BW25113 starting at the annotation origin with the sense and antisense coding sequences of BW25113 shown in blue, respectively, and the position and frequency of sequenced transposon insertion events shown in pink. (B) Comparison of genes identified by bimodal analysis as significantly underrepresented by transposon mutants in each library. (C) The insertion profile of *rodZ* in the Δ*yhcB* library, with RodZ domains shown below in blue. (D) The $\log_2$ fold-change of read depth per gene between the parent BW25113 and the Δ*yhcB* transposon library. Genes enriched for transposon insertions (>2-fold) in the *yhcB* library are shown in blue, indicative of more fit mutants. Genes with a >2-fold decrease in insertions are shown in red, indicative of sick mutants. A Q-value threshold of 0.01 was used (red horizontal line) (E) Comparison of genes predicted to be essential (sparsely disrupted genes) with genes identified as having a >2-fold decrease in reads compared to the control library (genes that contribute to fitness). This is a comparison of the bi-modal and enrichment analyses outputs. The overlap of these analyses are the 87 identified synthetic lethal genes (pink).

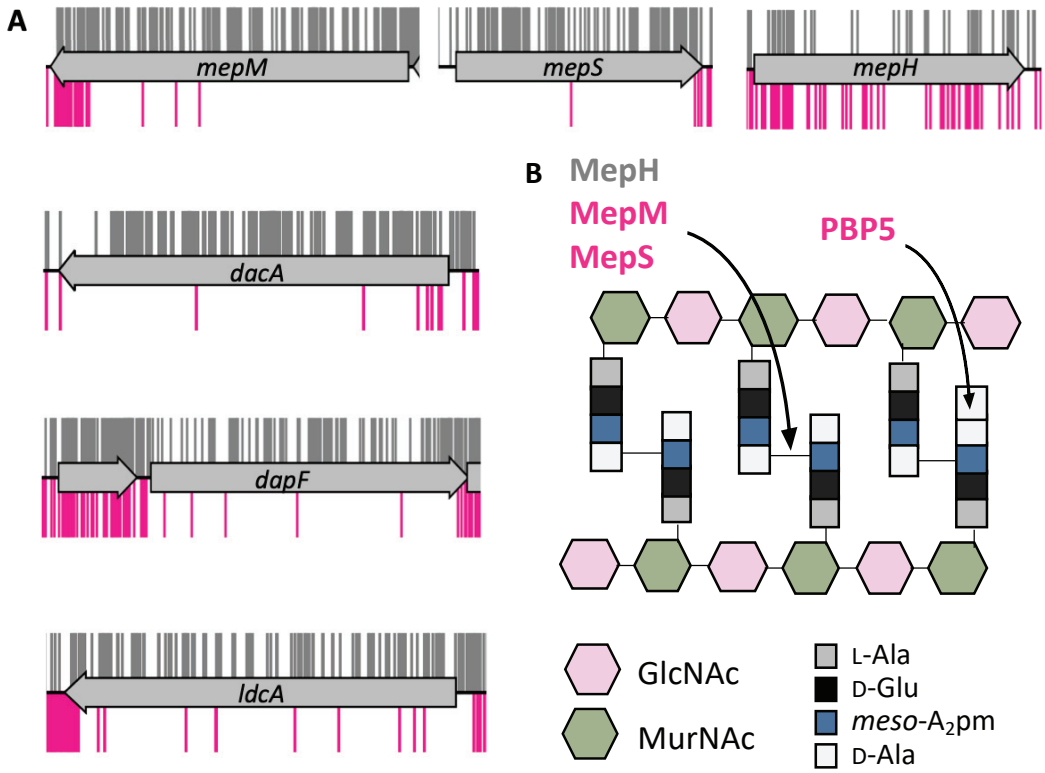


Figure 6. *yhcB* is synthetically lethal with components of PG synthesis and recycling pathways

(A) TIS data of the BW25113 library (grey) and BW25113Δ*yhcB* library (pink) were plotted above and below the gene track, respectively. Insertion sites are represented by vertical bars and are capped at a frequency of 1. (B) Schematic representation of the target sites of the endopeptidases and PBP5 (encoded by *dacA*). Abbreviations: *meso*-diaminopimelate (*meso*-A_2pm); N-acetylglucosamine (GlcNAc); N-acetylmuramic acid (MurNAc); L-Ala, L-alanine; D-Glu, D-glutamic acid; D-Ala, D-alanine.

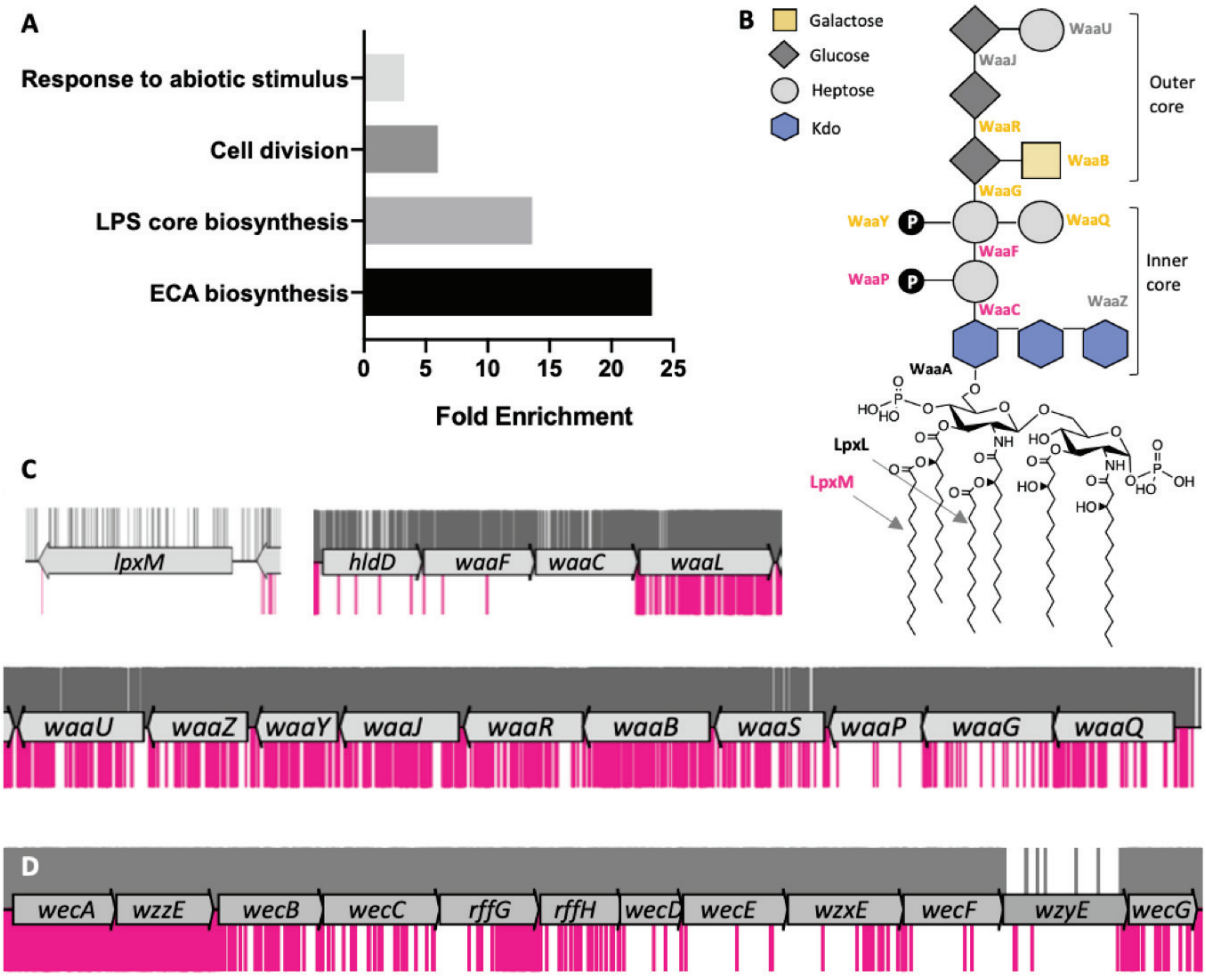


Figure 7. Functional enrichment among synthetic lethal genes
(A) Functional enrichment of cellular processes among the genes that are synthetically lethal with *yhcB*. Fisher's exact test with Bonferroni correction for multiple testing. Results displayed for $P < 0.05$. (B) A schematic of the structure of LPS. LPS biosynthesis enzymes are indicated next to the linkage they form (central column) or component they ligate (side branches). Synthetic lethal enzymes are labelled in dark pink, enzymes that contribute to fitness are labelled in orange, core essential enzymes in black and non-essential enzymes in grey. (C) TIS data for BW25113 (grey) and BW25113Δ*yhcB* (pink) libraries were plotted above and below the gene track, respectively. Insertion sites are represented by vertical bars and are capped at a frequency of 1. Insertions within genes required for LPS core biosynthesis, and *lpxM*, are significantly underrepresented. (D) The insertion profiles in genes of the enterobacterial common antigen biosynthetic pathway. Abbreviations: Lipopolysaccharide (LPS); Enterobacterial Common Antigen (ECA).

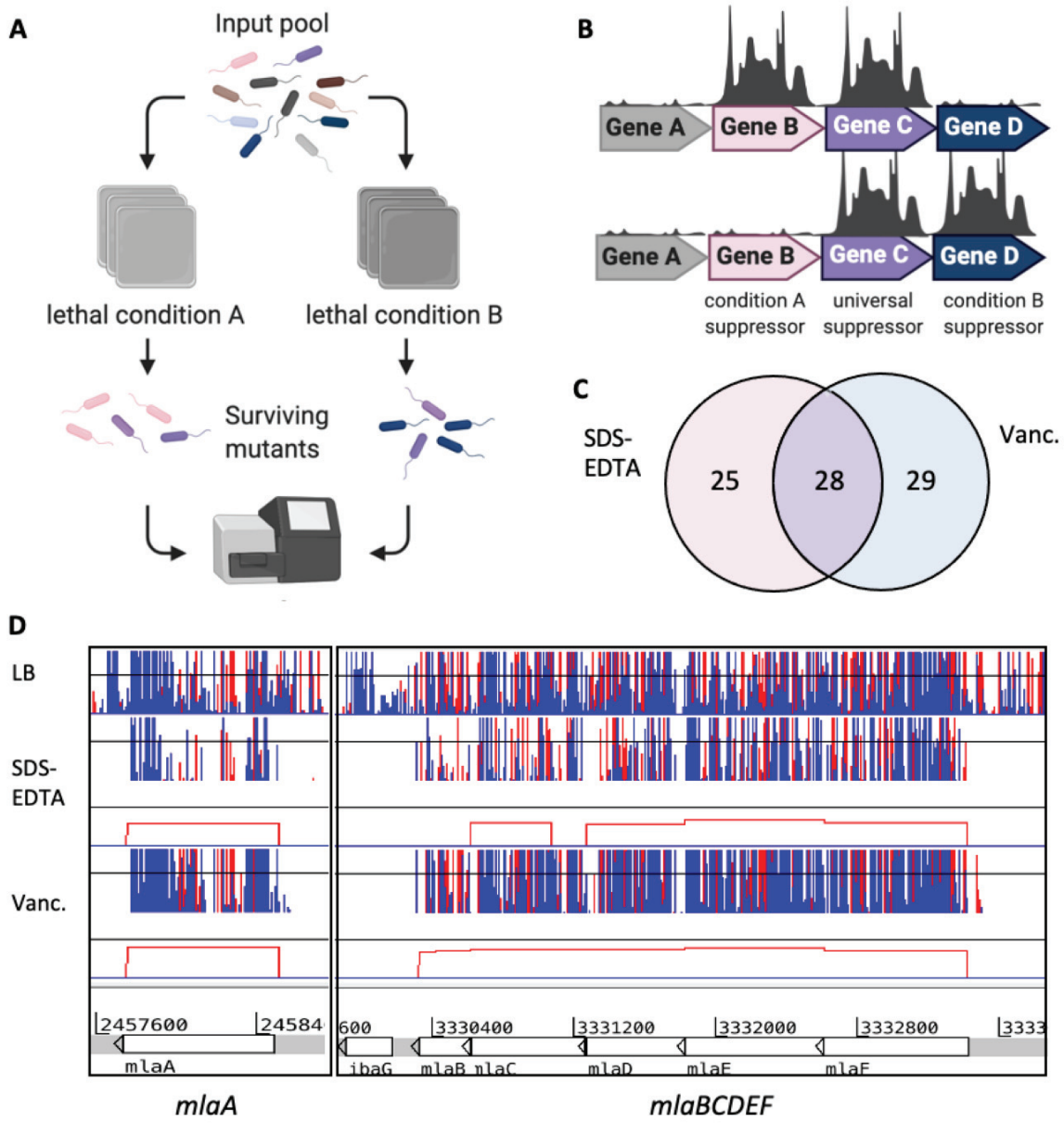


Figure 8. Identification of suppressor mutations that restore a *yhcB*-deletion defect (A) Experimental overview. (B) Cartoon of output. (C) Overlap between gene-deletion suppressors identified in the lower concentration vancomycin and SDS + EDTA screens. (D) Transposon insertion data for suppressor screens. Red and blue lines indicate the transposon insertion position corresponding with the transposon orientation at the point of insertion. The height of the bar corresponds with mapped sequencing read frequency. The red boxes underneath each suppressor dataset represent significant differential abundance of insertions of the condition compared to the control, identified by AlbaTraDIS. Panel A and B schematics created with BioRender.com, Panel D insertion plots visualised in Artemis (Rutherford et al. 2000).

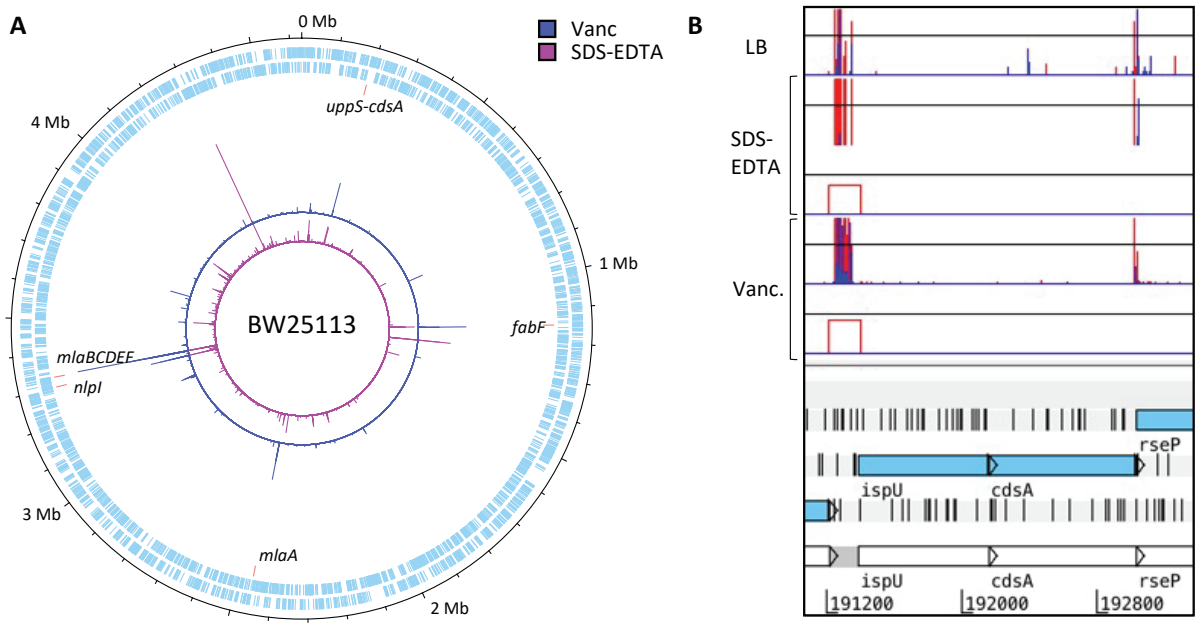


Figure 9. The *uppS-cdsA* operon

(A) Genome map showing the location and frequency of transposon insertion events that restore the viability of a *yhcB* mutant under toxic growth conditions: LB supplemented with vancomycin, or SDS and EDTA. (B) TIS data of the *uppS (ispU)* and *cdsA* operon. (Note that *uppS* is annotated as *ispU* in the BW25113 reference genome). Red and blue data correspond with the transposon orientation at the point of insertion. The height of the bar corresponds with mapped sequencing read frequency, capped at 20 in these images. The red boxes underneath each suppressor dataset represent significant differential abundance of insertions of the condition compared to the control, identified by AlbaTraDIS.

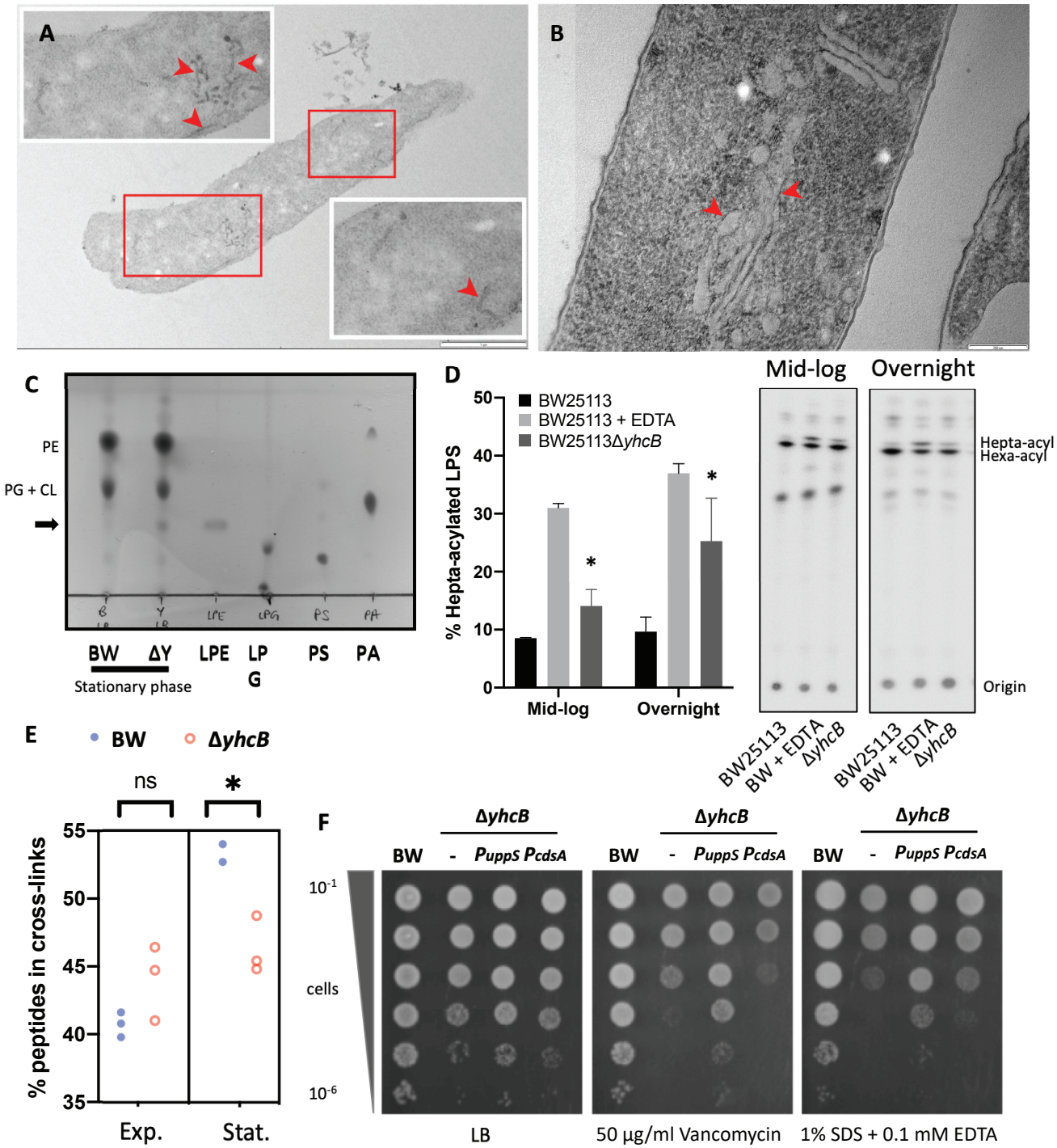


Figure 10. The *yhcB* mutant contains extra membrane and has an altered cell envelope

(A) Transmission electron micrograph (TEM) of a Δ*yhcB* mutant cell with membrane ruffles indicated by red arrows. Scale bar = 1 µm. Additional whole cell images are in Fig. S10. (B) Representative TEM image of a Δ*yhcB* mutant cell processed without primary fixation by fast freezing and freeze substitution. Internal membrane structures indicated by red arrows. Scale bar = 200nm. (C) Phospholipid species extracted from BW25113 (BW) and the Δ*yhcB* mutant (ΔY) grown in LB broth until stationary phase and separated by thin layer chromatography (TLC) using a chloroform:methanol:water (65:25:4) solvent system. Standards of lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), phosphatidylserine (PS) and phosphatidic acid (PA) were loaded alongside for

1413 comparison. The additional spot in the ΔY sample is indicated with an arrow. (D)
1414 TLC/autoradiographic analysis of [^{32}P]-labeled lipid A extracted from mid-logarithmic
1415 growth and overnight growth cultures of BW25113 and Δ*yhcB* strains grown in LB. As a
1416 positive control for lipid A palmitoylation, BW25113 cells were treated with 25 mM EDTA
1417 for 10 min prior to extraction. The mean percentage of hepta-acylated lipid A and standard
1418 deviation were calculated from samples prepared in triplicate. Student's *t*-tests: *$P < 0.05$
1419 compared with BW25113. (E) Comparison of the percentage of peptides in peptidoglycan
1420 crosslinks between strains at two stages of growth. Unpaired t-test with Welch's
1421 correction: *$P < 0.05$. Exponential phase cells were collected at an OD of 0.4, Stationary
1422 phase cells were harvested after 16 h at an OD of ~4.0. (F) Day cultures of BW25113;
1423 Δ*yhcB;* Δ*yhcB PuppS* and Δ*yhcB PcdsA* strains grown in LB for 5 h, normalised to an
1424 OD_{600} of 1.00 and 10-fold serially diluted before inoculating LB agar plates supplemented
1425 with vancomycin or SDS and EDTA. Abbreviations: Phosphatidylethanolamine (PE);
1426 Phosphatidylglycerol (PG); Cardiolipin (CL); Exponential phase (Exp.); Stationary phase
1427 (Stat.).
1428

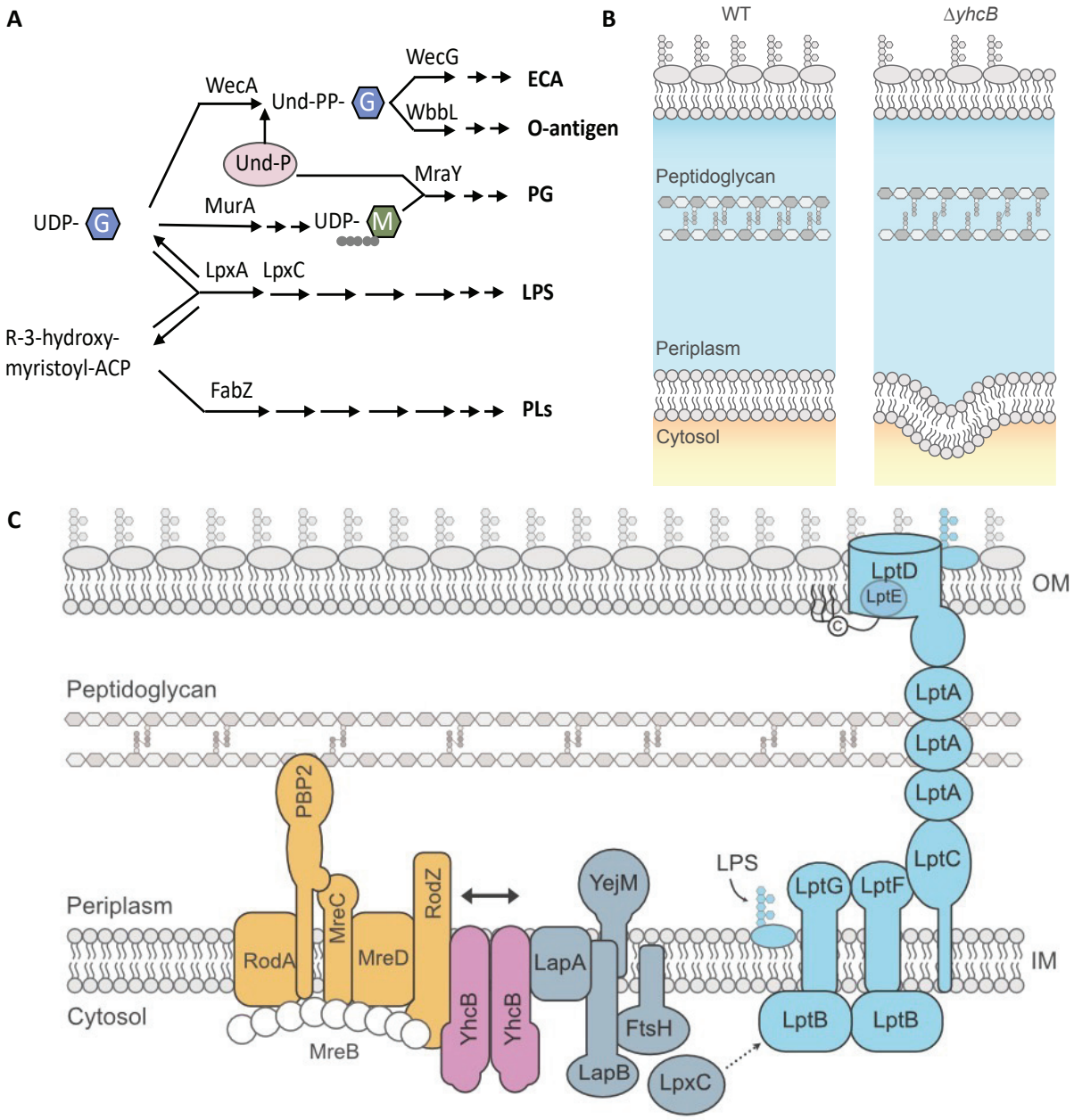
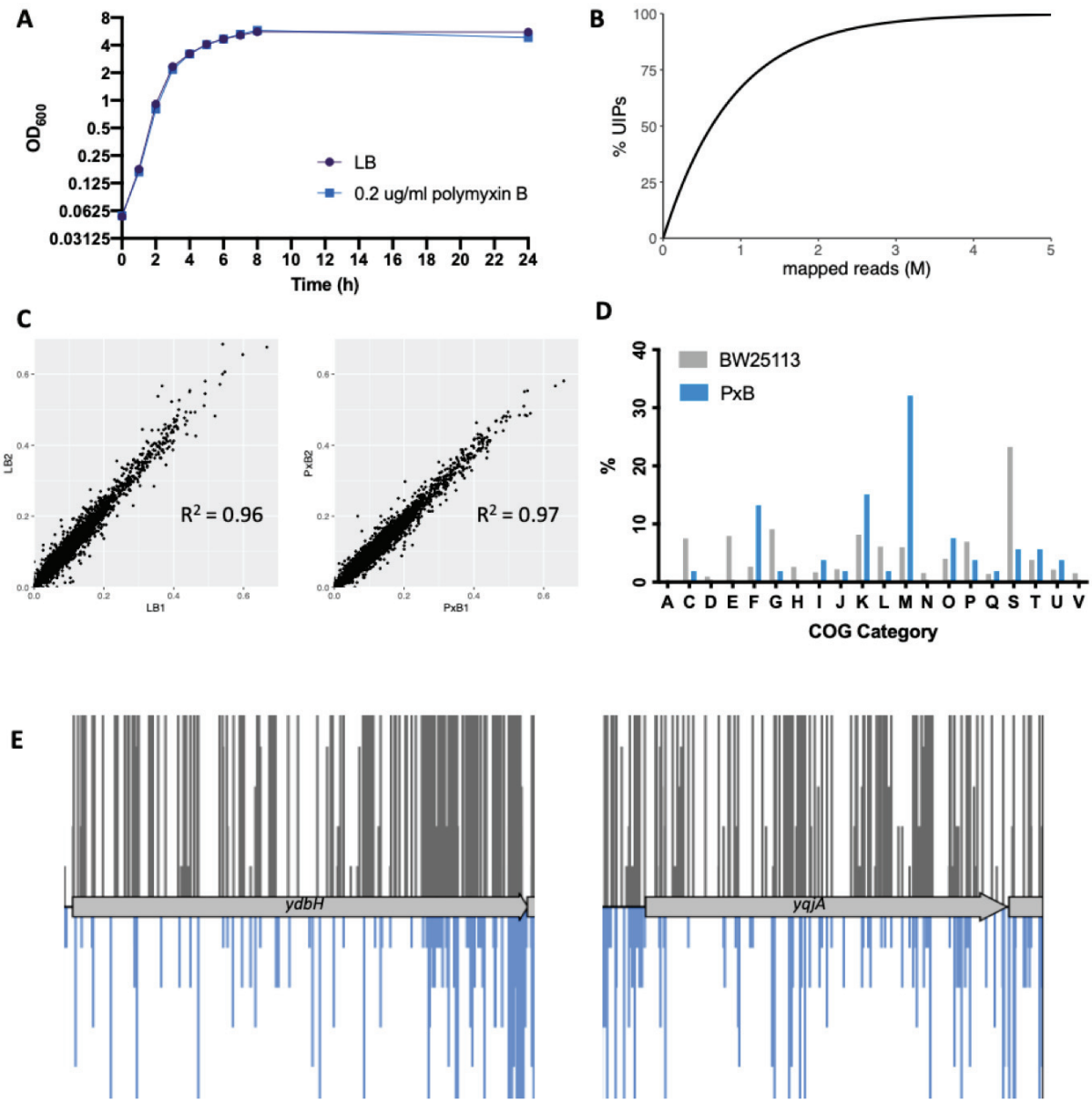


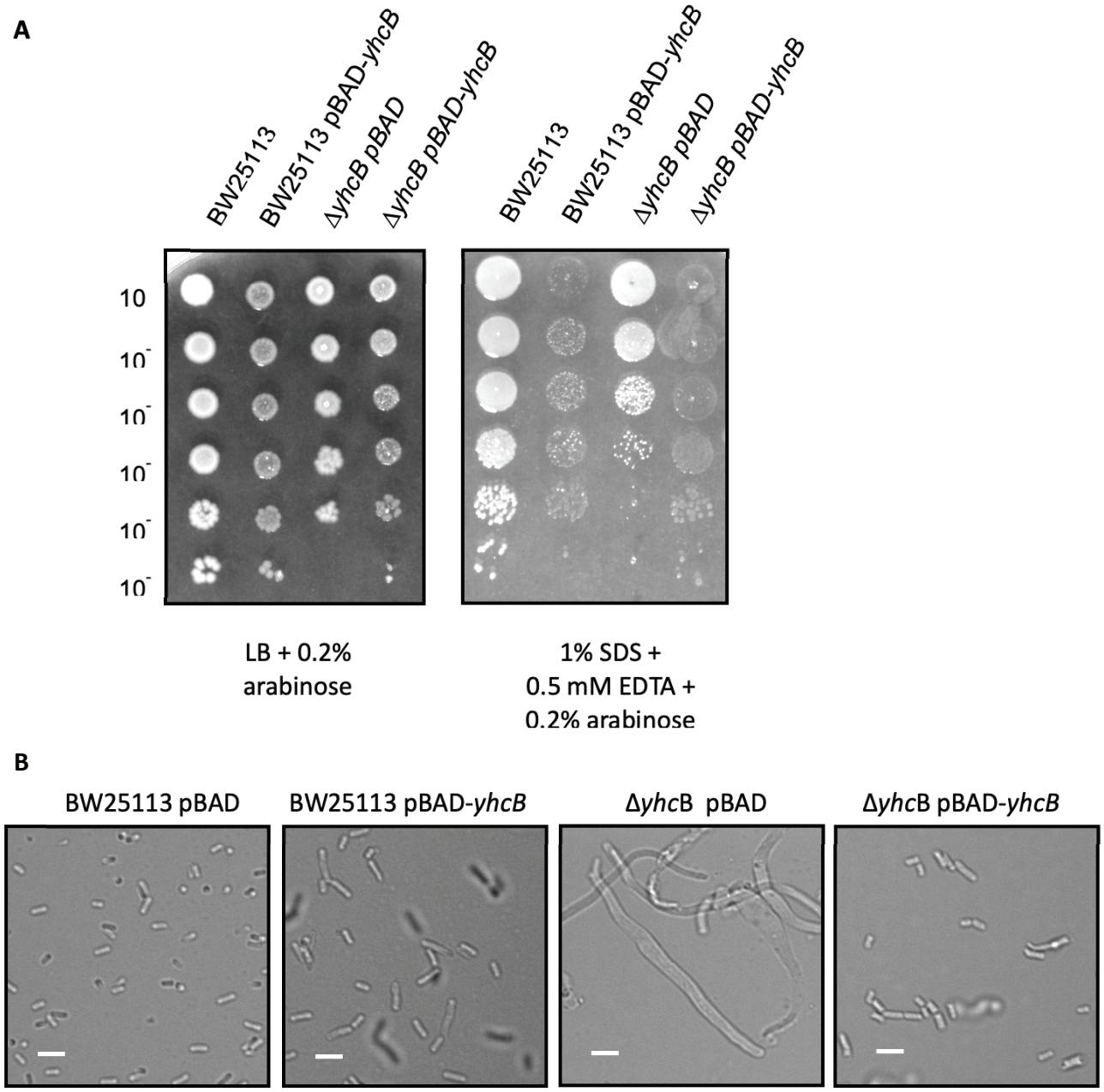
Figure 11. The pathways of cell envelope biosynthesis and relationship with YhcB (A) Schematic overview of the relationships between the biosynthetic pathways of individual components of the cell envelope. (B) A schematic of the imbalanced cell envelope of a *yhcB* mutant in comparison to wildtype (WT). The *yhcB* mutant suffers from defects in OM asymmetry, excessive phospholipid synthesis and altered crosslinking in the peptidoglycan layer. (C) The positional context of YhcB in conjunction with the reported protein interaction partners of YhcB *in situ*. YhcB interacts with both the elongasome (depicted here as mediated via RodZ, but YhcB is reported to interact with RodA, MreD and MreC in addition to RodZ) and LapA from the complex that regulates LpxC degradation. YhcB is spatially positioned to coordinate peptidoglycan expansion with LPS export. Abbreviations: Enterobacterial Common Antigen (ECA); Lipopolysaccharide (LPS); Peptidoglycan (PG); Phospholipid (PL); Undecaprenyl phosphate (Und-P); Undecaprenyl diphosphate (Und-PP); Uridine diphosphate (UDP); N-acetylglucosamine (G); N-acetylmuramic acid (M); Acyl Carrier Protein (ACP).



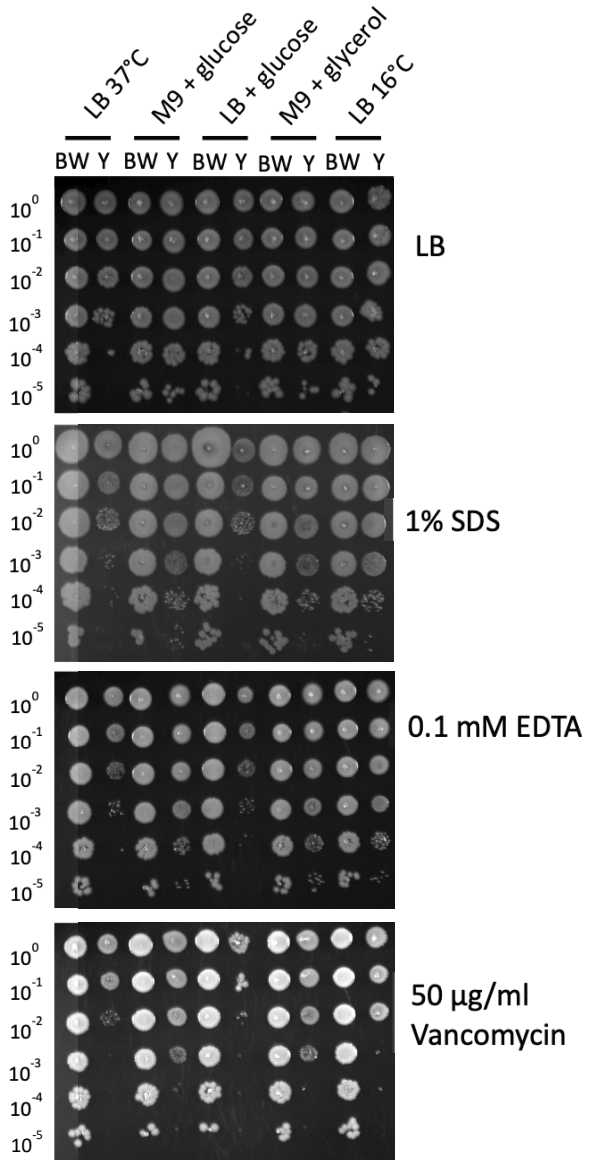
Supplementary Figure 1. Screening the BW25113 transposon library in sub-inhibitory concentrations of polymyxin B

(A) 0.2 µg/ml of polymyxin B in 50 ml LB does not inhibit growth of *E. coli* BW25113, under the conditions used to screen the library. One representative growth curve is shown, consistent with 5 repeats. (B) Calculation of the number of mapped reads needed to sample a given percentage of the transposon library. The equation $\mathrm{I} = s - s(\frac{s-1}{s})^n$ was used to estimate the number of mapped reads required to sample a given proportion of the dataset. s = 1,000,000 was taken as the total number of possible mutants (total transposon insertion sites). I = insertions identified, n = number of mapped reads. This data was used to calculate the approximate percentage of unique insertions identified for a given number of mapped reads, for a library of 1,000,000 unique mutants. (C) Comparison of insertion index scores (a measure of insertion density) per gene for each replicate of the library either exposed to LB only, or LB supplemented with polymyxin B. The scatter plots show the correlation coefficient for insertion density of each gene between replicates. (D) The relative abundance of Cluster of Orthologous Groups (COG) categories for all genes of BW25113 (grey) and for the 54 genes identified as required for growth in sub-inhibitory concentrations of polymyxin B (PxB; blue). (E) The transposon

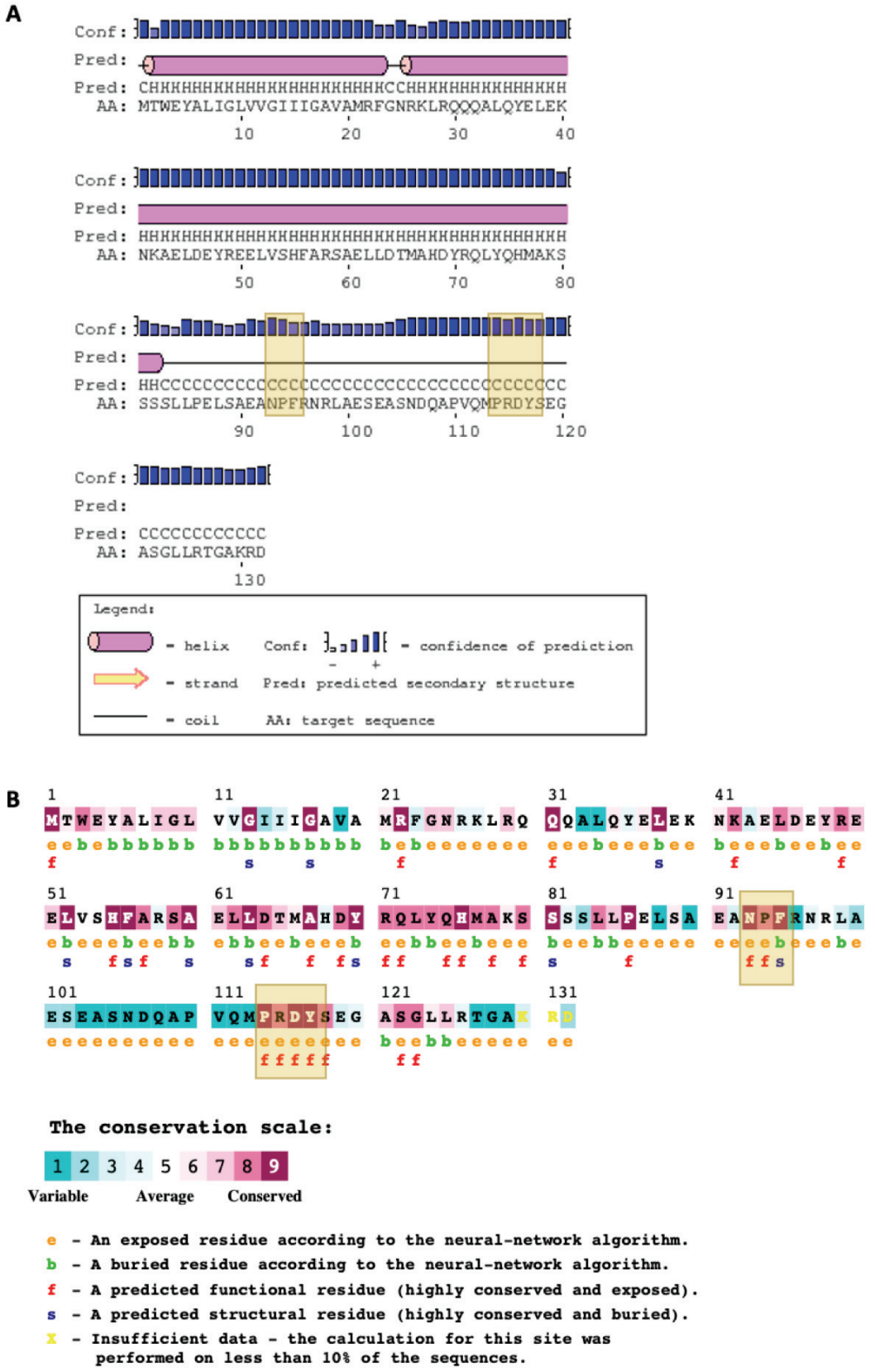
1463 insertion profiles of *yqjA* and *ydbH* following outgrowth in LB only (grey, above) or in LB
1464 supplemented with polymyxin B (blue, below). The transposon insertion position along
1465 each gene is marked by a vertical line. The vertical line size corresponds with read depth,
1466 with visibly fewer transposon-insertion sites identified within *ydbH* and *yqjA* following
1467 outgrowth in polymyxin B.
1468



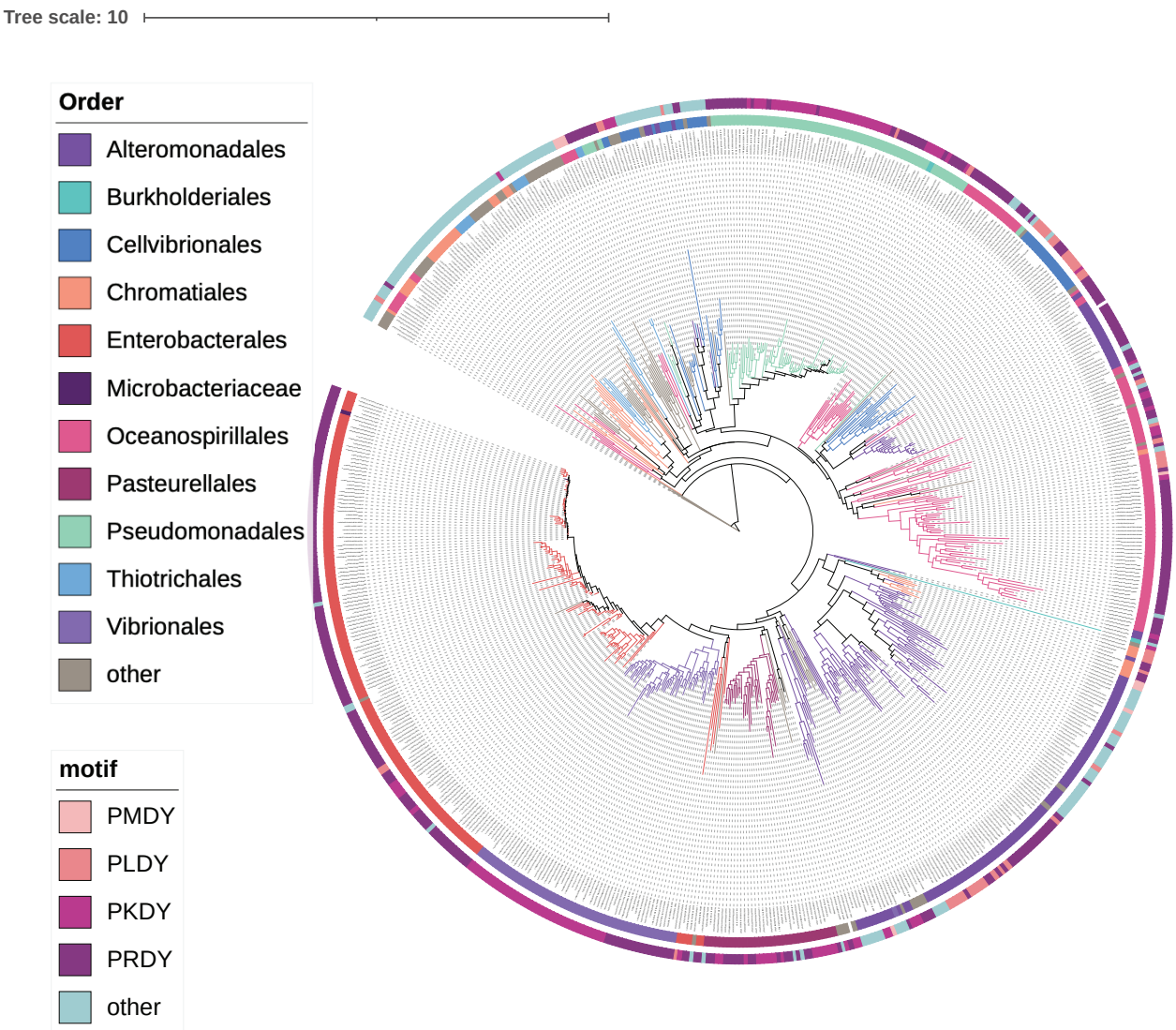
Supplementary Figure 2. Complementation of a Δ*yhcB* defect
(A) 10-fold serial-dilution of overnight cultures grown in LB, inoculated onto LB agar plates supplemented with 0.2% arabinose, with and without 1% SDS + 0.5 mM EDTA. Strains are carrying a pBad-Myc-His-A with or without the *yhcB* CDS under the control of an arabinose promoter. (B) DIC microscopy images of day cultures grown in LB supplemented with 0.4% arabinose and 100 µg/ml carbenicillin (to maintain plasmids), with a 5 µm scale bar for reference.



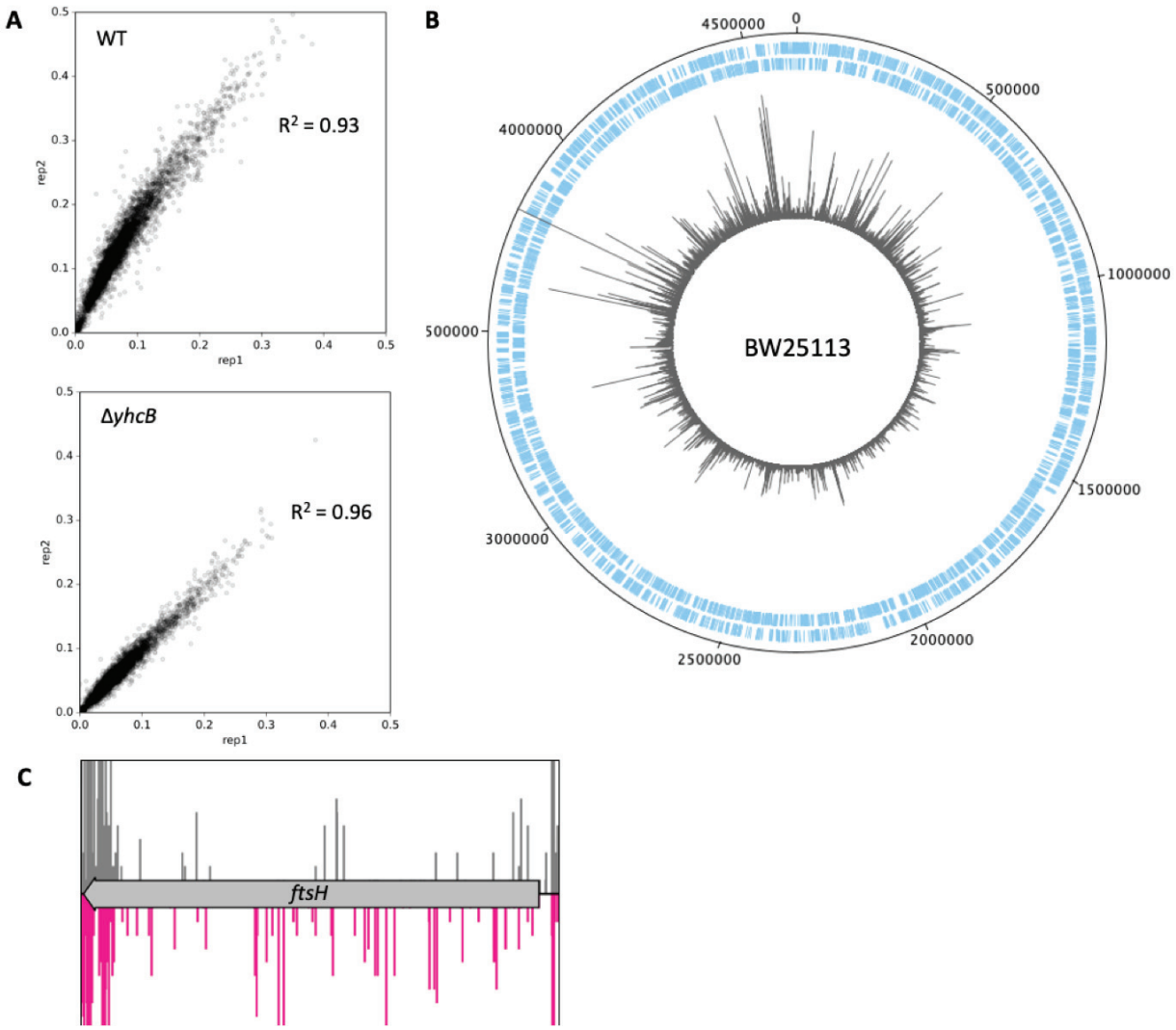
Supplementary Figure 3. The effect of different growth media on phenotype
10-fold serial-dilution of overnight cultures of the parent strain *E. coli* BW25113 (BW) and Δ*yhcB* strain (Y) grown to late stationary phase under different conditions (LB at 37˚C; M9 + 0.4% glucose at 37˚C; LB + 0.4% glucose at 37˚C; M9 + 0.4% glycerol at 37˚C; LB at 16˚C) normalized to an OD_{600} of 1.00 and inoculated onto LB agar plates supplemented with and without 1% SDS; 0.1 mM EDTA or 50 µg/ml vancomycin.



Supplementary Figure 4. Domains and conserved motifs of YhcB
(A) PSIPRED secondary structure prediction of YhcB (B) Conserved residues of YhcB predicted by ConSurf. Conserved 'NPF' and 'PRDY' motifs are highlighted with amber boxes in both panels.

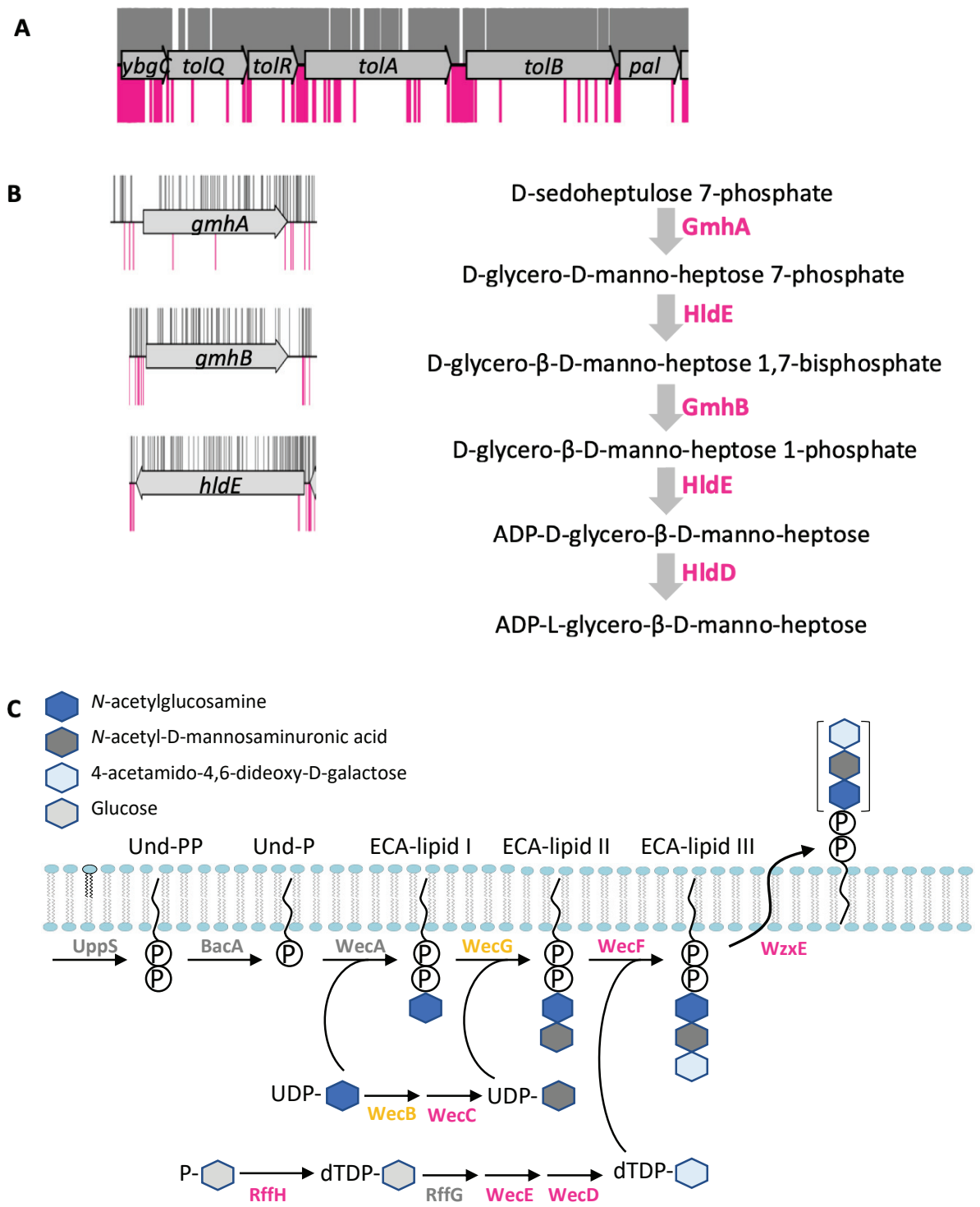


Supplementary Figure 5. Conservation of the PRDY domain
Phylogenetic analysis displaying conservation of YhcB in bacterial reference genomes. Branches of the tree, and second outermost track, are coloured according to taxonomic Order. The outermost track is coloured according to the amino acid residues within the 'PXDY' domain conserved among species. The label 'other' represents those that had a different sequence to the four listed.

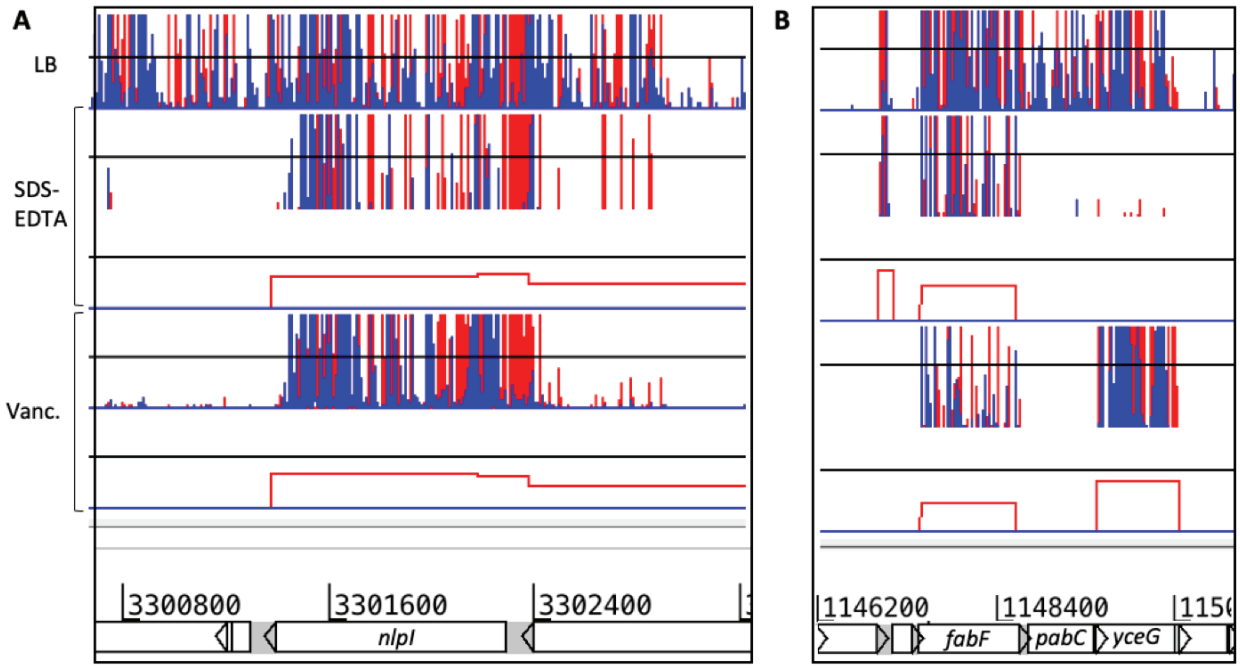


Supplementary Figure 6. Construction of a transposon library in BW25113 parent strain

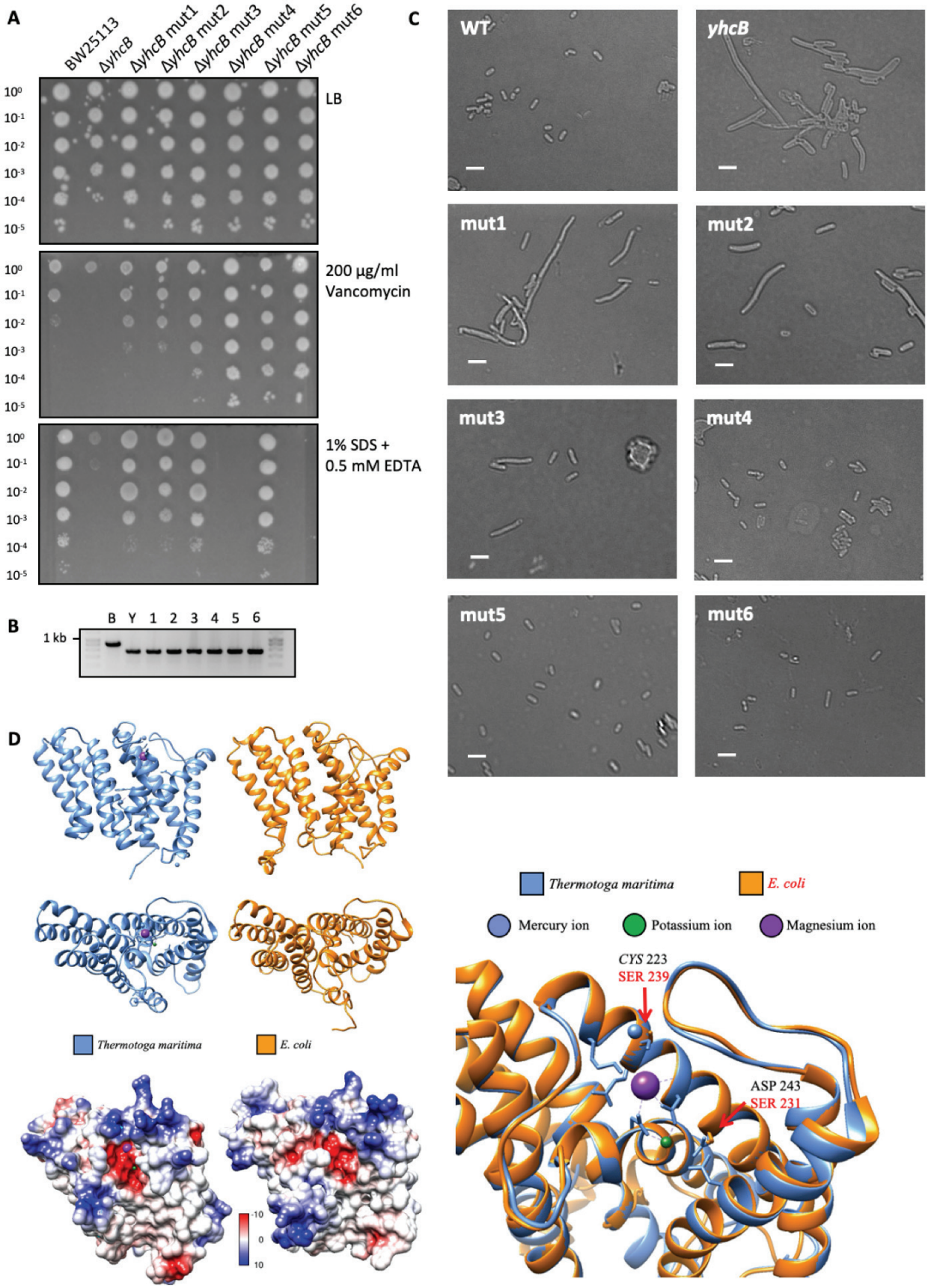
(A) Comparison of insertion index scores (IIS) between technical replicates of each library. (B) The BW25113 transposon library. A genome map of BW25113 starting at the annotation origin, with the sense and antisense coding sequences of BW25113 shown in blue, respectively, and the position and frequency of sequenced transposon insertion events shown in grey. (C) The transposon insertion profile of *ftsH* shown for both libraries. Peaks represent the abundance of detected transposon insertion events for each library: WT (grey, above) and *yhcB* (pink, below) with read frequency capped at 10.



Supplementary Figure 7. Biosynthetic pathways required in a Δ*yhcB* background Transposon insertion data of the yhcB library shown in pink, below, control library shown in grey, above, the gene track with all insertion data capped at a frequency of 1. (A) Transposon insertion profile of the *tol-pal* operon. (B) The heptose biosynthetic pathway, required for LPS core biosynthesis. (C) Schematic of the ECA biosynthesis pathway adapted from Jorgenson *et al*. (2016). Abbreviations: Enterobacterial Common Antigen (ECA); Undecaprenyl phosphate (Und-P); Phosphate (P); Adenosine diphosphate (ADP); deoxythymidine diphosphate (dTDP); Uridine diphosphate (UDP).



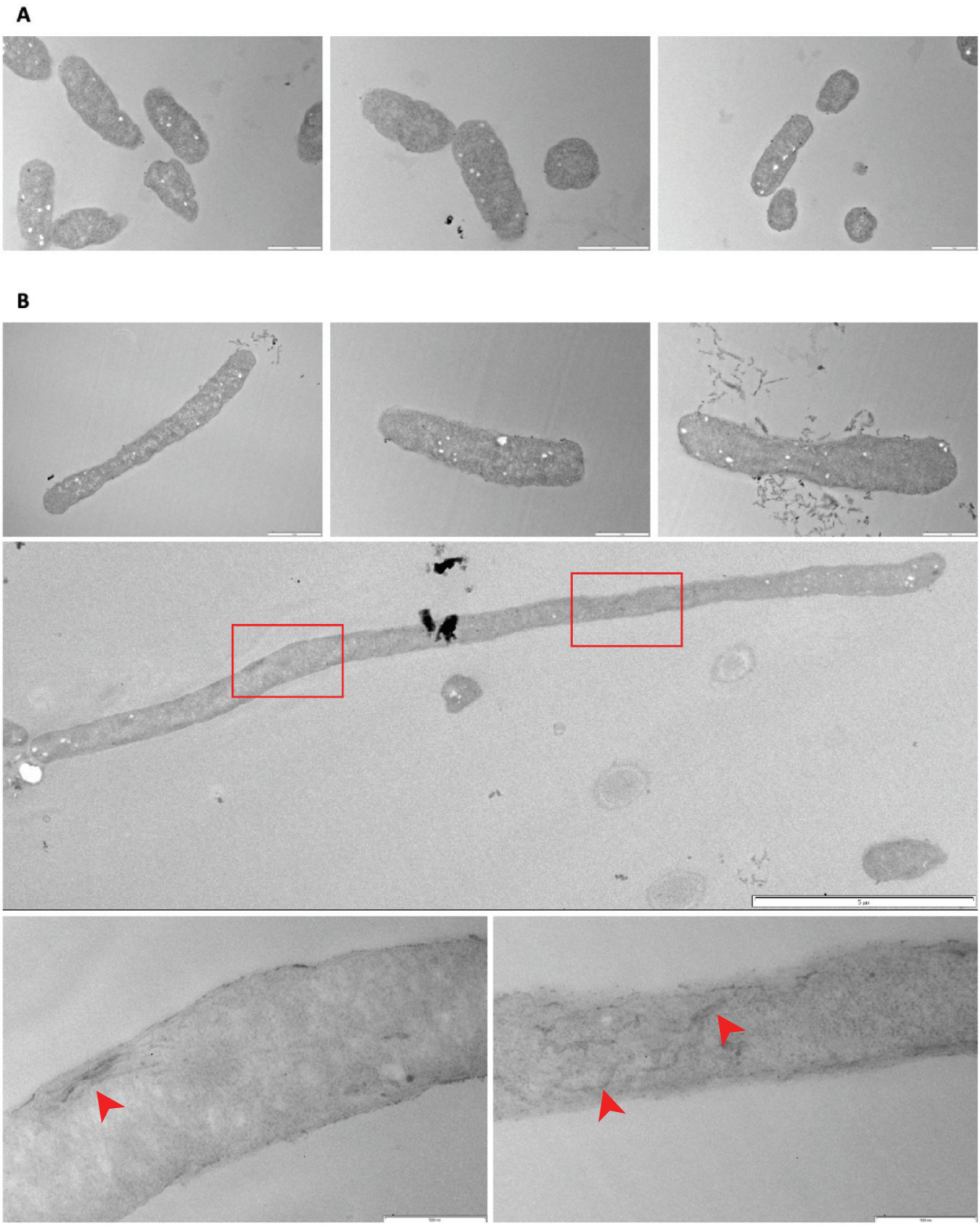
Supplementary Figure 8. Transposon insertion sites within *nlpI* and *fabF* that restore resistance of a *yhcB* mutant to vancomycin or SDS and EDTA.
Transposon insertion data for suppressor screens. Red and blue vertical lines indicate the transposon insertion position and correspond with the transposon orientation at the point of insertion. The height of the bar corresponds with mapped sequencing read frequency. The top track represents data for the library plated on LB. The tracks underneath represent the Δ*yhcB* transposon library plated on LB supplemented with SDS and EDTA, or vancomycin, at lethal doses to the BW25113Δ*yhcB* parent strain. The red boxes underneath each suppressor dataset represent significant differential abundance of insertions in the condition sample compared to the control, identified by AlbaTraDIS.



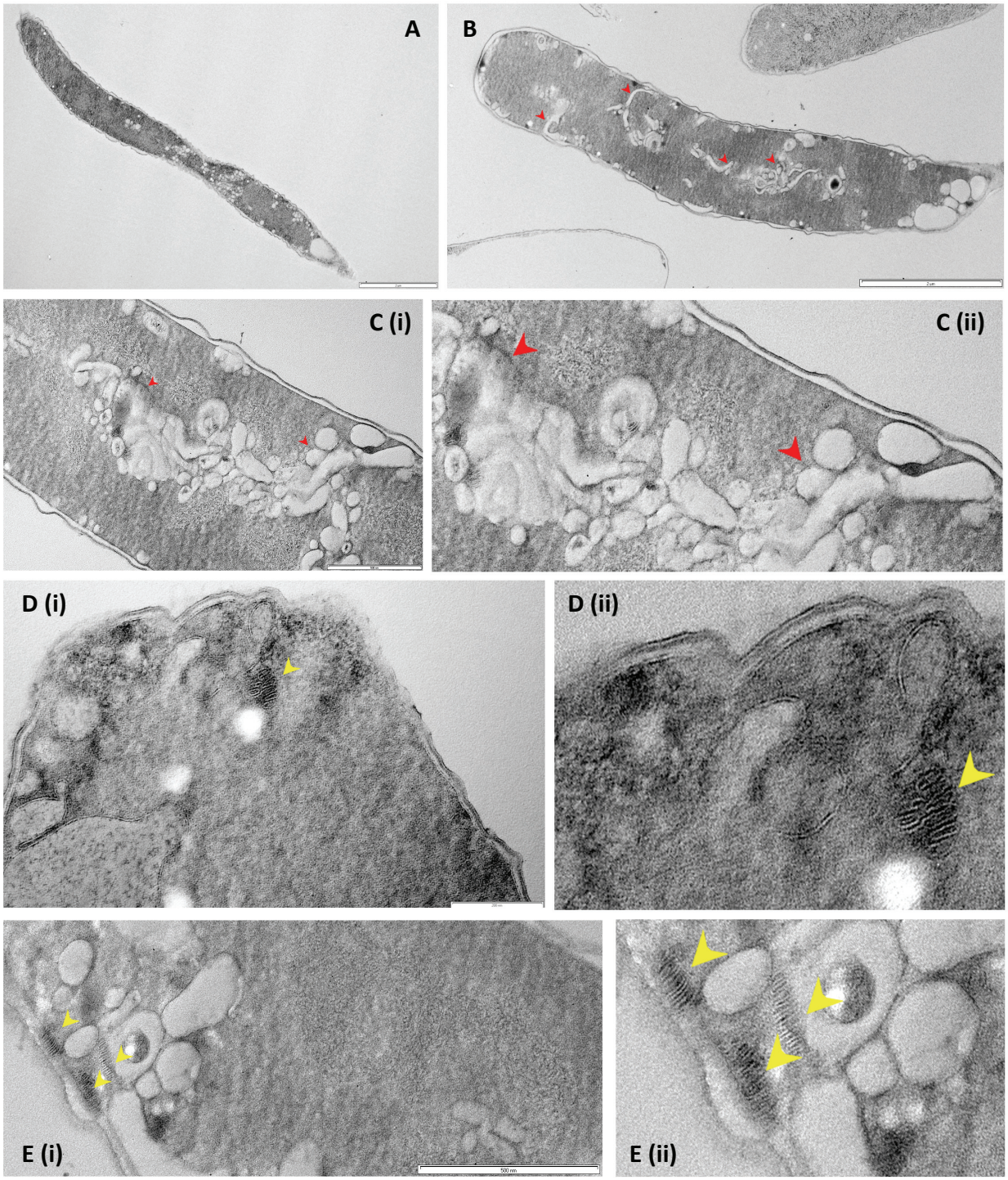
Supplementary Figure 9. Natural suppressor mutations that are restorative in a *yhcB* mutant

(A) Validation of natural suppressor mutants. 10-fold serial dilutions of E. coli K-12 BW25113, BW25113Δ*yhcB* and six BW25113Δ*yhcB* revertant suppressor mutants,

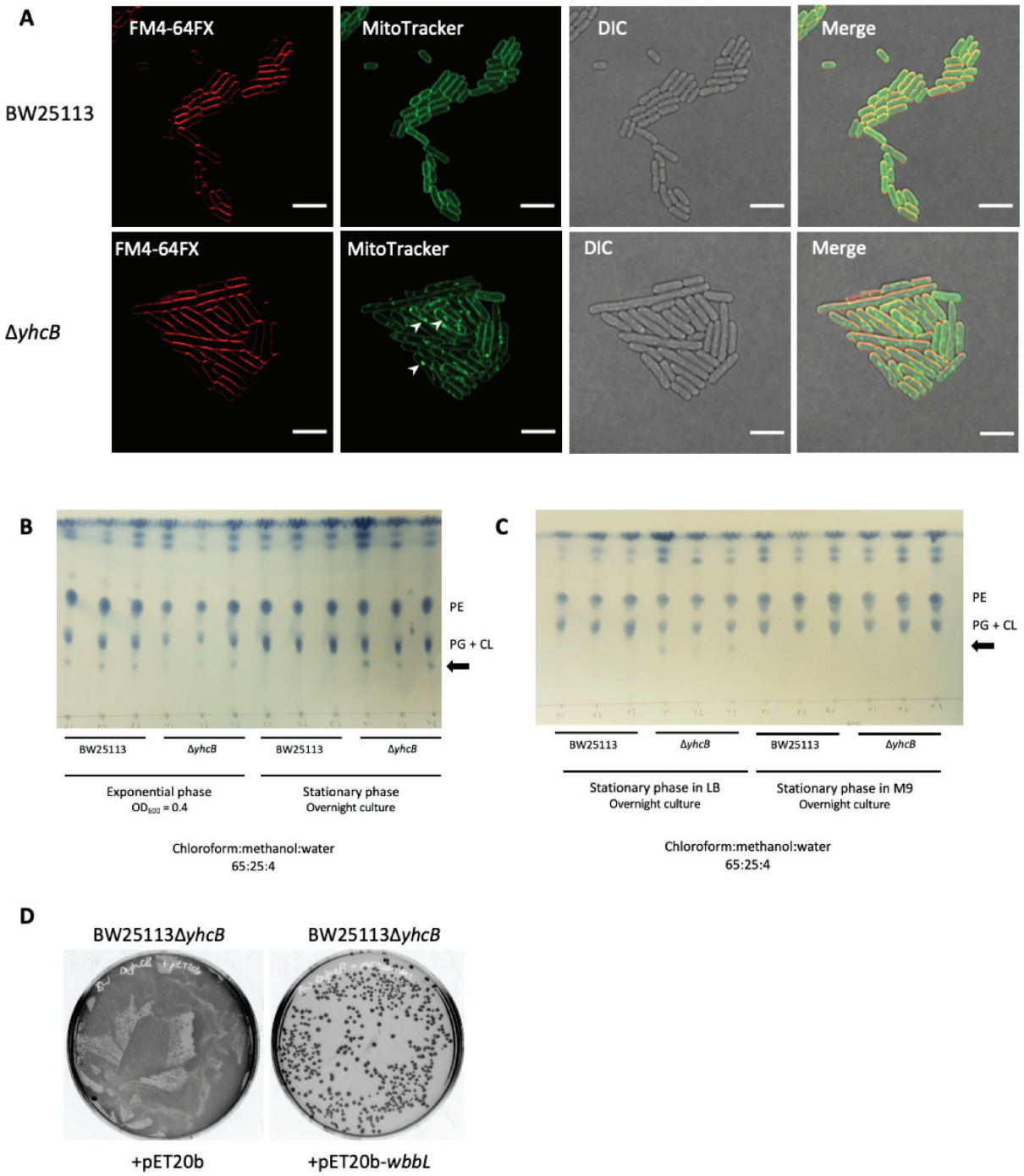
grown on LB, LB supplemented with 200 µg/ml vancomycin, or 1% SDS + 0.5 mM EDTA. (B) PCR amplification of the *yhcB* locus to confirm *yhcB*-deletion in these strains. (C) DIC images of BW25113, BW25113Δ*yhcB* and six BW25113Δ*yhcB* revertant suppressor mutants grown overnight in LB. Scale bar of 5 µm. (D) Solved structure of *Thermatoga maritima* CdsA (blue) and the predicted structure of *E. coli* CdsA (orange), with the surface electrostatic potential of each shown below. The deep red and deep blue colours indicate electronegative and electropositive regions at −10 and 10 $kT\,e^{-1}$, respectively. An overlay of the cation-binding pocket of CdsA from *T. maritima* and *E. coli* is shown in the bottom right panel. The equivalent mutated residues are annotated: shown in black, above for *T. maritima* and red, below for *E. coli*. Note the S223C residue mutated in *T. maritima* to achieve a resolved structure.



Supplementary Figure 10. TEM images of BW25113 and the Δ*yhcB* mutant
Transmission electron micrographs of (A) BW25113 and (B) the Δ*yhcB* mutant cells (standard fixation and processing). Scale bar = 1 µm in the top six images. A large Δ*yhcB* mutant cell is shown with a scale bar of 5 µm and two regions of excess or ruffled membrane structures are highlighted by red boxes (annotated in Fig. 10A); enlarged images of these sections are shown underneath with a scale bar of 500 nm each.

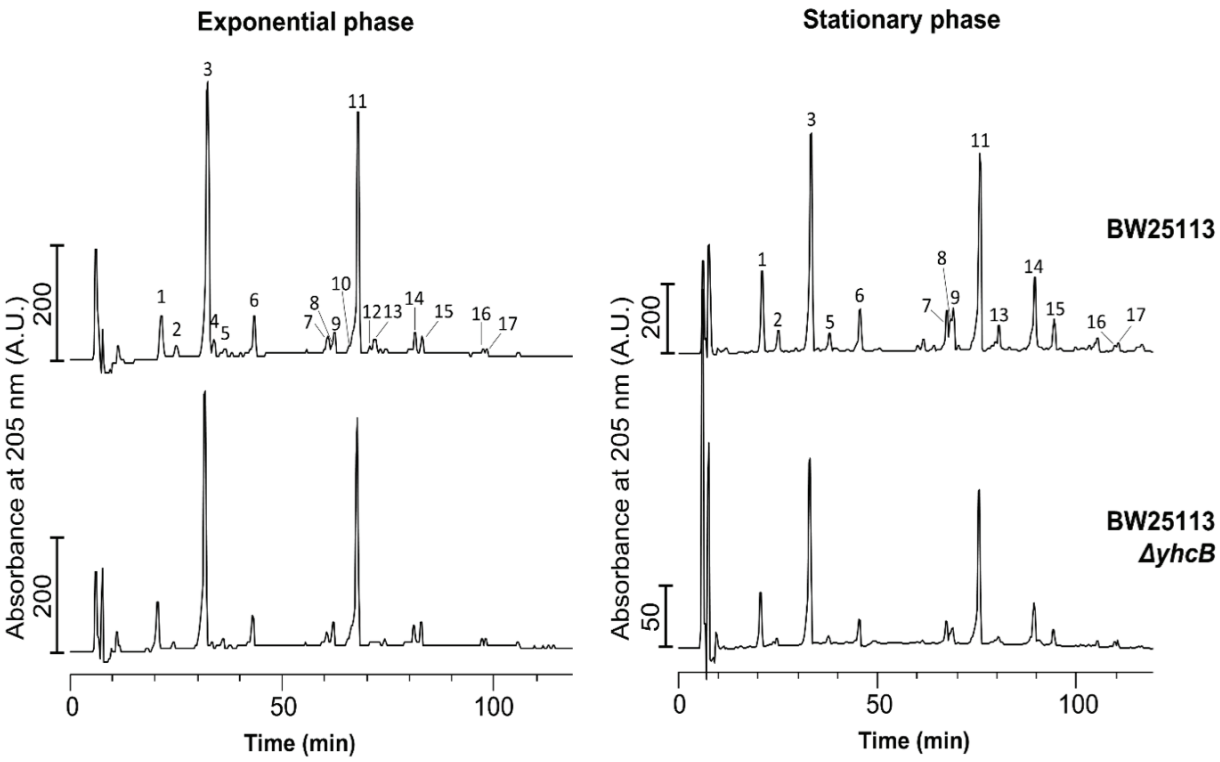


Supplementary Figure 11. Images of a Δ*yhcB* mutant cells
Electron micrographs of fast frozen/freeze-substituted *yhcB* mutant cells. Internal membranes indicated by red arrowheads; stacked membrane arrays indicated by yellow arrowheads. Bars are: (A) 2 µm; (B) 2 µm; (C) 500 nm; (D) 200 nm; (E) 500 nm. Note that panels C-E (ii) are higher magnification views from C-E (i), respectively.

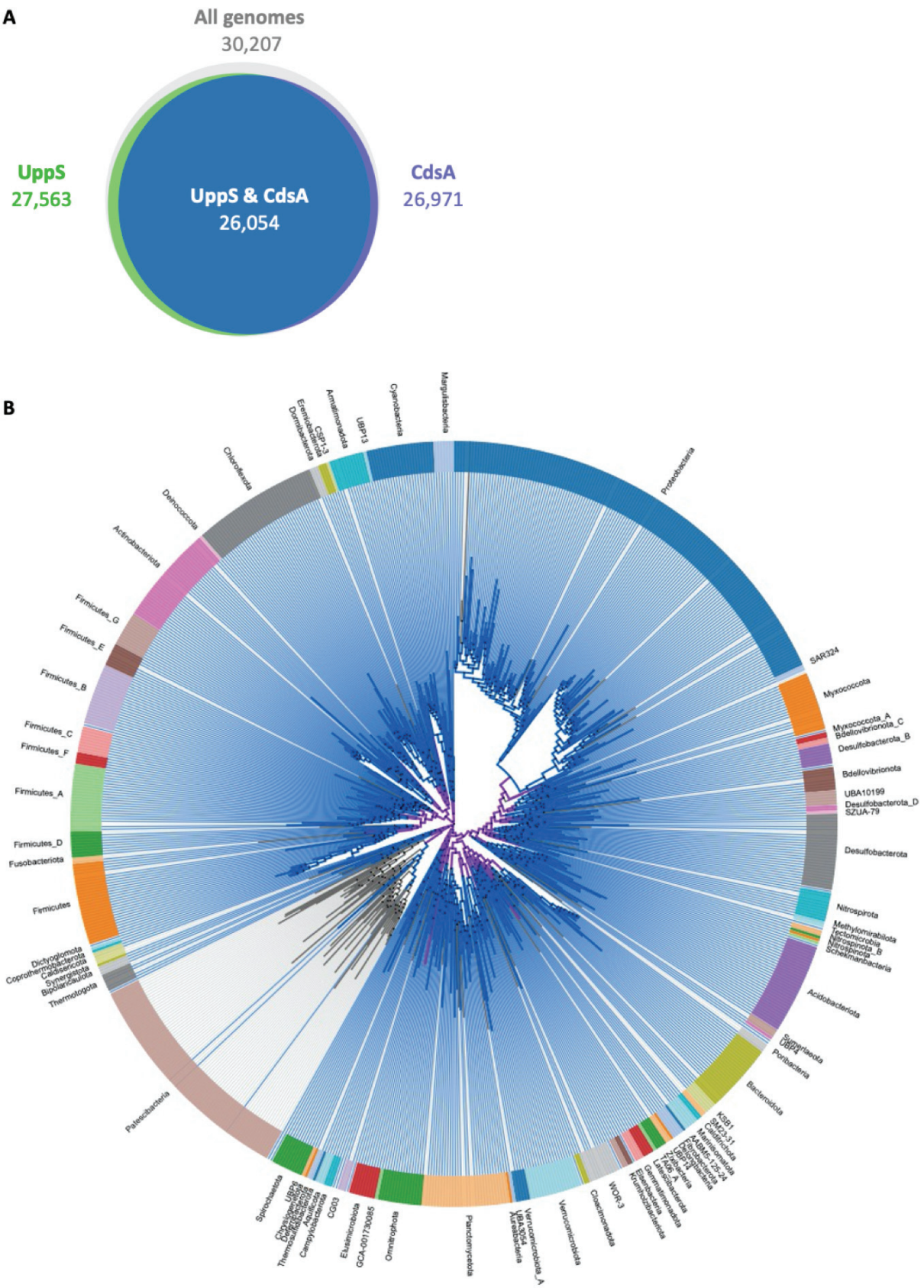


Supplementary Figure 12. The *yhcB* mutant contains extra membrane, has an altered cell envelope and restoration of O-antigen is toxic

(A) *E. coli* K-12 BW25113 and BW25112Δ*yhcB* grown in LB, labelled with FM4-64FX and MitoTracker Green FM. Images were taken at 3 h and are representative of n = 3 experiments. Lipid accumulation is indicated by the white arrows. Scale bar of 5 µm. (B-C) Thin layer chromatography (TLC) separation of phospholipid species in chloroform:methanol:water (65:25:4) solvent system, and stained with PMA. (B) Samples grown in LB and collected at two stages of growth. (C) Samples grown overnight in LB or M9-glucose. (D) Transformation efficiency of pET20b constructs +/- *wbbL* for restoring O-antigen in a Δ*yhcB* mutant. Abbreviations: Phosphatidylethanolamine (PE); Phosphatidylglycerol (PG); Cardiolipin (CL).

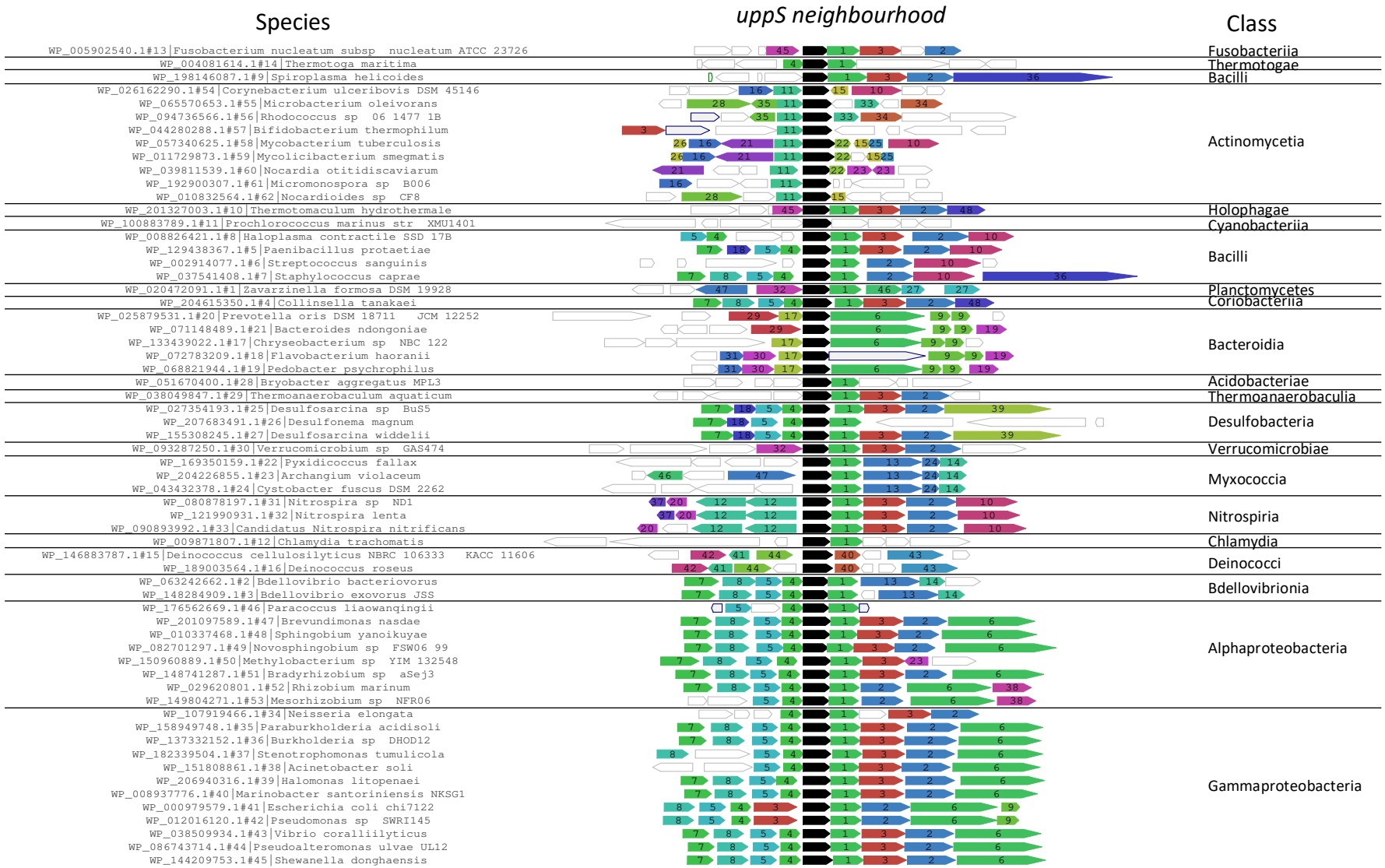


Supplementary Figure 13. Analysis of the peptidoglycan composition
Representative HPLC chromatograms showing the muropeptide composition of BW25113 and BW25113Δ*yhcB* at exponential and stationary phase. Purified peptdoglycan was digested with cellosyl and the resulting muropeptides were reduced with sodium borohydride and separated by HPLC. The major muropeptides (No. 1-17) are quantified in Table S5.



Supplementary Figure 14. UppS and CdsA conservation

(A) The number, and overlap, of bacterial genomes containing a UppS (K00806) or CdsA (K00981) homolog obtained from AnnoTree (v1.2.0) using the KEGG identifiers and the following search criteria: % identity: 30; E value: 0.00001; % subject alignment: 70; % query alignment: 70. (B) Tree representation of the phylogeny of bacterial genomes with species containing both UppS and CdsA highlighted in blue.



Supplementary Figure 15. *uppS* and *cdsA* neighbourhood conservation across the bacterial kingdom

The conservation of the *uppS* gene neighbourhood represented by 62 diverse bacterial species. *uppS* is depicted in black, and *cdsA* in green (1). The remaining gene identifiers can be found in Supplementary Table 9. Data calculated using FlaGs (Saha et al. 2020).

1599 **Supplementary Tables**
1600
1601 Supplementary Table 1. Polymyxin B TIS screen data
1602 Supplementary Table 2. Transposon library construction metrics
1603 Supplementary Table 3. Insertion index scores of the *yhcB* and WT transposon libraries
1604 Supplementary Table 4. Gene fitness in a *yhcB* mutant strain
1605 Supplementary Table 5. Peptidoglycan composition
1606 Supplementary Table 6. PANTHER analysis
1607 Supplementary Table 7. AlbaTraDIS identification of suppressor mutations
1608 Supplementary Table 8. Mutations identified in spontaneous revertant suppressor
1609 mutants
1610 Supplementary Table 9. FlaGs gene identifiers