An Exhaustive Multiple Knockout Approach to Understanding Cell Wall Hydrolase Function in *Bacillus subtilis*

Sean Wilson[1,2], Ethan Garner[1,2,*]

[1] Department of Molecular and Cellular Biology, Harvard University, Cambridge, United States.

[2] Center for Systems Biology, Harvard University, Cambridge, United States.

Corresponding Author: Ethan C. Garner

NW 445.20, Northwest Building, 52 Oxford Street, Cambridge, MA 02138

*E-mail: egarner@g.harvard.edu

ABSTRACT

Most bacteria are surrounded by their cell wall, a highly crosslinked protective envelope of peptidoglycan. To grow, bacteria must continuously remodel their wall, inserting new material and breaking old bonds. Bond cleavage is performed by cell wall hydrolases, allowing the wall to expand. Understanding the functions of individual hydrolases has been impeded by their redundancy: single knockouts usually present no phenotype. We used an exhaustive multiple-knockout approach to determine the minimal set of hydrolases required for growth in *Bacillus subtilis.* We identified 42 candidate cell wall hydrolases. Strikingly, we were able to remove all but two of these genes in a single strain; this "Δ40" strain shows a normal growth rate, indicating that none of the 40 hydrolases are necessary for cell growth. The Δ40 strain does not shed old cell wall, demonstrating that turnover is not essential for growth.

The remaining two hydrolases in the Δ40 strain are LytE and CwlO, previously shown to be synthetically lethal. Either can be knocked out in Δ40, indicating that either

hydrolase alone is sufficient for cell growth. Environmental screening and zymography revealed that LytE activity is inhibited by Mg^{2+} and that RlpA-like proteins may stimulate LytE activity. Together, these results demonstrate that the only essential function of cell wall hydrolases in *B. subtilis* is to enable cell growth by expanding the wall and that LytE or CwlO alone is sufficient for this function. These experiments introduce the Δ40 strain as a tool to study hydrolase activity and regulation in *B. subtilis*.

IMPORTANCE

In order to grow, bacterial cells must both create and break down their cell wall. The enzymes that are responsible for these processes are the target of some of our best antibiotics. Our understanding of the proteins that break down the wall – cell wall hydrolases – has been limited by redundancy among the large number of hydrolases many bacteria contain. To solve this problem, we identified 42 cell wall hydrolases in *Bacillus subtilis* and created a strain lacking 40 of them. We show that cells can survive using only a single cell wall hydrolase; this means that to understand the growth of *B. subtilis* in standard laboratory conditions, it is only necessary to study a very limited number of proteins, simplifying the problem substantially. We additionally show that the Δ40 strain is a research tool to characterize hydrolases, using it to identify 3 'helper' hydrolases that act in certain stress conditions.

INTRODUCTION

Most bacterial cells are surrounded by a peptidoglycan (PG) cell wall – a load-bearing structure that protects cells from lysing due to their high internal turgor (1). Bonds must be broken in the PG for cells to expand during growth (2). PG is built from disaccharide subunits linked to stem peptides. As new PG is inserted into the wall, the

47 disaccharides are polymerized into long chains, and their stem peptides are crosslinked
48 into the existing wall (3).
49 The enzymes that break PG bonds are termed cell wall hydrolases (hereafter
50 'hydrolases'). Hydrolases fall into several broad categories with different chemical
51 specificities (4). Amidases cleave the stem peptide from the sugar subunit.
52 Endopeptidases cleave bonds between peptides within the stem peptide. Lytic
53 transglycosylases (LTGs), lysozymes/muramidases, and glucosaminidases cleave
54 between sugar subunits. A wide array of different protein domains are capable of
55 hydrolase activity – for example, there are at least 7 distinct domains with LTG activity
56 and well over 100 distinct domains with hydrolase activity discovered thus far (5, 6).
57 Hydrolase activity is essential: without the breakage of PG bonds, the cell wall
58 cannot expand to accommodate the accumulating biomass it contains (2). Hydrolases
59 are also involved in a variety of other processes that require modification of the cell wall:
60 turning over old PG, cell separation, and sporulation, conjugation, and motility (4, 7).
61 Perhaps owing to the multiple cellular functions that require hydrolases, many bacteria
62 have a large number of hydrolases. *Bacillus subtilis* and *Escherichia coli,* for example,
63 each contain at least 20 hydrolases (4, 8). The large number of hydrolases in each
64 bacterium, combined with a high degree of functional and enzymatic redundancy
65 between them, has made it difficult to identify specific cellular functions for many
66 hydrolases. Single knockouts rarely present clear phenotypes due to compensation by
67 other hydrolases (4, 9). However, multiple-knockout approaches in *B. subtilis* have been
68 successful in revealing the importance of LytE and CwlO for cell growth, uncovering the

69 role of LytC and LytD in cell wall turnover, and identifying LytE, LytF, and CwlS as cell
70 separation hydrolases (4, 10, 11).

71 *lytE* and *cwlO* had been previously shown to be synthetically lethal when both
72 are deleted in *B. subtilis* (10, 12). The requirement of LytE or CwlO for cell growth was
73 demonstrated via microscopy: upon depletion of LytE in a *cwlO* null mutant, or vice
74 versa, cell elongation slows and then stops completely before cells lyse (12). To test
75 whether any other hydrolases were essential for *B. subtilis* growth, we employed an
76 exhaustive multiple-knockout approach. We created a minimal hydrolase strain that
77 allows the study of hydrolases in isolation, making it easier to assign functions to
78 uncharacterized hydrolases. Using this multiple hydrolase knockout strain, it is
79 straightforward to assay the biochemical activity and determine the effect of hydrolases
80 alone or in any desired combination on phenotypes like cell width, cell wall turnover, cell
81 growth, or any other process.

82 **RESULTS**

83 **Construction of a multiple hydrolase knockout strain**

84 To identify the minimal set of hydrolases required for growth in *B. subtilis* PY79,
85 we constructed a strain in which we sequentially removed as many hydrolases as
86 possible. First, we used PHMMER to screen the *B. subtilis* proteome for proteins
87 homologous to known hydrolases (4, 5, 8, 13, Table S2). We filtered our results to
88 identify candidates for hydrolases involved in growth with the following criteria:
89 candidates needed to: 1. have a functional hydrolase domain, 2. be secreted (enabling
90 access to the cell wall) and 3. be endo-acting, meaning that they can cleave internal
91 bonds rather than acting only on the ends of the stem peptide or glycan chains (exo-

92 acting) (4, 14), as exo-acting hydrolases, like the D,D-carboxypeptidase DacA or the
93 glucosaminidase NagZ, cannot contribute to cell growth because their activity does not
94 cause PG expansion. Candidates with the potential to be endo-acting were assumed to
95 be endo-acting unless proven otherwise. Candidate hydrolases that fit all above criteria
96 are indicated in Table 1. Cell wall hydrolases not present in PY79, our wild-type (WT)
97 background, are included for completeness.

98 We next generated single knockouts for each of the candidate hydrolases by
99 replacing the gene with an antibiotic resistance cassette flanked by loxP sites. We then
100 sequentially combined all knockouts into a single strain, using Cre-lox mediated loop
101 outs to remove markers when necessary (Figure 1). After each loop out step, we
102 verified deletion of all modified loci by PCR. After all knockouts had been combined into
103 a single strain, whole-genome sequencing was used to confirm all deletions and to
104 identify any genomic rearrangements or mutations that occurred during the construction
105 process. Despite the multiple rounds of transformation and loop outs this strain was
106 subjected to, we found no evidence of genomic rearrangements, and only 8 SNPs
107 leading to 5 point mutations in genes involved in unrelated processes (Table S1).

108 Ultimately, this effort produced a strain lacking 40 hydrolases, which we termed
109 "Δ40". The Δ40 strain is lacking all the identified hydrolases that met our criteria save
110 two - LytE and CwlO, two synthetically lethal endopeptidases previously shown to be
111 essential for growth (10). We were able to further knock out either *lytE* or *cwlO* in the
112 Δ40 strain, but not both, due to their synthetic lethality.

113 **Hydrolase activity is greatly reduced in the Δ40 strain**

114 To assess whether any other unidentified hydrolases remained in the Δ40 strain,
115 we first used zymography. Zymography is a renaturing SDS-PAGE assay for that
116 detects hydrolase activity (15), where cells wall binding proteins are extracted from cells
117 and separated by size via SDS-PAGE using a gel impregnated with purified cell walls.
118 Following separation by PAGE, proteins in the gel are renatured, and their activity
119 cleaves the purified cell wall in the gel, leading to clear "bands" in the gel which do not
120 stain with methylene blue, corresponding to the molecular weight of the protein with
121 hydrolase activity. It is important to note that zymography can only report on a subset of
122 hydrolases: hydrolases must successfully refold after denaturation and have *in vitro*
123 activity in the absence of any co-factors. Because either LytE or CwlO must be present
124 for viability, we conducted zymograms on the Δ40, Δ40 Δ*lytE,* and Δ40 Δ*cwlO* strains
125 (Figure 2A). The Δ40 strain showed only a single band of hydrolase activity, and this
126 band disappeared upon deletion of *lytE*. Thus, LytE is the only hydrolase detectable via
127 zymography in the Δ40 strain. We note that, although CwlO is also present in Δ40, we
128 do not expect to detect it by zymography, as previous zymography experiments have
129 suggested that full-length CwlO is inactive *in vitro* (16), an effect likely arising from
130 CwlO's auto-inhibitory coiled-coil domain (17).
131 To detect the activity of hydrolases not visible via zymography, we conducted PG
132 profiling of both wild type (WT) cells and the Δ40 strain (18), allowing us to determine
133 the abundance of hydrolase products in their cell walls (Figure 2B). During sample
134 preparation for PG profiling, we omitted teichoic acid removal to prevent sample
135 degradation: normally, teichoic acids are removed by treatment with a strong acid (18,
136 19), but this treatment also caused some hydrolysis of amide bonds in the PG,

137 complicating our analysis (Figure S1). The omission of the teichoic acid removal step
138 has been previously shown not to affect the results of PG profiling other than the
139 disappearance of phosphate-containing muropeptides, species not included in our
140 analysis (18).

141 Our PG profiling assay has limitations: as PG profiling relies on muramidase
142 digestion to yield soluble muropeptides for HPLC analysis, we could not use this assay
143 to detect hydrolases with muramidase activity. Likewise, D,D-endopeptidases cannot be
144 detected by PG profiling, as they produce products that are indistinguishable from
145 unmodified PG.

146 We compared the relative abundance of different PG hydrolase products in the
147 Δ40 and WT strains (Figure 2B). The Δ40 strain showed an absence of amidase activity
148 and a strong reduction of glucosaminidase activity, indicating that these classes of
149 hydrolases had been successfully reduced in the Δ40 strain. The residual
150 glucosaminidase activity could represent A) a yet unknown minor glucosaminidase with
151 a novel fold or B) sample degradation during PG purification. We observed an increase
152 in D,L-endopeptidase activity in the Δ40 strain, an expected result given Δ40 retains the
153 D,L-endopeptidases LytE and CwlO. In agreement with previous work (18), L,D-
154 endopeptidase activity was not detected in any strain.

155 Unexpectedly, the Δ40 strain also showed an increase in LTG activity (Figure
156 2B). We found that this remaining LTG activity required SweC, a recently characterized
157 membrane-bound CwlO co-factor homologous to the *E. coli* LTG MltG (20) (Table 1).
158 Removing *sweC* from the Δ40 strain eliminated all detectible LTG activity (Figure 2B).
159 However, as SweC's catalytic PG hydrolase domain is cytoplasmic (21), SweC cannot

160 target the cell wall directly and thus is unlikely to be the direct cause of the residual LTG
161 activity. *B. subtilis* also contains a second membrane protein homologous to MltG,
162 called YrrL. YrrL's catalytic domain is predicted to be extracellular, although YrrL is
163 likely too small to reach far enough into the cell wall space to directly participate in cell
164 wall expansion. Rather, YrrL may instead be involved in the insertion of new cell wall
165 material, as previously proposed for MltG in *E. coli* (20). If YrrL is indeed the source of
166 the residual LTG activity, its activity must require SweC; perhaps YrrL is activated by
167 interactions with SweC or SweD, similar to what has been seen for CwlO (21).

168 **Cell growth and morphology are similar in the Δ40 strain relative to wild type.**

169 We next characterized the growth rate of the Δ40 strain. The Δ40 strain grew at
170 the same rate as WT cells in both rich (CH) and synthetic media ($S7_{50}$ with glucose and
171 amino acids, see Methods for details) (Figure 3A and B). This indicates that the activity
172 of LytE and CwlO together are sufficient for normal cell growth. To investigate the
173 individual effects of each of these enzymes, we made knockouts of *lytE* and *cwlO* in
174 both WT and Δ40 backgrounds. Δ40 Δ*lytE* and Δ40 Δ*cwlO* both exhibited a ~10%
175 reduction in growth rate compared to Δ40. We observed cell lysis in both Δ40 Δ*lytE* and
176 Δ40 Δ*cwlO* strains in phase-contrast images, which could contribute to their slower
177 growth rates as measured in bulk by OD_{600} (Figure 3D). On the other hand, Δ*lytE* or
178 Δ*cwlO* in a WT background had the same growth rate as WT. This suggests either that
179 in WT cells, other hydrolases participate in but are not required for growth, or that LytE
180 and CwlO are not being expressed highly enough to maintain normal growth on their
181 own in the Δ40 background.

182 Next, we quantified cell dimensions in these strains using FM 5-95 membrane
183 stain. Δ40 cells had a WT cell length and were 3% wider ($p<0.0001$, unpaired t-test with
184 Welch's correction). Δ*cwlO* cells were 13% wider and 18% shorter than WT cells, a
185 phenotype that persisted in the Δ40 Δ*cwlO* strain ($p<0.0001$ for all comparisons:
186 unpaired t-test with Welch's correction for width comparisons, Mann-Whitney test for
187 length comparisons). Δ40 Δ*cwlO* cells were less able to control their width as compared
188 to Δ40 cells, having a 1.5x wider cell width distribution (7.5% vs. 11.33% coefficient of
189 variation, F test $p<0.001$). In contrast, Δ*lytE* cells were only slightly wider than WT cells
190 (1%, $p<0.0001$, unpaired t-test with Welch's correction), and Δ40 Δ*lytE* cells were
191 slightly narrower (1%, $p<0.0001$, unpaired t-test with Welch's correction) than Δ40 strain
192 alone, with a slight decrease in length (Figure 3C, $p<0.0001$, Mann-Whitney test). Thus,
193 CwlO appears to be involved in cell width maintenance, as removing *cwlO* causes
194 changes in cell width both in Δ40 and WT backgrounds, consistent with previous reports
195 (12). Furthermore, given that removing *cwlO* increases the cell width coefficient of
196 variation in the Δ40 background but does not increase the width variation when deleted
197 from WT cells, other hydrolases must also have a role in width homeostasis.

198 **Δ40 cells do not turn over their cell wall**

199 Hydrolases are involved in cell wall turnover, where old PG material is shed from
200 the cell wall (22). We measured the rate of cell wall turnover of both WT and Δ40 cells
201 using pulse-chase labeling with the radioactive cell wall precursor ^{3}H-N-
202 acetylglucosamine (^{3}H-GlcNAc). This revealed that, while WT cells turn over PG at a
203 rate of about 50% per generation in agreement with previous work (22), turnover in Δ40
204 strain was absent, with a rate not significantly different from zero ($p=0.4837$) (Figure

205 3A). These results demonstrate that LytE and CwlO, the only identifiable remaining
206 hydrolases in the Δ40 strain, do not contribute to cell wall turnover. Furthermore, this
207 data reveals that cell wall turnover is not an essential process: cell growth only requires
208 the cleavage of bonds so the cell can expand.

209 As hydrolases have been shown to be involved in the regulation of cell wall
210 thickness (23), we measured the cell wall thickness of the Δ40 strain by transmission
211 electron microscopy (TEM), finding it was not significantly different from WT (p=0.1382)
212 (Figure 3B). This suggests that cell wall turnover has no impact on cell wall thickness
213 and that none of the 40 hydrolases removed in the Δ40 strain are responsible for cell
214 wall thickness regulation. Given previous studies have demonstrated hydrolases
215 regulate cell wall thickness, the unchanged cell wall thickness thicknesses observed in
216 our Δ40 demonstrate that LytE and CwlO are the hydrolases controlling cell wall
217 thickness, as these hydrolases are the only non-integral membrane hydrolases
218 remaining.

219 **Δ40 Δ*cwlO* cells are sensitive to various stresses, including ionic stress**

220 Although the Δ40 strain grows normally under our standard lab conditions, we
221 wondered whether the absence of so many hydrolases would sensitize cells to stress
222 conditions. We used a spot dilution assay to measure the viability of our strains under a
223 variety of stress conditions: temperature, ionic stress, pH, and osmotic pressure (Figure
224 5). In all conditions, including our control (37˚C), Δ40 cells had fewer CFUs than WT.
225 This is expected because Δ40 cells grow in long chains, and thus cells are cannot
226 readily separate into individual CFUs; we do not believe that this reflects an overall
227 change in viability because Δ40 grows at the same rate as WT (Figure 3). In all stress

228 conditions Δ40 cells were similarly viable to WT cells, as were Δ*lytE,* Δ*cwlO,* and Δ40
229 Δ*lytE* cells. However, Δ40 Δ*cwlO* cells were susceptible to multiple stresses, including
230 low pH, low temperature, and ionic stress.

231 We were particularly intrigued by the susceptibility of Δ40 Δ*cwlO* to Mg^{2+}. Mg^{2+} is
232 coordinated between PG and teichoic acids (24), and this Mg^{2+} binding is thought to
233 give structural stability to the cell wall (25, 26). High levels of Mg^{2+} are often protective
234 against cell wall perturbations, including knockouts of hydrolases, PBPs, or components
235 of the rod complex (10, 27); thus, the Mg^{2+} sensitivity of the Δ40 Δ*cwlO* strain seemed
236 counterintuitive. Our experiments indicated Δ40 Δ*cwlO* cells were sensitive to both Ca^{2+}
237 and Mg^{2+}; growth was inhibited by the addition of 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 10
238 mM $CaCl_2$, but not by the addition of 20 mM NaCl, suggesting that the growth inhibition
239 was not due to changes in ionic strength or chloride ions. We did observe growth
240 inhibition due to ionic stress at far higher salt concentrations (500 mM NaCl). Notably,
241 cells were not sensitive to an equivalent osmotic stress (1M sorbitol), indicating the
242 sensitivity is to ionic stress, not osmotic.

243 As Δ*cwlO* mutants in the WT background were Mg^{2+} insensitive, we sought to
244 identify which hydrolases caused cells to be sensitive to Mg^{2+} when they were removed.
245 To find these hydrolases, we returned to intermediate strains used to construct the Δ40
246 strain, which are missing subsets of hydrolases. We transformed a *cwlO* knockout into
247 these intermediate strains, then screened these crosses for the same small colony
248 phenotype and the Mg^{2+} sensitivity seen in the Δ40 Δ*cwlO* strain. This identified two
249 genes: *yabE* and *ydjM*. Notably, during construction of the Δ40 strain, we had noticed
250 that *yocH* seemed significant – at several intermediate verification steps, a WT copy of

251 *yocH* had reintegrated itself. Furthermore, a Δ*ydjM* Δ*yocH* Δ*cwlO* mutant was previously
252 demonstrated to be sick, with short and sometimes anucleate cells (10). Because *yabE*,
253 *ydjM*, and *yocH* have similar hydrolase domains, and because *yocH* and *ydjM* had been
254 identified previously to be involved in a synthetic sick interaction with *cwlO,* we
255 additionally tested whether the removal of *yocH* contributed to the Δ40 Δ*cwlO* Mg^{2+}
256 sensitivity phenotype, and found that it did.

257 In total, we identified three genes, *yabE*, *ydjM*, and *yocH*, whose absence in a
258 Δ*cwlO* background caused the Mg^{2+} sensitivity: A Δ*yabE* Δ*ydjM* Δ*yocH* Δ*cwlO* strain
259 showed a similar stress profile to Δ40 Δ*cwlO*, including sensitivity to $MgCl_2$ and $CaCl_2$
260 (Figure 6A). *yabE*, *ydjM*, and *yocH* are 3 uncharacterized RlpA-like superfamily domain-
261 containing proteins expressed during exponential growth. Like *lytE* and *cwlO, yocH* and
262 *ydjM* are in the *walR* regulon, while *yabE* is regulated by *sigA* (Table 1). All are likely
263 lytic transglycosylases: *yocH* has been shown to have lytic activity and has homology to
264 the *E. coli* lytic transglycosylase *mltA* (28), and all three share a similar catalytic domain.
265 Because *yabE*, *ydjM*, and *yocH* all contain a RlpA-like protein domain, we refer to these
266 genes collectively as RLPAs, and to the triple deletion of all three genes as ΔRLPAs.

267 **LytE is inhibited by Mg^{2+} *in vitro* and *in vivo*, and RLPAs suppress Mg^{2+} lethality *in*
268 ***vivo***

269 Finally, we sought to identify the source of Mg^{2+} growth inhibition in the ΔRLPAs
270 Δ*cwlO* background. Because LytE is essential in the absence of CwlO, we hypothesized
271 that the sensitivity of the Δ40 Δ*cwlO* strain to Mg^{2+} (and, by extension, the sensitivity of
272 the ΔRLPAs Δ*cwlO* strain to Mg^{2+}) could be explained by Mg^{2+} inhibition of LytE. To
273 investigate this, we first characterized the response of Δ*cwlO* cells to the removal of

274 LytE. We constructed an otherwise wildtype strain with *cwlO* knocked out and *lytE*
275 under inducible control and monitored its growth by time-lapse phase-contrast
276 microscopy. When *lytE* was induced, cell growth was normal (Movie S1). When *lytE*
277 induction was removed, cell growth initially slowed, followed by a period of 'stuttery'
278 growth, where elongating cells intermittently shrank while showing accompanying
279 fluctuations in their phase contrast signal (Movie S2). Ultimately, cells lysed about 1-2
280 doubling times after the removal of *lytE* induction as previously observed (10, 12). Next,
281 we performed the same imaging in the ΔRLPAs Δ*cwlO* strain after the addition of 10
282 mM $MgCl_2$ and observed the same 'stuttery' phenotype, suggesting that LytE function
283 might be inhibited by Mg^{2+} (Movie S4). Without the addition of Mg^{2+}, cell growth of the
284 ΔRLPAs Δ*cwlO* strain was normal (Movie S3). In WT cells or Δ*cwlO* cells, the presence
285 of Mg^{2+} has no effect on cell viability or growth – growth is only inhibited in the absence
286 of the RLPAs. Thus, the RLPAs appear to allow LytE to maintain its activity in the
287 presence of Mg^{2+}.

288 To test whether LytE activity is directly inhibited by Mg^{2+}, we performed
289 zymography with the addition of Mg^{2+} to the renaturation buffer on the Δ40 Δ*cwlO* strain,
290 where LytE activity is easy to detect as it is the only remaining band (Figure 2A).
291 Indeed, LytE activity was strongly reduced in the presence of 25 mM $MgCl_2$ (Figure 6B).
292 Additionally, we reasoned that if the Mg^{2+}-sensitivity phenotype was due to direct
293 inhibition of LytE by Mg^{2+}, increasing the levels of LytE should protect cells from death
294 by increasing the total amount of LytE activity. Indeed, overexpression of LytE allowed
295 cells to survive in the presence of higher levels of Mg^{2+}, although 100 mM $MgCl_2$ still
296 inhibited growth (Figure 6C).

297 Thus, we conclude that LytE activity is inhibited by Mg^{2+} both *in vivo* and *in vitro*.
298 Furthermore, our data indicates that the RPLAs allow LytE to maintain normal function
299 in the presence of Mg^{2+}, though the specific mechanism is unclear. Whether the RLPAs
300 act directly or indirectly on LytE remains to be determined, but we anticipate that the
301 RLPAs interact with and activate LytE similar to what has been observed for the
302 *Mycobacterium smegatis* hydrolases RipA and RpfB: RipA's C-terminus (containing a
303 NLPC/P60 domain like LytE) interacts with RpfB's RlpA-like LTG domain (29), and RipA
304 and RpfB have synergistic activity *in vitro* (30). By analogy, LytE's catalytic NLPC/P60
305 domain may interact with the RlpA-like domains in YabE, YdjM, and YocH, leading to
306 increased LytE activity, allowing LytE to continue to function in the presence of Mg^{2+}.
307 The ΔRLPAs Δ*cwlO* strain also has increased sensitivity to ionic stress and low
308 temperatures, suggesting RLPAs might stimulate LytE activity under those conditions as
309 well.

310 **DISCUSSION**

311 Bacterial cell growth requires the action of PG hydrolases, but previous *in vivo*
312 hydrolase studies have been impeded by their diversity and redundancy. We
313 constructed and validated a *B. subtilis* strain lacking all hydrolases potentially involved
314 in cell growth besides LytE and CwlO. These deletions constitute 40 genes in total,
315 representing 10% of secreted proteins and 1% of all genes. The resulting Δ40 strain
316 enables the investigation of given hydrolases and the cellular contexts in which they
317 function, and in this work, allowed several new discoveries regarding their sufficiency,
318 regulation, and genetic interplay.

319 First, we found that not only is the Δ40 strain viable, it grows at the same rate as
320 WT cells under standard lab growth conditions. This demonstrates that LytE and CwlO
321 alone can function to expand the cell wall to allow cell growth. Furthermore, as single
322 knockouts of LytE and CwlO in the Δ40 strain are viable and allow growth (albeit at a
323 slightly reduced rates with some shape defects), this demonstrates *B. subtilis* requires
324 only one of these two hydrolases to grow.
325 Second, the Δ40 strain allowed us to discover a yet-to-be-identified LTG which
326 requires SweC for its activity in the Δ40 strain. Given that the only remaining candidate
327 LTG we identified in the Δ40 strain is YrrL, it is likely that SweC is required for YrrL
328 activity. As CwlO activity is dependent on SweC, and SweC forms a complex with
329 SweD, FtsE, and FtsX (21), YrrL might be an additional member of this complex, and
330 SweCD/FtsEX might regulate both YrrL and CwlO. These experiments demonstrate
331 how the Δ40 strain can be used to interrogating the regulation or direct biochemical
332 activity of any other *B. subtilis* hydrolase: as the majority of cell wall hydrolase activity is
333 removed in the Δ40 strain, PG profiling can be used to identify the specific activities of
334 individual hydrolases reintroduced back into the Δ40 background.
335 Likewise, our minimal hydrolase strain allowed us to show that RlpA-like lytic
336 transglycosylases enhance LytE activity *in vivo* and that this enhancement can be
337 important for growth under conditions where LytE activity is inhibited, including the
338 presence of divalent cations, ionic stress, and cold. Although the mechanism for LytE
339 enhancement is unclear, we hypothesize that RlpAs stimulate LytE activity via a direct
340 interaction, as has been observed in *M. smegmatis* (30). Synthetic lethal or synthetic
341 sick interactions are straightforward to identify and characterize in the Δ40 strain, giving

342 a useful tool to interrogate genetic relationships between different hydrolases or
343 between hydrolases and other genes of interest – such as those involved in cell wall
344 synthesis.

345 Surprisingly, the Δ40 strain grows normally under standard lab conditions. What,
346 then, is the function of these 40 hydrolases, and why does *B. subtilis* encode so many
347 of them? This multitude of hydrolases likely arises from the fact that hydrolases are
348 involved in other processes aside from cell growth such as sporulation (4) and cell
349 motility (31). Additionally, some hydrolases might be only be needed under nutrient
350 conditions not tested here, such as during phosphate limitation where teichoic acids are
351 not produced, where cells may require hydrolases that are not regulated by teichoic
352 acids (32–34). Finally, these other hydrolases may be important during non-exponential
353 growth states such as during stationary phase, where the recycling of cell wall turnover
354 products, lacking in the Δ40 strain, reduces cell lysis (35). Thus, a broader screen of the
355 sensitivity of the Δ40 strain in different nutrient and environmental conditions will allow
356 researchers to determine which hydrolases are useful for which conditions.

357 In summary, the Δ40 minimal hydrolase strain provides a powerful experimental
358 background to investigate the function, regulation, and interplay of hydrolases,
359 improving our understanding of precisely how these enzymes conduct their cellular
360 tasks. In the future, individual hydrolases can be reintroduced into the Δ40 strain to
361 investigate their specific activities in the absence of confounding contributions from the
362 other 39 genes. Using the Δ40 strain, PG profiling can determine the biochemical
363 activity of hydrolases. Uncovering synthetic genetic interactions between hydrolases
364 and other genes of interest – now easy to do for all 40 hydrolases at once – will allow us

365 to flesh out our understanding of bacterial cell growth. Understanding the function of cell
366 wall hydrolases is essential for a complete understanding of how bacteria grow, and the
367 Δ40 strain will allow rapid progress to this end.

368 **ACKNOWLEDGEMENTS**

369 We would like to thank Carl Wivagg, Alex Bisson, Matthew Holmes, Ferran
370 Garcia-Pichel, and Susanne Neuer for helpful advice and discussions, and Georgia
371 Squyres for both helpful advice, discussions, and reading of the manuscript. This work
372 was funded by National Institutes of Health Grants DP2AI117923-01 to ECG, as well as
373 support from the Volkswagen Foundation. This work was supported by the NSF-Simons
374 Center for Mathematical and Statistical Analysis of Biology at Harvard (1764269) and
375 the Harvard Quantitative Biology Initiative. Some work was performed at the Center for
376 Nanoscale Systems at Harvard University, supported by NSF ECS-0335765. HPLC-MS
377 work was performed by the Harvard Center for Mass Spectrometry Core Facility, and
378 sequencing was performed by the Bauer Core Facility at Harvard University.

379

380 **METHODS**

381 **Strains, media, and growth conditions**

382 Glycerol stocks stored at -80˚C were struck onto LB agar plates. For strain
383 bSW61 (*lytE::pSpac-lytE,* Δ*cwlO*), these plates were additionally top spread with 1 mM
384 IPTG. After incubation overnight at 37˚C, colonies were inoculated into 1 mL media and
385 grown on a roller at 37˚C until they reached mid-exponential-phase growth (OD ~0.2).
386 Cells were diluted 1:10 in prewarmed media and again grown until mid-exponential
387 phase; this process was repeated until the start of the experiment. Alternately, a 1:10
388 dilution series of cells were grown overnight in media on a roller at 25˚C. The next day,
389 the culture whose OD600 was nearest to 0.2 was diluted 1:10 and grown in media at
390 37˚C as above. $S7_{50}$AA indicates $S7_{50}$ media with added amino acids as in (36).

391 **Strain construction**

392 The wild type strain for this work was *B. subtilis* PY79. Strains used in this study
393 are listed in Table S3. Constructs were created using Gibson assembly of PCR
394 products. Linear Gibson assembly products were transformed into competent *B. subtilis*.
395 Transformants were selected on LB plates containing the appropriate antibiotic. The
396 resulting strains were verified by PCR. Constructs used in this study, as well as any
397 plasmids used to create each construct, are listed in Table S3. Primers, along with
398 strain construction details, are listed in Table S4. Resistance cassettes and promoters
399 were amplified from purified plasmids (listed in Table S3), all other fragments were
400 amplified from WT gDNA.

401 To combine knockouts, the parent strain was transformed with PCR product
402 containing the locus (homology arms + resistance cassette) or gDNA as indicated. All

403 resistance cassettes used have loxP sites flanking the cassette, allowing Cre-based
404 loop out using plasmid pDR244 (a gift from David Rudner) of the cassette to yield a
405 markerless knockout. Removal of the plasmid was accomplished by shifting streaks to
406 42˚C where it cannot be replicated due to a temperature-sensitive origin. Successful
407 loop outs were confirmed via loss of antibiotic resistance.

408 **PHMMER search**

409 We used pfamscan version 1.6 to search the *B. subtilis* 168 and PY79 proteomes
410 for all pfam domains using default parameters: e-value: 0.01, significance E-values [hit]:
411 0.03, significance bit scores [sequence]: 25, significance bit scores [hit]: 22. We then
412 filtered the list for domains of interest, found in Table S3, and removed any cytoplasmic
413 proteins.

414 **PG purification, HPLC conditions, and MS data analysis**

415 PG purification was conducted as in (37), with the addition of a protein digestion
416 step. Cells were grown in a baffled flask to an OD of ~0.5 in 50 mL of CH media. Cells
417 were mixed 50/50 with 50 mL of boiling 10% SDS and boiled for 15 min in a water bath,
418 then pelleted at 5000x g and washed 5x with ddH2O. Cells were then resuspended in 2
419 mL DNAse/RNase buffer (10 mM Tris pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) with 20 uL
420 DNAse I and 20 uL RNAse A, then incubated overnight at 37˚C and washed 3x with
421 ddH2O to remove nucleic acids. Next, cells were resuspended in 2 mL Proteinase K
422 buffer (10 mM Tris pH 7.5, 1 mM $CaCl_2$) with 20 uL Proteinase K, incubated overnight at
423 45C, and washed 3x with ddH2O to remove proteins. Purified PG at this point was
424 ready for use in zymography as substrate. For PG profiling, the PG was resuspended in
425 12.5 mM $NaHPO_4$ pH 5.5 with 5000 units of mutanolysin and digested overnight (16h)

426 at 37˚C on a roller to yield soluble muropeptides. Undigested material was pelleted by
427 spinning at 16000x g for 5 mins and the supernatant was transferred to a new tube.
428 Soluble muropeptides were reduced with sodium borohydride (1 mg/mL) for 30 mins
429 and the reaction was stopped by adding 10 uL 30% phosphoric acid. The pH was
430 adjusted to 4-6 using NaOH, and the reduced soluble muropeptides were characterized
431 by high-resolution LC-MS operating in both positive and negative mode. Soluble
432 reduced muropeptides were separated on a Waters column with the following method:
433 column temperature 52˚C, flow rate 0.5 mL/min, linear gradient of solvent A (0.1%
434 formate) to 10% solvent B (acetonitrile + 0.1% formate) over 80 min.
435 Mass spectrometry data was analyzed using a custom MATLAB program,
436 available at https://bitbucket.org/garnerlab/wilson_40_2020/. For each compound, at
437 each time point, theoretical m/z values were compared with observed m/z with a cutoff
438 of 0.05 Da. Charge was determined from the isotopic distribution. To be considered a
439 match, each compound needed to have the appropriate charge, isotopic distribution,
440 and m/z value, and we required that a compound be present on both the positive and
441 negative scans. We filtered out any compounds that were less than 1% the abundance
442 of the most abundant species at each time point. Finally, if multiple compounds were
443 found at the same time point, the less abundant ones were filtered out.
444 **Zymography**
445 Zymography was performed as in (38), but using PG prepared as detailed under
446 PG purification. Purified PG was sonicated at max power on a sonicator for 2 min. A
447 15% polyacryamide bis-Tris gel was prepared with the purified PG. 50 mL of cells were
448 grown in a baffled flask with shaking to an OD of ~0.5. Cells were concentrated by

449 centrifugation at 5000x g for 5 min, resuspended in 1 mL of supernatant, then further
450 concentrated by centrifugation at 16,000x g for 2.5 min. All media was aspirated, the
451 centrifuge tube turned around, and centrifuged again at 16,000x g for 2.5 min. Cell wall
452 binding proteins were extracted by resuspending the pellet in an equal volume of 8%
453 SDS. This mixture was heated at 95˚C for 2 min then centrifuged at 16,000x g for 2.5
454 min. The supernatant was transferred to a new tube, 6x SDS-PAGE Laemmli buffer was
455 added, and the extracted proteins were loaded on the gel. Gels were run in SDS-MOPS
456 buffer at constant amps (20 mA) for 4-6 hours, then washed with ddH2O, and placed in
457 renaturation solution (1% Triton-X 100, 25 mM Tris pH 7.5). The gel was incubated for
458 16h at 37C with gentle shaking, then stained 4h with a methylene blue staining solution
459 (1% methylene blue, 0.1% KOH), washed several times with ddH2O, and destained
460 overnight in 500 mL ddH2O with gentle shaking. Gels were photographed using a
461 Canon SC1011 scanner in a standard plastic page protector.

462 **Growth rates**

463 Cells were grown to an OD of ~0.3-0.5 on a roller drum at 37˚C and diluted to an
464 OD of ~0.05 in baffled flasks in a water bath shaker at 37˚C. Samples were withdrawn
465 at 5 min intervals and OD was measured in a plastic cuvette using a Biowave Cell
466 Density Meter CO8000. T vs. OD curves were fit to a single exponential ($OD = Ae^{BT}$) to
467 extract a growth rate (B).

468 **Turnover rates**

469 Cells were grown in $S7_{50}AA$ to an OD of ~0.3-0.5 on a roller drum at 37˚C and
470 diluted to an OD of ~0.05 in 3 mL of prewarmed media containing 1 uCi of ^{3}H-N-
471 acytylglucosamine (specific activity: 20 Ci/mmol, American Radiolabeled Chemicals,

472 Inc., St. Louis, MI, USA) in 25mm wide test tubes in a water bath shaker at 37˚C. Cells
473 were labeled for 3 generations (OD ~0.4), then filtered, washed twice with prewarmed
474 media, and resuspended in 25 mL of prewarmed media. Samples were withdrawn at 5
475 min intervals and OD was measured in a plastic cuvette using a Biowave Cell Density
476 Meter CO8000. Samples were mixed 50:50 with ice cold 10% TCA + 20 mM unlabeled
477 GlcNAc, incubated on ice for 10 mins, then filtered and washed. Filters were dried,
478 resuspended in Ultima Gold LSC cocktail (PerkinElmer, Waltham, MA, USA) and
479 radioactivity was measured using a scintillation counter (Tri-Carb 2100 TR,
480 PerkinElmer). Counts/min vs. OD plots were fit to a single exponential (CPM = Ae^{BT}) to
481 extract a turnover rate (B).

482 **Cell dimensions**

483 Cells were grown to an OD of ~0.3-0.5 in a water bath shaker at 37˚C. 1 mL of
484 culture was stained with FM 5-95 and concentrated to 100 uL by centrifugation at 2000x
485 g and resuspension. 5 uL of concentrated cells were spotted under 2% agarose pads in
486 CH containing 0.5 ug/mL FM 5-95. Images were collected on a Nikon Ti-E microscope
487 using a Nikon CFI Plan Apo DM Lambda 100X Oil objective, 1.45 NA, phase ring Ph3
488 using an ORCA-Flash4.0 V2 sCMOS camera. Analysis was performed using
489 Morphometrics v1.1 (39). Zero length or width cells were discarded, as well as any cells
490 with width greater than length. Outliers were removed using Graphpad Prism ROUT
491 with default parameters (1%).

492 **Electron microscopy and cell wall thickness**

493 Electron microscopy was performed as in (40). Briefly, exponentially growing
494 cells were fixed in 100 mM MOPS buffer pH 7 containing 2% paraformaldayde, 2.5%

495 gluteraldehyde, and 1% dimethyl sulfoxide overnight at 4°C, washed, stained with 2%
496 osmium tetroxide in 100 mM MOPS for 1 hr, washed, and stained overnight with 2%
497 uranyl acetate. The cells were then dehydrated and embedded in Embed 812 resin.
498 Serial ultrathin sections (80 nm) were cut with a Diatome diamond knife (EMS,
499 PA) on a Leica Ultracut UCT (Leica Microsystems, Germany) and collected on 200-
500 mesh thin-bar formvar carbon grids. Sections were imaged on a Hitatchi HT7800
501 transmission electron microscope.
502 Images collected were segmented (inner cell wall, outer cell wall) using DeepCell
503 (41), and cell wall thickness was measured using a custom Matlab program available at
504 https://bitbucket.org/garnerlab/wilson_40_2020/. Briefly, the distance between the inner
505 and outer cell wall was measured every 10 nm along a user-defined line, and the mean
506 of that measurement was taken to be the cell's cell wall thickness.
507 **Spot dilution assay**
508 Cells were grown to an OD of 0.5 and diluted 1:10 into 100 uL of LB media in a
509 96 well plate. A 1:10 serial dilution series was made, and 3 uL of each dilution was
510 spotted onto the plate using a multichannel pipettor. The plates were allowed to dry and
511 incubated in at 37˚C or 42˚C as indicated for 18h. Plates incubated at 25˚C or 18˚C
512 were left for additional time (24h and 48h, respectively). Plates were photographed
513 using a Canon SC1011 scanner with the lid open.
514 For the colony morphology assay in Figure 1, this protocol was followed except
515 that a colony of cells of each strain were simply resuspended in 100 uL of media using a
516 toothpick (omitting the broth culture step).
517 **Data availability**

518 All custom software used in this work is available at

519 https://bitbucket.org/garnerlab/wilson_40_2020/. Raw HPLC-MS data for PG profiling

520 experiments available from ftp://massive.ucsd.edu/MSV000086886

521 (doi:10.25345/C5R21D). Raw and error corrected sequencing reads for whole genome

522 sequencing available at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA702153

523 (BioProject PRJNA702153).

524

525

Name (alias)	PY79	UniProt	Locus tag	Regulons	e-value	References	Activity
				AMIDASE			
				Amidase_2 (PF01510)			
cwlA	Y/KO	P24808	BSU25900		5.60E-19	(42–44)	
cwlH (yqeE)	Y/KO	P54450	BSU25710	gerE^*, sigK^*	7.50E-25	(45)	
xlyA	Y/KO	P39800	BSU12810	xpf*	1.00E-23	(46)	
xlyB (yjpB)	Y/KO	O34391	BSU12460		2.20E-18	similarity; xlyA	
blyA (yomC)	N	O31982	BSU21410		2.30E-18	(47)	
				Amidase_3 (PF01520)			
cwlC	Y/KO	Q06320	BSU17410	sigK^*	3.4E-44	(48, 49)	
cwlD	Y/KO	P50864	BSU01530	lexA^*, sigE^*, sigG^*	1.10E-48	(19, 50, 51)	
lytC (cwlB)	Y/KO	Q02114	BSU35620	sigA^*, sigD^*, sinR^*, slrR^*, yvrHB^*	6.4E-54	(7, 49, 52–54)	
yqiI	Y/KO	P54525	BSU24190	sigA*	5.50E-56	(55)	
yrvJ	Y/KO	O32041	BSU27580	sigH^	2.30E-44	similarity; lytC	
				Amidase_6 (PF12671)			
yhbB (ygaQ)	Y/KO	O31589	BSU08920	sigE^*	1.50E-39	uncharacterized	
yjcM	Y/KO	O31635	BSU11910	abrB*, sigD*	1.30E-23	uncharacterized	
				SpoIIP (PF07454)			
spoIIP	Y/KO	P37968	BSU25530	sigE^*, sigF^*, sigG^*, spoVT^*	9.70E-79	(56, 57)	amidase, DDEP
				Beta-lactamase (PF00144)			
pbpE	Y/KO	P32959	BSU34440	sigW^*	3.40E-61	(58)	not shown
pbpX	Y/KO	O31773	BSU16950	sigM^*, sigV^*, sigW^, sigX^*	7.90E-54	similarity; pbpE	
amiE (ybbE)	Y	O05213	BSU01670	murR*	7.40E-76	does not hydrolyse PG (14)	
				N-ACETYLGLUCOSAMIDASE/LYTIC TRANSGLYCOSYLASE			
				Glyco_hydro_3 (PF01915)			
nagZ (yzbA, ybbD)	Y	P40406	BSU01660	murR*	6.50E-131	does not hydrolyse PG (14)	
				Glyco_hydro_18 (PF00704)			
yaaH (sleL)	Y/KO	P37531	BSU00160	sigB^*, sigE^*, spoIIID^*	1.20E-26	cleaves small fragments (59–61)	
ydhD	Y/KO	O05495	BSU05710	sigE^*	9.60E-30	similarity; yaaH	
ykvQ	Y/KO	O31682	BSU13790	sigK^*	2.50E-23	similarity; yaaH	
yvbX	Y/KO	O32258	BSU34020		1.10E-33	similarity; yaaH	

				Glucosaminidase (PF01832)			
lytD (cwlG)	Y/KO	P39848	BSU35780	sigD^*, sigG^	3.30E-11	(7, 62, 63)	
lytG	Y/KO	O32083	BSU31120		6.80E-24	(64)	
				3D (PF06725)			
yabE	Y/KO	P37546	BSU00400	sigA*	1.80E-22	similarity; yuiC, yocH	probably LTG
yocH	Y/KO	O34669	BSU19210	abrB^*, sigA^*, spo0A^*, walR^*	3.50E-22	(28)	probably LTG
yuiC	Y/KO	O32108	BSU32070	codY^*, sigF^*	9.40E-21	(65)	probably LTG
yorM	N	O31901	BSU20330		7.90E-11	similarity; yuiC, yocH	probably LTG
				Hydrolase_2 (PF07486)			
cwlJ (ycbQ)	Y/KO	P42249	BSU02600	sigE^*, sigK^*, spoIIID^*	1.60E-18	(66)	probably LTG
sleB (ypeA)	Y/KO	P50739	BSU22930	sigG^*	1.20E-25	(67–69)	probably LTG
ykvT	Y/KO	O31685	BSU13820	walR*	5.80E-28	similarity; sleB, cwlJ	probably LTG
				SLT (PF01464)			
xkdO	Y/KO	P54334	BSU12680	xpf*	6.20E-20	similarity; cwlQ, cwlP	probably LTG
cwlQ (yjbJ)	Y/KO	O31608	BSU11570	sigD^*	1.70E-34	(70, 71)	LTG + muramidase
yqbO	Y/KO	P45931	BSU26030		5.70E-24	similarity; cwlQ, cwlP	probably LTG
cwlP (yomI)	Y/KO	O31976	BSU21350		1.80E-32	(72)	muramidase, DDEP
				Lysozyme_like (PF13702)			
yocA	Y/KO	O34636	BSU19130		4.30E-59	similarity; CwlT	probably muramidase
cwlT (yddH)	N	P96645	BSU04970	immR*	2.10E-42	(73)	muramidase, DLEP
				DPBB_1 (PF03330)			
ydjM (yzvA)	Y/KO	P40775	BSU06250	phoP*, walR*	1.00E-09	similarity; PaRlpA (74)	probably LTG
yoaJ (EXLX1)	N	O34918	BSU18630	fur*	2.90E-07	does not hydrolyse PG (75)	
				SpoIID (PF08486)			
spoIID (spoIIC)	Y/KO	P07372	BSU36750	sigE^*, spoIIID^*	1.30E-23	(56, 76)	LTG
lytB (cwbA)	Y	Q02113	BSU35630	sigA^*, sigD^*, sinR^*, slrR^*, yvrHB^*	3.90E-23	does not hydrolyse PG (77, 78)	
				YceG (PF02618)			
yrrL (mltG)	Y	O34758	BSU27370	spo0A^*	3.20E-90	similarity; EcMltG (20)	probably LTG
sweC (yqzC)	Y	O32023	BSU24940	spo0A^*	1.40E-07	similarity; EcMltG (20)	

PEPTIDASE							
DL-endopeptidase							
				NLPC/P60 (PF00877)			
cwlO (yzkA,yvcE)	Y	P40767	BSU34800	sigA*, walR*	4.40E-29	(16)	DLEP
cwlS (yojL)	Y/KO	O31852	BSU19410	abh^*, abrB^*, ccpA^*, sigD^*, sigH^*	4.70E-29	(11)	DLEP
lytE (papQ, cwlF)	Y	P54421	BSU09420	sigA^*, sigH^*, sigI^*, spo0A^*, walR^*	3.30E-28	(79, 80)	DLEP
lytF (cwlE, ydhD)	Y/KO	O07532	BSU09370	sigD^*, sinR^*, slrR^*	1.40E-28	(81, 82)	DLEP
pgdS (ywtD)	Y	P96740	BSU35860	sigD^*	1.00E-22	does not hydrolyze PG (83)	
ykfC	Y/KO	O35010	BSU12990	codY*	1.80E-29	(84)	DLEP
cwlT (yddH)		P96645	BSU04970	immR*	3.50E-34	(73)	
				Peptidase_M14 (PF00246)			
yqgT	Y/KO	P54497	BSU24830		2.70E-27	similarity to B. sphaericus EP1, (85)	probably DLEP
				Peptidase_C92 (PF05708)			
yycO	Y/KO	Q45607	BSU40280	sigK^*	5.0E-5		
LD-endopeptidase							
				Peptidase_M15 (PF01427)			
cwlK (ycdD)	Y/KO	O34360	BSU02810		3.10E-20	(86)	LDEP
				Peptidase_M23 (PF01551)			
lytH (yunA, yutA)	Y/KO	O32130	BSU32340	sigK^*	1.80E-21	(87)	LDEP
spoIIQ	Y	P71044	BSU36550	sigF^*	1.00E-24	no evidence for PG hydrolysis	
spoIVFA	Y	P26936	BSU27980	sigE^*, spoIIID^*	3.40E-12	no evidence for PG hydrolysis	
cwlP (yomI)	N	O31976	BSU21350		1.80E-27	(72)	muramidase, DD-EP

526 **Table 1: List of cell wall hydrolases in *Bacillus subtilis* identified using PHMMER.**

527 Cell wall hydrolases were identified via a PHMMR (13) search with default parameters
528 of the *B. subtilis* subsp. 168 and *B. subtilis* subsp. PY79 proteome for PFAM domains
529 associated with known cell wall hydrolases (Table S2). For each hydrolase, we report its
530 name (and any aliases), whether it is knocked out in the Δ40 strain (KO) or present in
531 PY79 (Y/N), its UniProt accession number, its locus tag, any reported regulons it is a
532 member of (^ indicates source Faria et al. 2016, * indicates source SubtiWiki), the
533 PHMMR search significance e-value, and any relevant references showing its
534 biochemical activity. Abbreviations: DLEP, D,L-endopeptidase; LDEP, L,D-
535 endopeptidase; LTG, lytic transglycosylase.
536

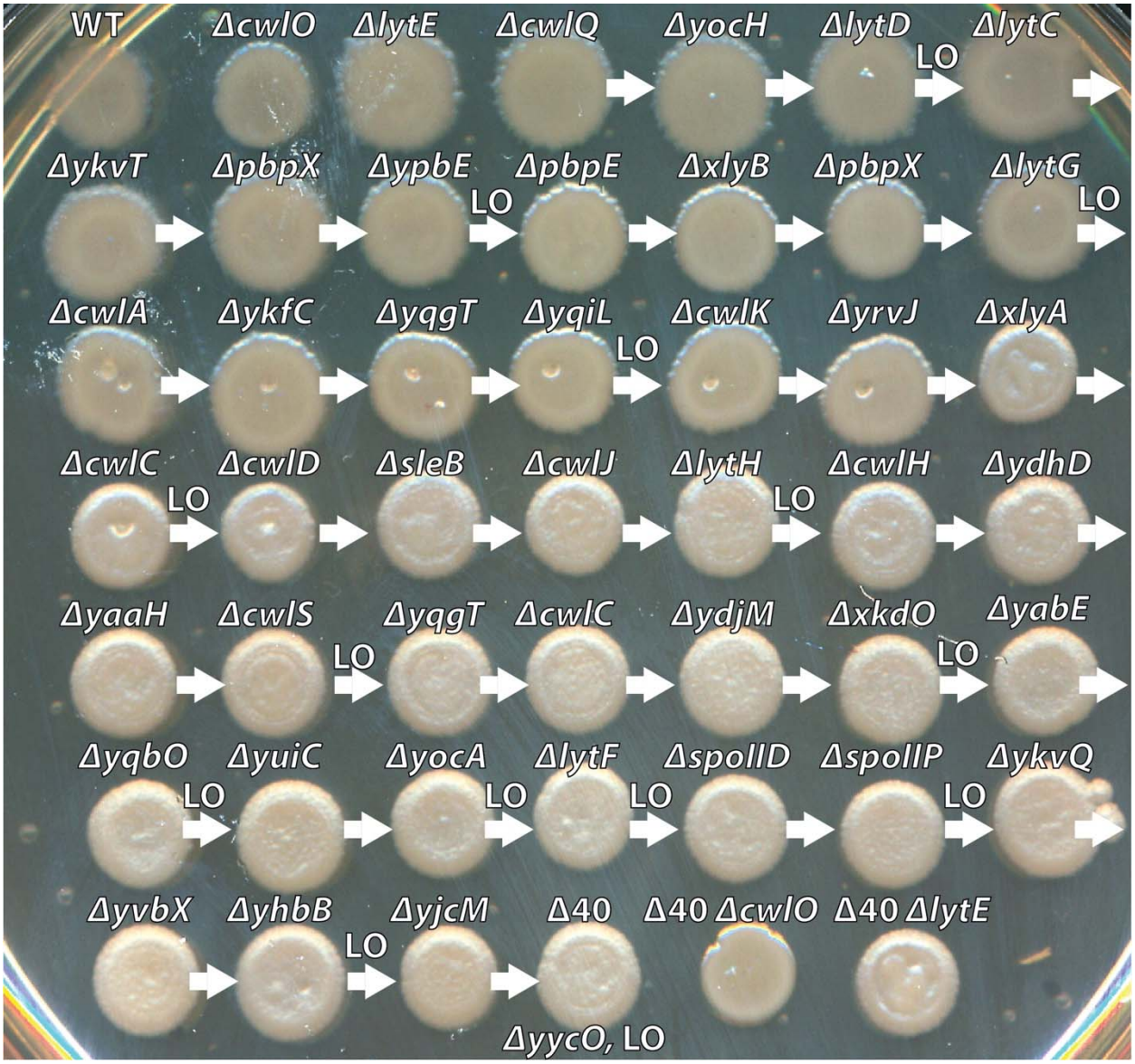
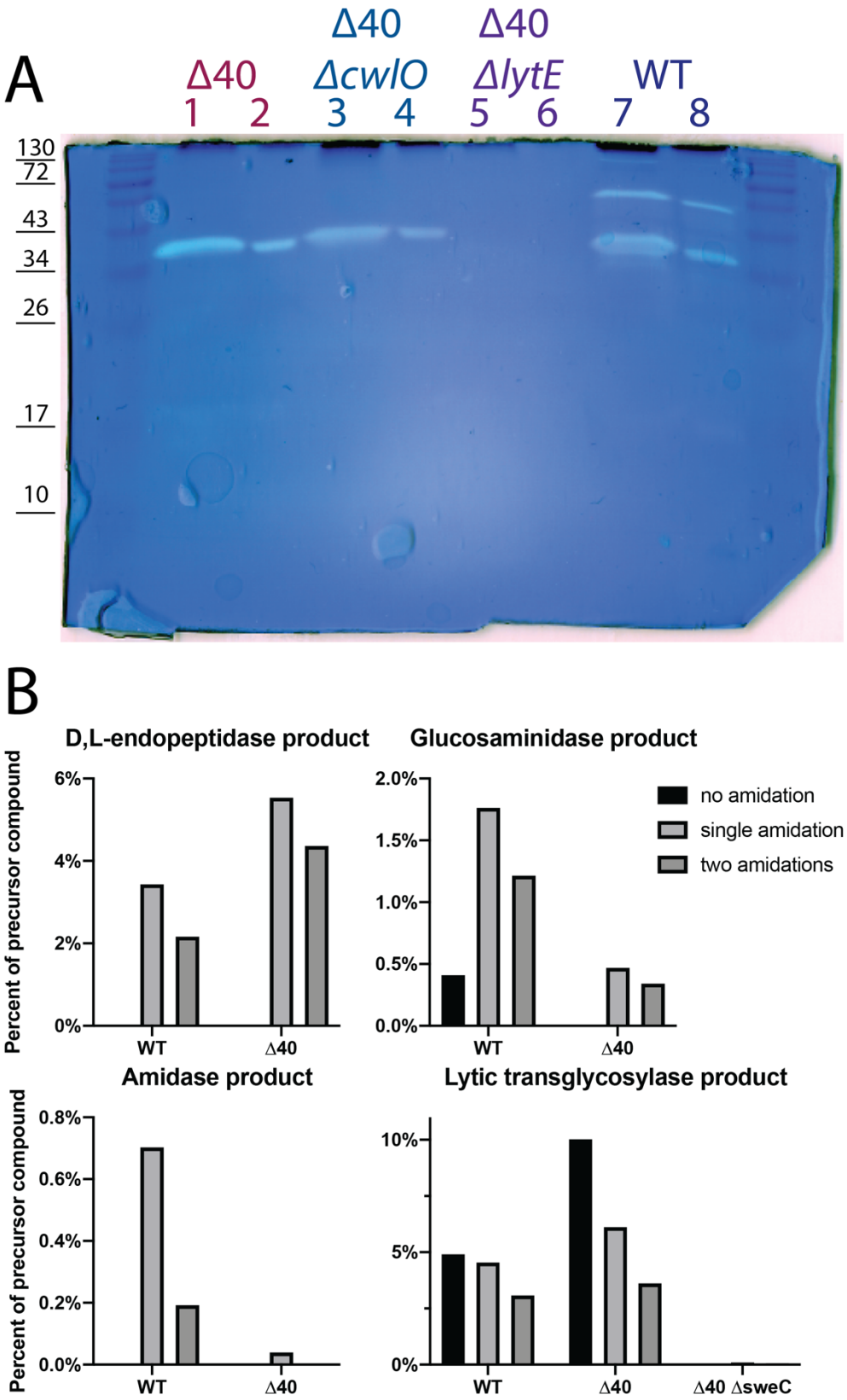
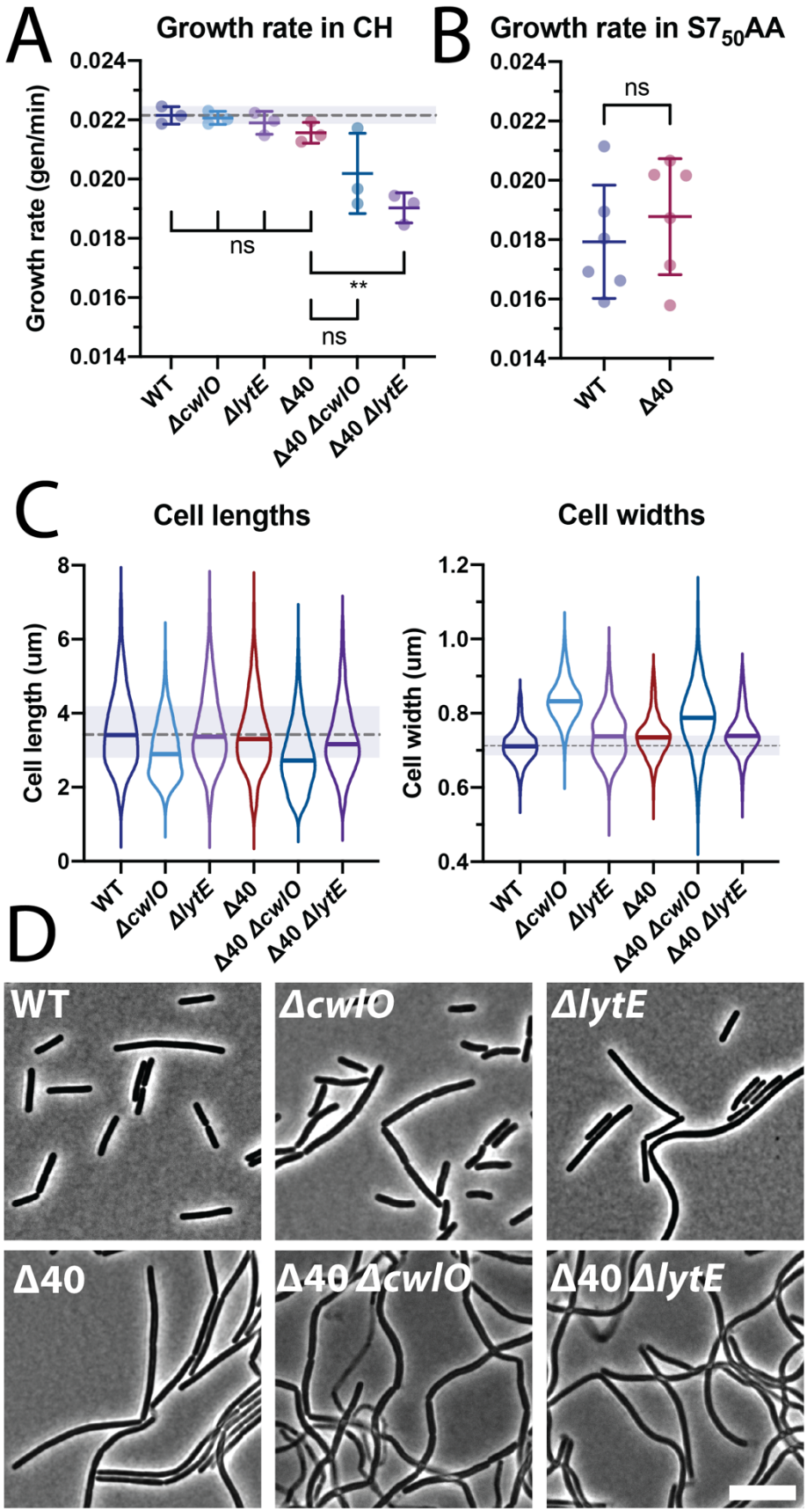


Figure 1: Construction of the Δ40 strain via sequential knockout and loopout. Colony morphology of each cloning intermediate for the Δ40 strain. WT cells were transformed with a series of resistance-cassette-marked knockouts (starting with Δ*cwlQ*). Periodically, antibiotic resistance cassettes were removed via Cre-loxP mediated loopout (indicated by LO). Arrows indicate sequential integrations (e.g., the strain indicated by Δ*yocH* contains Δ*yocH* and Δ*cwlQ*). Dense cell suspensions were spotted and incubated overnight to visualize colony morphology.


A
Δ40
Δ40
ΔcwlO
ΔlytE
WT
1 2 3 4 5 6 7 8
130
72
43
34
26
17
10
B
D,L-endopeptidase product
Glucosaminidase product
Percent of precursor compound
6%
4%
2%
0%
2.0%
1.5%
1.0%
0.5%
0.0%
no amidation
single amidation
two amidations
WT
Δ40
Amidase product
Lytic transglycosylase product
0.8%
0.6%
0.4%
0.2%
0.0%
10%
5%
0%
Δ40 ΔsweC

546 **Figure 2: The Δ40 strain has a substantially reduced cell wall hydrolytic**
547 **complement**
548 **A: Only LytE activity is visible by zymography in the Δ40 strain.** Zymography
549 (renaturing SDS-PAGE gel using purified *B. subtilis* cell walls as substrate) was
550 performed as described in Methods. The activity of cell wall hydrolases causes clearing
551 of the gel. In each pair of lanes, the first was loaded with 20 μL of cell wall binding
552 protein extract, and the second with 5 μL. Several major and minor bands of cell wall
553 hydrolytic activity are visible in the wild type strain (strain PY79, lanes 7 and 8). Only
554 one band of cell wall hydrolytic activity is visible in the Δ40 mutant (strain bSW431,
555 lanes 1 and 2). This band is still visible when *cwlO* is knocked out in the Δ40 strain (Δ40
556 Δ*cwlO*, strain bSW433, lanes 3 and 4) but disappears in the reciprocal *lytE* knockout
557 (Δ40 Δ*lytE*, strain bSW435, lanes 5 and 6). Thus, this band represents LytE activity.
558 **B: Identification of cell wall hydrolase products in WT and Δ40 cells by**
559 **peptidoglycan profiling.** Purified cell walls were digested to yield soluble
560 muropeptides which were separated and characterized via HPLC-MS (see Methods for
561 details). The abundance of each cell wall hydrolase product was normalized by the
562 abundance of its precursor compound; each product contains two mDAP residues, and
563 each mDAP can be amidated, so these species were compared separately (for
564 example, the abundance of singly amidated glucosaminidase product was divided by
565 the abundance of singly amidated crosslinked PG, its immediate biochemical
566 precursor). D,L-endopeptidase products are still present as expected, because the
567 strain retains the D,L-endopeptidases LytE and CwlO. Amidase products are largely
568 absent. Glucosaminidase products are substantially reduced in abundance. Lytic

569 transglycosylase products are still present in the Δ40 strain but disappear if *sweC* is
570 additionally knocked out. Thus, the Δ40 strain is deficient for several major classes of
571 cell wall hydrolase activity. Strains used: PY79, WT; bSW431, Δ40; bSW537, Δ40
572 Δ*sweC*.


A
Growth rate in CH
Growth rate (gen/min)
0.024
0.022
0.020
0.018
0.016
0.014
ns
**
ns
WT
ΔcwlO
ΔlytE
Δ40
Δ40 ΔcwlO
Δ40 ΔlytE
B
Growth rate in S7$_{50}$AA
ns
WT
Δ40
C
Cell lengths
Cell length (um)
8
6
4
2
0
Cell widths
Cell width (um)
1.2
1.0
0.8
0.6
0.4
D
WT
ΔcwlO
ΔlytE
Δ40
Δ40 ΔcwlO
Δ40 ΔlytE

573

574 **Figure 3: The Δ40 strain grows at a similar rate as WT cells and has mild shape**
575 **defects.**
576 **A: The Δ40 strain has a similar growth rate to WT in rich media.** Cultures were
577 grown in CH media at 37˚C to an OD of ~0.3-0.5, diluted to an OD of 0.05, and samples
578 were collected every 6 minutes for 1.5 hrs (~3 doublings). OD_{600} vs time plots were fit to
579 a single exponential to obtain the growth rate. Each point represents the doubling time
580 from a single experiment, and solid lines show mean and standard deviation. The dotted
581 line shows the mean WT growth rate, for comparison. While Δ40 has a similar growth
582 rate to WT, *lytE* and *cwlO* knockouts grow more slowly in this background than in a WT
583 background. Strains used: PY79, WT; bSW23, Δ*cwlO*; bSW295, Δ*lytE*; bSW431, Δ40;
584 bSW433, Δ40 Δ*cwlO*; bSW435, Δ40 Δ*lytE*.
585 **B: The Δ40 strain has a similar growth rate to WT in minimal media.** Cultures were
586 grown in $S7_{50}$AA media at 37˚C. Samples were collected and data was analyzed as in
587 (A). Strains used: PY79, WT; bSW431, Δ40.
588 **C: Cell lengths (left) and widths (right) in hydrolase mutants.** Cells were labeled
589 with membrane stain and imaged by epifluorescence microscopy. Cell dimensions were
590 measured from these images using Morphometrics. Solid lines in violins show medians.
591 Dashed line outside violins shows WT median for comparison. Shaded region outside
592 violins shows WT quartiles. Strains used: PY79, WT; bSW23, Δ*cwlO*; bSW295, Δ*lytE*;
593 bSW431, Δ40; bSW433, Δ40 Δ*cwlO*; bSW435, Δ40 Δ*lytE*.
594 **D: Representative phase contrast images of hydrolase mutant strains.** Δ40 cells
595 primarily form long chains, Δ40 Δ*cwlO* cells have variable widths, and Δ40 Δ*lytE* cells
596 sometimes have phase-light, lysed cells still attached to their poles (see Figure S2 for

TEM images). Both Δ40 Δ*cwlO* and Δ40 Δ*lytE* have a population of phase-light, lysed cells. Scale bar is 10 µm.

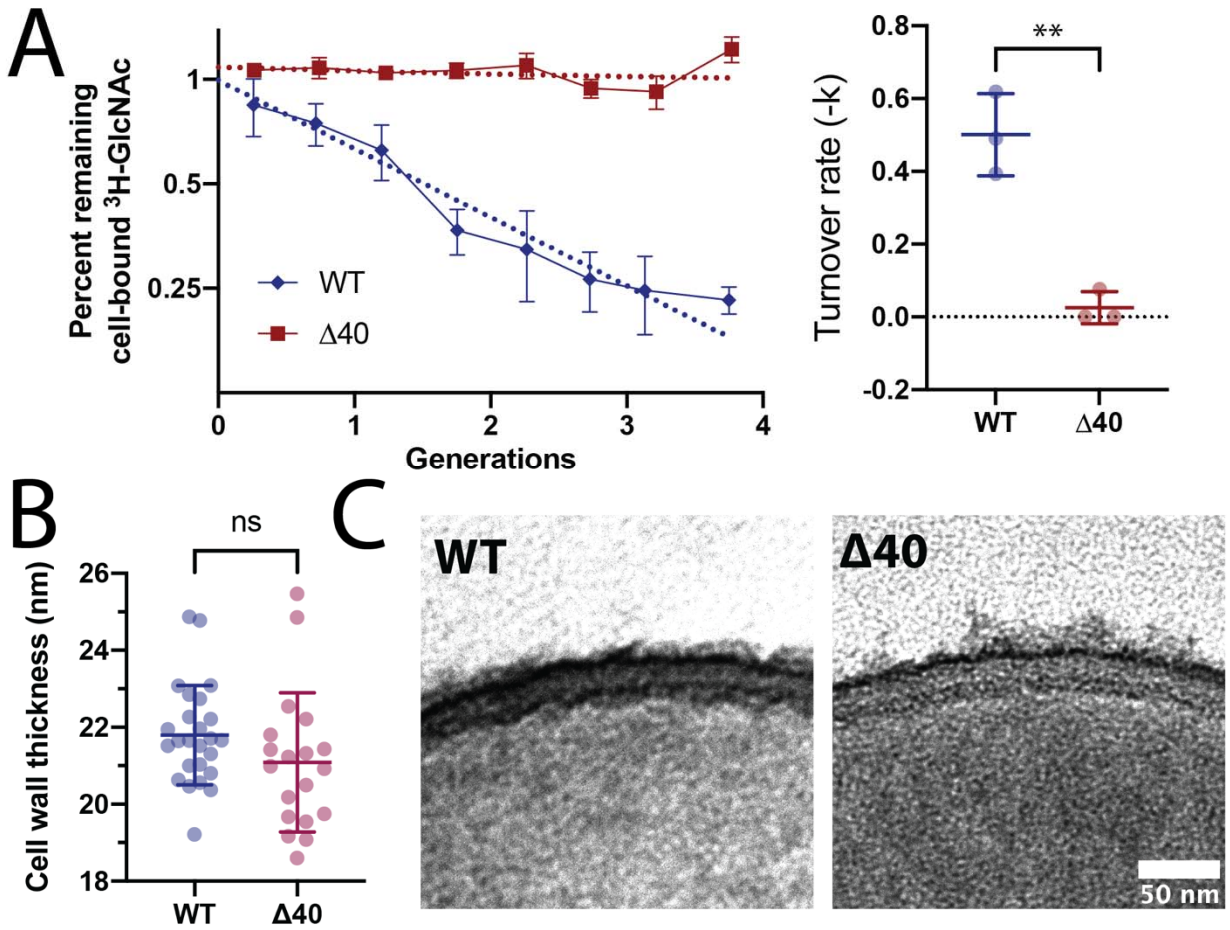


Figure 4: The Δ40 strain has no increase in cell wall thickness and does not turn over cell wall.

A: Cell wall turnover rate is negligible in the Δ40 strain. *Left*: Pulse-chase radiolabel measurements were used to determine the cell wall turnover rate. Cells were labeled with H^3-GlcNAc, which incorporates into the cell wall. The ^{3}H-GlcNAc was then washed out and radioactivity was subsequently measured for 3 generations. A decrease in radioactivity indicates that material is being removed from the cell wall, e.g. that cell wall is turning over. Each experiment was replicated at least 3 times. Dotted lines show

608 single exponential fit to mean data. *Right*: Single exponential fits to each experiment at
609 left. Each point represents the time constant (-k) obtained from a fit to a single
610 experiment. Error bars show SD. The Δ40 turnover rate is not significantly different from
611 zero (one sample t test, p=0.4837). Strains used: PY79, WT; bSW431, Δ40.
612 **B: Cell wall thickness in the Δ40 strain is similar to WT.** Cell wall thickness was
613 measured via transmission electron microscopy as described in Methods. Briefly,
614 exponentially growing cells were fixed, osmicated, stained with uranyl acetate,
615 embedded in Embed 812, sectioned, and imaged without additional staining. Each point
616 is the mean cell wall thickness measured for a single cell. Error bars show SD. Strains
617 used: PY79, WT; bSW431, Δ40.
618 **C: Representative images of cell wall thickness.** Representative images of cell wall
619 thickness analyzed in C. Strains used: PY79, WT; bSW431, Δ40. Scale bar is 50 nm.
620

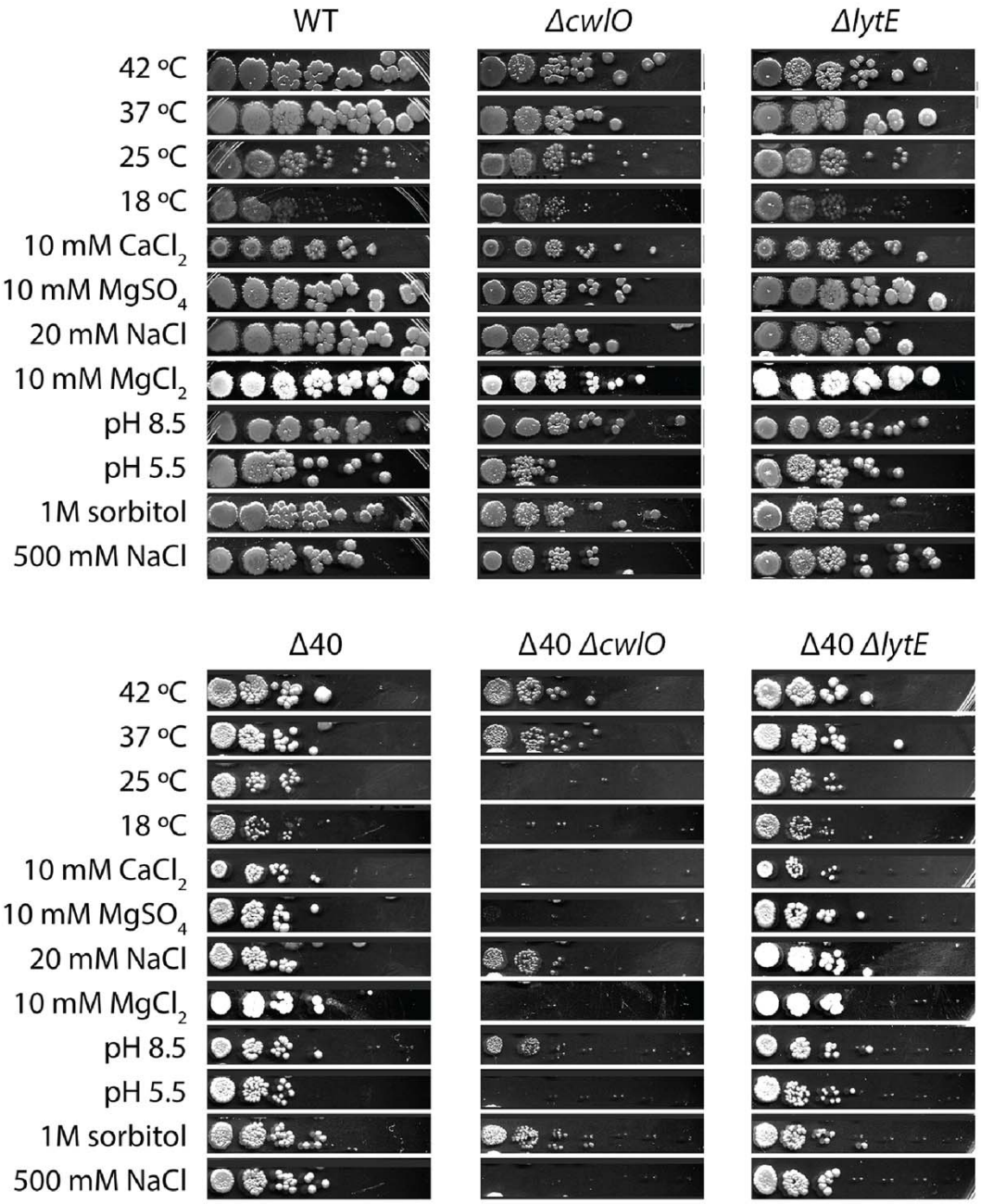


Figure 5: The Δ40 strain has similar viability to WT in a range of stress conditions, but Δ40 Δ*cwlO* is sensitive to ionic, cold, and low pH stress. Spot dilution assays of different strains under various stress conditions. Cultures of each strain were plated in a 1:10 dilution series onto LB plates containing various stressors and grown overnight at the specified temperature, or at 37˚C if not indicated. Most conditions supported normal

627 growth, but growth of the Δ40 Δ*cwlO* strain was inhibited at 25˚C, pH 5.5, or with the

628 addition of 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM $CaCl_2$, or 300 mM NaCl. Strains used:

629 PY79, WT; bSW23, Δ*cwlO*; bSW295, Δ*lytE*; bSW431, Δ40; bSW433, Δ40 Δ*cwlO*;

630 bSW435, Δ40 Δ*lytE*.

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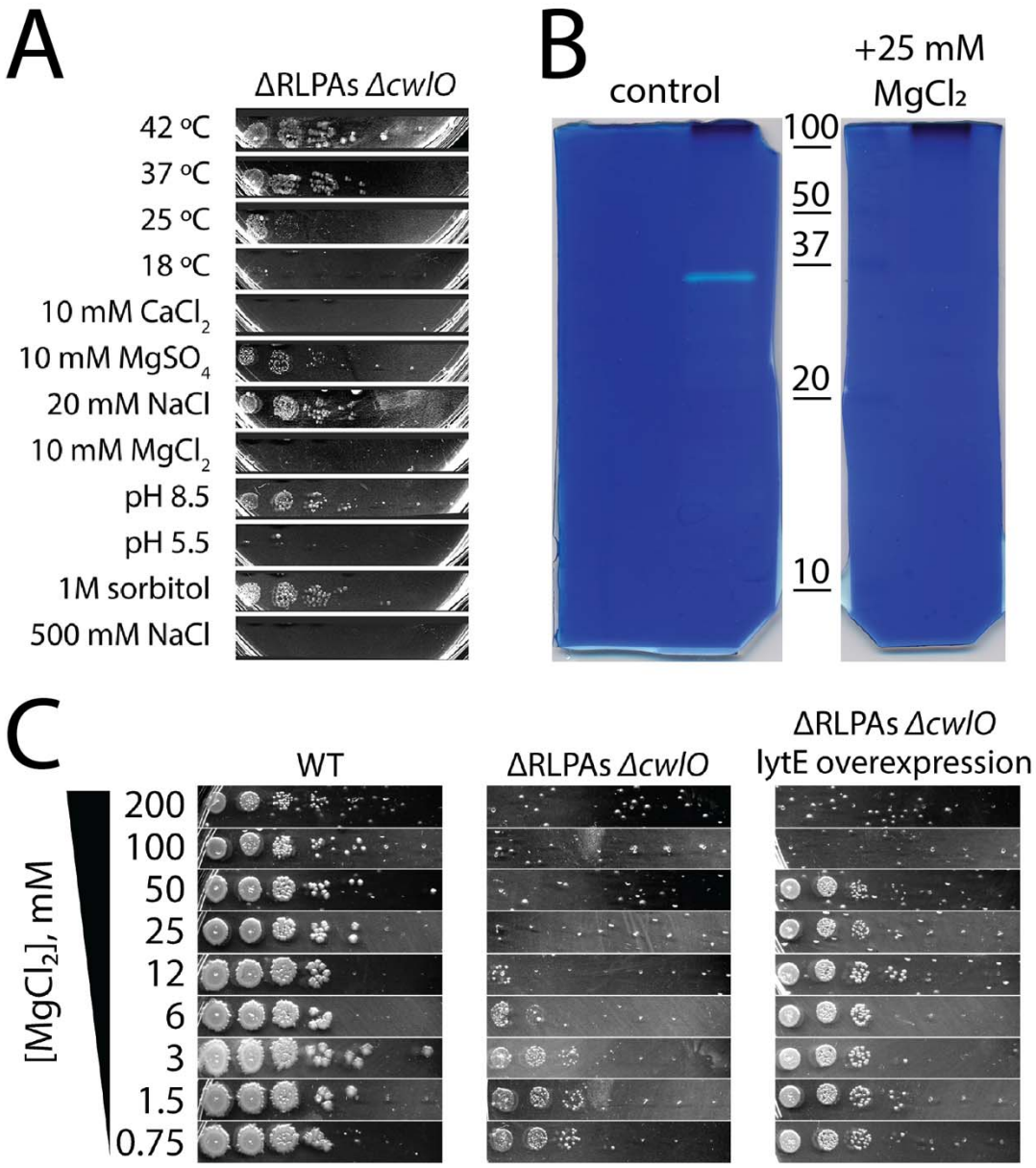

634

635 **Figure 6: Three uncharacterized RlpA-like proteins stimulate LytE activity in the**

636 **presence of divalent cations.**

637 **A: The removal of three RlpA-like proteins makes Δ*cwlO* cells stress-sensitive.**

638 Spot dilution assays were performed as in Figure 4. Δ*yabE* Δ*yocH* Δ*ydjM* (ΔRLPAs)

639 Δ*cwlO* showed the same stress sensitivity profile as Δ40 Δ*cwlO*, except that 10 mM

640 $MgSO_4$ and 25˚C only partially inhibited growth. Strain used: bSW490, Δ*cwlO* Δ*yabE*

641 Δ*yocH* Δ*ydjM*.

642 **B: Mg^{2+} directly inhibits LytE activity.** Zymography was performed on Δ40 Δ*cwlO*

643 cells as in Figure 1A, but with the addition of the indicated concentration of $MgCl_2$ to the

644 renaturation buffer. 25 mM $MgCl_2$ almost completely inhibited the activity of LytE. Strain

645 used: bSW433, Δ40 Δ*cwlO.*

646 **C: LytE overexpression rescues Mg^{2+} sensitivity in the ΔRLPAs Δ*cwlO***

647 **background.** Spot dilutions were performed as in Figure 5A, with the indicated

648 concentration of $MgCl_2$ and the addition of 1 mM IPTG to drive LytE overexpression.

649 Strains used: PY79, WT; (ΔRLPAs) Δ*cwlO*, bSW490, Δ*cwlO* Δ*yabE* Δ*yocH* Δ*ydjM*;

650 (ΔRLPAs) Δ*cwlO* lytE overexpression, bSW519, Δ*cwlO* Δ*yabE* Δ*yocH* Δ*ydjM*

651 *amyE::pHyperSpank-lytE*.

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