Title: Induction of AmpC-mediated β-lactam resistance requires a single lytic transglycosylase in *Agrobacterium tumefaciens*

Authors:

Wanda M. Figueroa-Cuilan[1*], Matthew Howell[1&], Christopher Richards[1], Amelia Randich[1^], Akhilesh K. Yadav[2$], Felipe Cava[2], and Pamela J.B. Brown[1#]

Affiliations:

[1]Division of Biological Sciences, University of Missouri-Columbia, Columbia, Missouri, 65202

[2]Laboratory for Molecular Infection Medicine, Department of Molecular Biology, Umeå University, Umeå, Sweden

Running title: AmpC-mediated β-lactam resistance of *A. tumefaciens*

[#]Corresponding author:

brownpb@missouri.edu

(573) 884-0384

Present addresses:

*Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

[&]Department of Biology, Westminster College, Fulton, Missouri 65251

[$]Analytical Chemistry Division, CSIR-Indian Institute of Toxicology Research, Lucknow-226001, India

[^]Department of Biology, The University of Scranton, Scranton, Pennsylvania,18510

ABSTRACT

The remarkable ability of *Agrobacterium tumefaciens* to transfer DNA to plant cells has allowed the generation of important transgenic crops. One challenge of *A. tumefaciens*-mediated transformation is eliminating the bacteria after plant transformation to prevent detrimental effects to plants and the release of engineered bacteria to the environment. Here we use a reverse genetic approach to identify genes involved in ampicillin resistance with the goal of utilizing these antibiotic-sensitive strains for plant transformations. We show that treating *A. tumefaciens* C58 with ampicillin led to increased β-lactamase production, a response dependent on the broad-spectrum β-lactamase AmpC and its transcription factor AmpR. Loss of the putative *ampD* orthologue, *atu2113,* led to constitutive production of AmpC-dependent β-lactamase activity and ampicillin resistance. Finally, one cell wall remodeling enzyme, MltB3, was necessary for the AmpC-dependent β-lactamase activity and its loss elicited ampicillin and carbenicillin sensitivity in the *A. tumefaciens* C58 and GV3101 strains. Furthermore, GV3101 *ΔmltB3* transforms plants with comparable efficiency to wildtype but can be cleared with sub-lethal concentrations of ampicillin. The functional characterization of the genes involved in the inducible ampicillin resistance pathway of *A. tumefaciens* constitutes a major step forward in efforts to reduce the intrinsic antibiotic resistance of this bacterium.

IMPORTANCE

Agrobacterium tumefaciens, a significant biotechnological tool for transgenic plant lines, is highly resistant to a wide variety of antibiotics, posing challenges for various applications. One challenge is the efficient elimination of *A. tumefaciens* from transformed plant tissue without

using levels of antibiotics that are toxic to the plants. Here, we present the functional characterization of genes involved in β-lactam resistance in *A. tumefaciens.* Knowledge about proteins that promote or inhibit β-lactam resistance will enable the development of strains to improve the efficiency of *Agrobacterium*-mediated plant genetic transformations. Effective removal of *Agrobacterium* from transformed plant tissue has the potential to maximize crop yield and food production, improving the outlook for global food security.

Keywords

Antibiotic resistance, *Agrobacterium tumefaciens*, β-lactamases, anhydro amidases, membrane-bound lytic transglycosylases, ampicillin, plant transformation

INTRODUCTION

Rhizobiaceae is a family of bacteria that include soil-dwelling and plant associated bacteria. While some species of this family have the ability to establish symbiotic relationships with plants, others are pathogenic such as the genus *Agrobacterium*. Members of this genus are responsible for a number of diseases, including the cane gall disease (*Agrobacterium rubi*), hairy root disease (*Agrobacterium rhizogenes*), crown gall disease of grapes (*Agrobacterium vitis*), and crown gall disease to flowering plants and woody shrubs (*Agrobacterium tumefaciens*) (1–5). In nature, *A. tumefaciens* causes crown gall by adhering to wounded plants and injecting a section of a bacterial DNA plasmid (Transfer DNA [T-DNA]) that integrates into the plant chromosomes (1–3, 6–11). The T-DNA segment encodes for metabolic and hormone-producing enzymes that hijack the plant to produce custom energy sources that only *Agrobacterium* can use (9, 10). The increased abundance of energy

71 sources, such as phytohormones, within plant cells leads to their over-proliferation and
72 eventual gall formation (6). Gall formation on plants and trees leads to crop damage, and
73 significant economic losses have been attributed to this issue every year (2, 3).
74 While the genus *Agrobacterium* exhibits pathogenicity against plants, the natural ability of
75 *Agrobacterium* to transfer DNA to plants has been exploited to produce transgenic plants
76 through genetic engineering (6, 9, 11–13). However, one challenge for *A. tumefaciens*-
77 mediated plant transformations is the elimination of the bacteria from the transformed plant
78 tissue. Elimination of recombinant *A. tumefaciens* from plant tissues is crucial to prevent
79 detrimental effects to plants and to reduce the risk of releasing engineered bacteria into the
80 environment (14–16). β-lactam antibiotics are frequently applied during plant transformations
81 to eliminate *A. tumefaciens* from plant tissues and are preferred over other classes of
82 antibiotics (17–19). This is because β-lactams target cell wall synthesis, a process unique to
83 bacteria, and therefore are less toxic to eukaryotic plant cells than antibiotics that inhibit
84 protein or nucleic acid synthesis (20, 21). However, the natural resistance of *A. tumefaciens*
85 to β-lactams can be only overcome with toxic levels (~200-1000 mg/L), which has been
86 shown to cause embryogenic tissue necrosis, or to affect plant tissue growth and regeneration
87 rates in a wide variety of plants (17, 18, 22–28). Moreover, depending on the concentration
88 and class of β-lactam, clearing *Agrobacterium* from embryos can take up to 60 days, yet, in
89 some cases, complete elimination of *A. tumefaciens* is not achieved (29). Thus, currently,
90 there is a need for the identification and understanding of regulatory pathways and enzymes
91 involved in β-lactam resistance in *A. tumefaciens.* Functional characterization of bacterial
92 enzymes involved in β-lactam resistance will permit the development of tools that could

93 improve the efficiency of plant genetic transformations, and therefore maximize crop yields
94 and food production.
95 β-lactam antibiotics target the bacterial cell wall by inhibiting the activity of Penicillin
96 Binding Proteins (PBPs); the enzymes involved in the synthesis of the bacterial peptidoglycan
97 (PG) cell wall (30–38). The bacterial PG cell wall is an essential polymer consisting of
98 alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) sugars
99 crosslinked through peptide bridges (39–44). Because the PG cell wall is a covalently-
100 enclosed polymer, its expansion not only requires cell wall synthesis but also remodeling. Cell
101 wall remodeling is mediated by PG degradation enzymes such as the lytic transglycosylases
102 (LT) (45–48). To allow cell wall expansion, LTs cleave between the Mur*N*Ac and Glc*N*Ac
103 sugar strands resulting in the formation of 1,6-anhydroMurNAc Glc*N*Ac on glycan strands and
104 the liberation of 1,6-anhydromuropeptides (AnhMP) cell wall degradation fragments. The
105 liberated AnhMP fragments are transported to the bacterial cytoplasm for cell wall recycling
106 (42, 49, 50). In the cytoplasm, the recycling of AnhMP fragments keeps the concentration of
107 these products low (51, 52). However, cell wall stressors such as treatment with β-lactam
108 antibiotics, or mutations that inhibit the cell wall recycling pathway result in the accumulation
109 of AnhMP cell wall degradation fragments and derepression of β-lactamases (34, 50, 53–56).
110 In bacterial organisms including *Pseudomonas aeruginosa* and *Enterobacter cloaceae,* the
111 AnhMP cell wall degradation fragments are transcriptional activators of inducible β-
112 lactamases, which are enzymes that cleave and inactivate β-lactam antibiotics (57–63).
113 In the soil environment, many soil microorganisms produce antibiotics to compete for
114 survival, selecting for intrinsic resistance pathways in soil pathogens. For example, the
115 genomes of many soil bacteria contain β-lactamases, such as the cephalosporinase AmpC

116 (34, 64). As a cephalosporinase, AmpC is known to destroy β-lactam antibiotics including
117 monobactams, cephalosporins, and penicillins (34). The AmpC consensus protein sequence
118 consists of a signal sequence for periplasmic transport and a β-lactamase catalytic domain
119 **(Fig. S1A)**. The regulation of AmpC expression varies across bacterial organisms. In
120 *Escherichia coli*, AmpC is a non-inducible β-lactamase that is expressed at low levels and
121 regulated by a promoter and a growth rate-dependent attenuator mechanism (65–67). In
122 contrast, in *P. aeruginosa* and other enterobacteria, AmpC is normally expressed at low
123 levels, but is inducible and can be derepressed during exposure to β-lactams (34, 60, 68). In
124 these cases, AmpC expression is regulated by AmpR, a LysR-type transcriptional regulator
125 found in an operon with AmpC (60). AmpR consists of two domains; a Helix-Turn-Helix DNA-
126 binding domain (DBD) that binds the intergenic region between AmpC and AmpR, and a LysR
127 effector-binding domain (EBD), which contains the regulatory region of AmpR (**Fig. 1A, Fig.**
128 **S1A**) (57). AmpR is a bifunctional transcriptional regulator that controls both the activation
129 and repression of AmpC (60). The induction mechanism of AmpC by AmpR in response to β-
130 lactams is linked to bacterial cell wall synthesis, remodeling, and recycling (34, 50, 53, 57, 62,
131 69). Indeed, the AnhMur cell wall degradation fragments released by LTs during cell growth,
132 are AmpR-activating molecules. In contrast, cell wall building blocks such as UDP-Glc*N*Ac
133 Mur*N*Ac pentapeptides bind to AmpR and repress *ampC* transcription **(Fig. 1A)**. A block in
134 bacterial cell wall synthesis after exposure to β-lactams results in accumulation of AnhMur cell
135 wall degradation products in the bacterial cytoplasm, displacement of the AmpR repressor
136 UDP-Glc*N*Ac Mur*N*Ac pentapeptide, activation of AmpR, and transcription of *ampC* **(Fig. 1A).**
137 The genus *Agrobacterium* is naturally resistant to β-lactams and the molecular basis for
138 this resistance is poorly understood. A study that screened for β-lactamase production in *A.*

139 *tumefaciens* detected cephalosporinase production (70) and the *A. tumefaciens* genome
140 encodes one putative cephalosporinase, an AmpC homolog (*atu3077)*. In addition, similar to
141 *Enterobacteriaceae* and *Pseudomonadales, ampC* is found in an operon with *ampR* in most
142 *Agrobacterium* genomes **(Fig. 1A)**. Here, we sought to determine if AmpC is a functional β-
143 lactamase*,* if AmpC is inducible, and if the natural ampicillin resistance observed in *A.*
144 *tumefaciens* is dependent on AmpC.

145 We present a functional characterization of proteins involved in intrinsic ampicillin
146 resistance in *A. tumefaciens.* We found that AmpR is required for AmpC-dependent β-
147 lactamase activity and that loss of the anhydro-amidase AmpD (*atu2113*, misannotated as an
148 AmiD homolog in the genome (71)) led to increased resistance to ampicillin, a process
149 dependent on AmpC. We suggest that AmpD is required for proper recycling of cell wall
150 degradation products and its loss results in the accumulation of cell wall degradation products
151 and activation of AmpC by AmpR. Furthermore, we found that deletion of a single LT, the
152 membrane-bound lytic transglycosylase B3 (MltB3), is necessary for AmpC-dependent β-
153 lactamase activity and its loss led to ampicillin sensitivity in the *A. tumefaciens* C58 and
154 GV3101 strains. Finally, transformation of *Arabidopsis thaliana* utilizing a *ΔmltB3* GV3101
155 strain required significantly lower concentrations of ampicillin while exhibiting similar WT
156 transformation efficiency. This work underscores the significance of understanding the β-
157 lactam resistance pathway of *A. tumefaciens* with the aim of expanding tools for the *A.*
158 *tumefaciens*-mediated transformations.

159

160 **RESULTS AND DISCUSSION**

161 **The AmpC-AmpR operon is responsible for inducible ampicillin resistance in *A.***

162 ***tumefaciens* C58**

163 To begin our characterization, we first assessed the susceptibility of *A. tumefaciens* to
164 different concentrations of ampicillin around the minimum inhibitory concentration (MIC)
165 reported for *A. tumefaciens* on solid and liquid media (72). We found that cells grown in LB
166 with 25 or 50 µg/mL ampicillin (AMP 25 or AMP 50, respectively) for 24 h displayed slow
167 growth in liquid medium in comparison to cells grown in LB without ampicillin (No AMP) **(Fig.**
168 **1B)**. To better understand the cause of this growth defect, we performed phase-contrast
169 microscopy of cells treated with AMP 25 for 2 h **(Fig. S1B)**. We found that treatment with AMP
170 25 causes a significant increase in the median cell length **(Fig. S1C)** and 23.5% of were
171 undergoing cell lysis **(Fig. 1C, Fig. S1B)**, confirming that the bactericidal effect of AMP 25 on
172 WT *A. tumefaciens* is the cause of the overall decrease in optical density. Similarly, WT cells
173 grown on AMP 25 solid medium for 36 h have a viability defect in comparison to WT cells
174 grown in LB No AMP **(Fig. 1D)**. The increased sensitivity of WT *A. tumefaciens* to ampicillin in
175 the presence of sulbactam, a broad spectrum β-lactamase inhibitor, suggests that β-
176 lactamase production is responsible for the observed ampicillin resistance **(Fig. 1E)**. Finally,
177 to determine if β-lactamase production is induced, we treated WT cells with AMP 25 for 2 h,
178 generated whole cell lysates, and performed nitrocefin assays on total protein content **(Fig.**
179 **1F)**. Nitrocefin is a chromogenic substrate related to the cephalosporins that undergoes a
180 color change when is hydrolyzed by β-lactamases (73). After treatment of WT cells with AMP
181 25 for 2 h, the activity of β-lactamases is readily detected in nitrocefin assays **(Fig. 1F).**
182 Together, these results suggest that *A. tumefaciens* C58 β-lactamase production is induced in
183 the presence of β-lactams such as ampicillin. To assess the contributions of putative enzymes
184 involved in ampicillin resistance, we employed a reverse genetics approach (74).

185 The *ampC* ortholog of *A. tumefaciens* C58 (*atu3077)* encodes the only putative
186 inducible β-lactamase in the genome of *A. tumefaciens* C58 (34)*. ampC* is syntenic with
187 *ampR* and the *A. tumefaciens* AmpC and AmpR proteins are 74.7% and 85.9% similar to their
188 respective orthologs from *P. aeruginosa***.** To determine the role of AmpC, we deleted *ampC*
189 (*atu3077*) from the *A. tumefaciens* C58 genome. Deletion of *ampC* does not impact cell
190 growth and cell viability **(Fig. 1B, 1D)** or cell morphology **(Fig. S1B)**, beyond a slight increase
191 in cell length **(Fig. S1C).** To pinpoint the contribution of *ampC* to ampicillin resistance, we
192 assessed the growth dynamics of *ΔampC* cells in the presence of AMP in liquid **(Fig. 1B)**.
193 *ΔampC* cells treated with AMP 25 or AMP 50 for 24 h showed a severe growth defect
194 indicating that *ΔampC* is required for ampicillin resistance. *ΔampC* cells treated with AMP 25
195 exhibited cell division defects (34.8%) and cell lysis (15.8%) **(Fig. 1C, Fig. S1B).** The
196 increased cell length of *ΔampC* treated with AMP 25 is likely due to the observed cell division
197 defects **(Fig. S1B-C, Fig 1C).**
198 To confirm that ampicillin resistance is mediated by the AmpC β-lactamase, we used
199 the disc diffusion assay to compare resistance levels to ampicillin in the presence and
200 absence of the broad-spectrum β-lactamase inhibitor, sulbactam **(Fig. 1E)**. As expected,
201 *ΔampC* led to increased sensitivity to ampicillin and the presence of sulbactam did not result
202 in large increases in the zone of growth inhibition **(Fig. 1E).** Furthermore, monitoring the rates
203 of nitrocefin hydrolysis showed that production of β-lactamase is readily detected in WT cells
204 treated with AMP 25 but is undetectable in *ΔampC* **(Fig. 1F)***.* Together, these observations
205 suggest that the natural resistance to ampicillin depends on the presence of AmpC, which
206 functions as an inducible β-lactamase.
207 We hypothesized that if transcription of *ampC* is strictly controlled by AmpR, deletion of

208 *ampR* should mimic deletion of *ampC.* To test this hypothesis, we deleted *ampR* (*atu3078*)
209 from the genome of *A. tumefaciens* C58. Deletion of *ampR* led to normal cell growth **(Fig.**
210 **1B)**, morphology **(Fig. S1B),** cell length **(Fig. S1C)** and cell viability **(Fig. 1D).** Low
211 concentrations of ampicillin in either liquid or solid medium was lethal to *ΔampR* and resulted
212 in similar cell division defects and cell lysis as *ΔampC* **(Fig. 1B-C, Fig. S1B)**. Like *ΔampC,*
213 *ΔampR* is sensitive to ampicillin treatment **(Fig. 1D-E)** and fails to produce detectable β-
214 lactamase activity when treated with AMP 25 **(Fig. 1F, Fig. S1D).** These results suggest that
215 AmpR is necessary for induction of the AmpC β-lactamase in the presence of ampicillin.
216
217 **Loss of AmpD derepresses β-lactamases in *A. tumefaciens* C58.**
218 The finding that AmpC and AmpR are necessary for ampicillin resistance in *A. tumefaciens*
219 C58 led us to explore how the pools of different cell wall fragments would alter the AmpC-
220 mediated β-lactamase induction. Similar to exposure to β-lactams, loss of cell wall recycling
221 amidases has been shown to increase the AmpR-activating fragments (cell wall degradation
222 fragments) in the cytoplasm, resulting in the transcriptional derepression of *ampC* and β-
223 lactam resistance (48, 75–78). The genome of *A. tumefaciens* contains one 1,6-anhydro
224 amidase ortholog, *atu2113*, reannotated as AmpD. The domain organization of AmpD
225 consists of the Amidase_2 (Ami_2) catalytic domain and a PG-binding domain (PBD) that
226 facilitates the interaction with cell wall products **(Fig. S2A)** (45, 48). *A. tumefaciens* AmpD
227 exhibits 64.4% sequence similarity to AmpDh2, one of three broad-spectrum 1,6-anhydro
228 amidase AmpDh paralogs found in *Pseudomonas aeruginosa* (54, 56, 77, 79).
229 Given that in *A. tumefaciens* ampicillin triggers the AmpC-dependent production of β-
230 lactamases, we hypothesized that if AmpD was an anhydro amidase involved in the recycling

231 of cell wall degradation fragments, its loss should result in increased AmpR-activating
232 fragments in the cytoplasm, β-lactamase induction, and ampicillin resistance. First, we found
233 that *ΔampD* cells exhibit normal cell viability **(Fig. 2A)**, cell growth **(Fig. 2B),** and morphology
234 **(Fig. S2B-C).** *ΔampD* is highly resistant to ampicillin **(Fig. 2A)**. Indeed, WT cells spotted on
235 AMP 160 were not viable, whereas *ΔampD* spotted on AMP 160 only displayed a ~10-fold
236 decrease in viability compared to untreated cells. In liquid, *ΔampD* cells continued to grow
237 normally, even in the presence of AMP 100 **(Fig. 2B)** and ampicillin treatment did not trigger
238 obvious morphological changes or cell lysis **(Fig. S2B-C, Fig. 2C)**. Nitrocefin assays of
239 *ΔampD* indicated readily detectable amounts of β-lactamase production in both the presence
240 and absence of ampicillin **(Fig. 2D, Fig. S2D).** The increased zone of inhibition observed in
241 the presence of ampicillin and sulbactam is consistent with the high level of ampicillin
242 resistance observed in *ΔampD* being mediated by a β-lactamase **(Fig. 2E)**. Together, these
243 results indicate loss of AmpD leads to derepression and overproduction of β-lactamases. Our
244 findings are consistent with other bacterial models such as *P. aeruginosa*, where deletion of
245 1,6-anhydro amidases involved in the recycling of AmpR-activating ligands leads to increased
246 β-lactamase expression (53–56) due to the build-up of activating ligands in the cytoplasm.
247

248 **AmpC is constitutively produced in *ΔampD***

249 We have shown that AmpC and AmpR are required for ampicillin resistance **(Fig. 1)**, and loss
250 of AmpD leads elevated β-lactamase production and ampicillin resistance in *A. tumefaciens*
251 C58 **(Fig 2)**. To confirm that AmpC is the β-lactamase produced by the *ΔampD* strain, we
252 deleted *ampC* or *ampR* in the *ΔampD* background **(Fig. 3)**. In the absence of ampicillin, we
253 found that *ΔampCΔampD* and *ΔampRΔampD* display normal cell viability and growth **(Fig.**

254 **3A, Fig S3A).** We found that treatment with AMP 25, on either solid medium or liquid medium,
255 is lethal to *ΔampCΔampD* or *ΔampRΔampD* **(Fig. 3A, Fig. S3A).** Treatment of *ΔampCΔampD*
256 or *ΔampRΔampD* with AMP 25 for 2 h resulted in lysis of 22.9% and 33.8% of the cells in
257 comparison to untreated cells (No AMP), where lysis was not readily observed **(Fig. 3B, Fig**
258 **S3B)**. In comparison to *ΔampC* or *ΔampR*, where cell division defects were observed in >20%
259 of the population, very few *ΔampCΔampD* or *ΔampRΔampD* cells exhibited cell division
260 defects when treated with AMP 25 **(Fig. 3B)***.* The low incidence of cell division defects
261 observed in *ΔampCΔampD* or *ΔampRΔampD* suggest that the activity AmpD contributes to
262 the inefficient cell division of *ΔampC* and *ΔampR* cells following ampicillin treatment. Finally,
263 to assess whether the *ΔampD* strain could induce β-lactamase production in the absence of
264 *ampC* or *ampR*, we performed nitrocefin assays. *ΔampCΔampD* and *ΔampRΔampD* failed to
265 produce detectable levels of β-lactamase in the absence or presence of AMP 25 **(Fig. 3D,**
266 **Fig. S3C).** Together, these results suggest that induction of AmpC is the main cause for the
267 elevated resistance to ampicillin observed in *ΔampD*. This data is consistent with the current
268 model for β-lactam resistance in *P. aeruginosa,* where the loss of anhydro amidases leads to
269 accumulation of cell wall degradation products that activate AmpR leading to derepression of
270 AmpC (54–56, 69).
271

272 **Absence of MltB3 (*Δatu3779*) leads to a failure of AmpC-dependent induction of β-**
273 **lactamases**

274 LTs are likely to function as the enzymes that generate the AmpR-activating fragments.
275 Different families of LTs have been linked to β-lactam resistance in several bacterial
276 organisms (80, 81). For instance, in *Caulobacter crescentus* deletion of *sdpA*, a soluble LT,

277 led to reduced sensitivity to ampicillin (82). In *P. aeruginosa,* loss of several *mltBs* and/or *slt*
278 led to a decrease in the β-lactam MIC, cell viability, and increased outer membrane
279 permeability (83, 84). Thus, we sought to determine if LTs contribute to β-lactam resistance of
280 *A. tumefaciens*. The *A. tumefaciens* genome consists of 8 putative LTs belonging to 3
281 families: family 1) the soluble lytic transglycosylases (Slt), 2) Membrane-bound lytic
282 transglycosylase A (MltA), and 3) Membrane-bound lytic transglycosylase B (MltB) **(Fig. S4A)**.
283 We found that single deletions of LTs did not affect cell viability, suggesting a wide
284 redundancy of functions between LTs **(Fig. S4B)**.

285 Despite the potential for functional redundancy, we found that deletion of a single, family 3,
286 membrane-bound lytic transglycosylase, MltB3 (*atu3779)*, led to ampicillin hypersensitivity
287 **(Fig. S4B, Fig. 4A)**. Treatment of *ΔmltB3* with AMP 25 for 2 h led to a severe cell lysis defect
288 (28.8%) **(Fig. 4A-B)**. In addition, *ΔmltB3* cells grown in AMP 25 for 24 h had a severe growth
289 defect indicating that MltB3 is required for ampicillin resistance **(Fig. 4C)**. Finally, *ΔmltB3*
290 exhibited reduced production of β-lactamase after AMP 25 treatment for 2 hours **(Fig. 4D,**
291 **Fig. S4C).** Together, these results suggest that in *A. tumefaciens,* MltB3 is a specialized
292 enzyme that functions in the AmpR-AmpC β-lactamase pathway. These observations contrast
293 with the *P. aeruginosa* model*,* in which the β-lactam sensitivity of LT mutants are due to
294 increased outer membrane permeability rather than β-lactamase production (83)**.**

295 The finding that *Δmltb3* led to ampicillin sensitivity and reduced β-lactamase induction in
296 *A. tumefaciens* 58C prompted us to ask whether *Δmltb3* could be sensitive to other β-lactams
297 or if MltB3 performs the same function in other *A. tumefaciens* strains, especially in those
298 commonly used for plant transformations. To this end, we deleted *mltB3* in *A. tumefaciens*
299 GV3103 and found that this mutation made the strain susceptible to AMP 25 **(Fig 5A)**. In

300 addition, deletion of *mltB3* in GV3101 led to sensitivity to carbenicillin 15 (CARB 15), an
301 antibiotic commonly used in plant tissue culture systems **(Fig. 5A)**. To confirm that the
302 absence of MltB3 (*ΔmltB3*) prevented the induction of β-lactamase production in the GV3101
303 strain after ampicillin treatment, we performed nitrocefin assays using lysates of cells treated
304 with AMP 25 for 2 h. While β-lactamase activity is readily detected from WT GV3101-treated
305 cells with AMP 25, the *ΔmltB3* mutant produced relatively low levels of inducible β-lactamase
306 **(Fig. 5B, Fig. S5)**. Together, we conclude that MltB3 is the major LT in *A. tumefaciens* C58
307 and GV3101 that is required for the *A. tumefaciens* natural resistance to ampicillin and other
308 β-lactams such as carbenicillin.
309
310 **Plant transformation with *Δmltb3* cells requires a low concentration of ampicillin for**
311 **the elimination of bacteria**
312 We reasoned that ampicillin sensitivity observed in *ΔmltB* GV3101 could prove to be
313 beneficial during plant transformation. First, we confirmed that *ΔmltB3 A. tumefaciens*
314 GV3101 could effectively transform *Arabidopsis thaliana* using the standard floral dip
315 technique (85) with an efficiency comparable to WT **(Fig. 5C)**. Next, we asked whether
316 elimination of the bacteria could be performed using low concentrations of ampicillin (AMP
317 25). Seeds transformed with WT *A. tumefaciens* GV3101 contained similar bacterial loads
318 compared to seeds transformed with *Δmltb3 A. tumefaciens* GV3101 when plated on solid
319 media without ampicillin **(Fig. 5D)**. When plated on media containing AMP 25, seeds
320 transformed with *Δmltb3* exhibited a significant drop in bacterial load. In contrast, this low
321 level of ampicillin did not reduce the bacterial load of seeds transformed with WT GV3101
322 **(Fig. 5D)**. These results demonstrate that the *Δmltb3* GV3101 strain can be utilized to

323 transform *Arabidopsis thaliana* and elimination of the bacteria can be performed using lower
324 concentrations of ampicillin. Together, this shows the significance of understanding the cell
325 biology of *A. tumefaciens* as a means to improve genetic engineering approaches.
326

327 **CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

328 The natural ability of *A. tumefaciens* to transform plants has allowed the production of
329 transgenic crops of incredible economic importance for the past four decades. One challenge
330 of the *A. tumefaciens*-mediated plant transformation is natural resistance of *A. tumefaciens* to
331 antibiotics, which requires toxic concentrations of antibiotics to eliminate *A. tumefaciens* from
332 transformed tissues. Here we show that *A. tumefaciens* induces β-lactamase activity in
333 response to ampicillin exposure. Indeed, induction of β-lactamase activity upon exposure to
334 ampicillin is dependent on the β-lactamase AmpC and the transcription factor AmpR.
335 Moreover, we found that deletion of a single LT, MltB3, sensitizes *A. tumefaciens* to the β-
336 lactams.

337 We propose that during *A. tumefaciens* growth and remodeling, there is a delicate balance
338 between the synthesis and degradation of the bacterial cell wall. PBPs insert precursor
339 cytoplasmic monomers into the growing cell wall polymer (**Fig 6, steps 1-2**). During
340 remodeling, cell wall hydrolytic enzymes such as such as endopeptidases and LTs, including
341 MltB3, liberate cell wall degradation products, which are transported back into the cytoplasm
342 of *A. tumefaciens* for their recycling (**Fig 6, steps 3-4**). In the cytoplasm, hydrolytic enzymes
343 including L,D-carboxypeptidases (LD-CPases), amidases such as AmpD, and glycosidases
344 limit the pool of cell wall fragments by allowing their recycling **(Fig 6, steps 5-8).** When cell
345 wall degradation fragments accumulate during β-lactam treatment, the AmpR-dependent

346 production of the AmpC β-lactamase is increased **(Fig 6, step 9)**. Overall, the identification
347 and contributions of genes conferring ampicillin resistance in *A. tumefaciens* will be beneficial
348 for improving the design of *A. tumefaciens*-mediated genetic engineering.
349

350 **MATERIALS AND METHODS**

351 ***Bacterial strains and growth conditions.*** *Agrobacterium tumefaciens* C58 and derived
352 strains were grown in Luria-Bertani (LB) medium (10 g/l tryptone, 10 g/l yeast extract and 5 g/l
353 NaCl) at 28°C with shaking. *E. coli* DH5α and S17-1 were routinely cultivated in LB medium at
354 37°C with shaking and the antibiotic kanamycin was added at a concentration of 50 μg/ml and
355 25 μg/ml to maintain plasmids in DH5α and S17-1 strains, respectively.
356

357 ***Construction of expression plasmids and strains****.* All strains and plasmids used are listed
358 in **Table 1**. Synthesized DNA primers are listed in **Table 2**. To construct deletion vectors, the
359 corresponding coding sequences were amplified from purified *Agrobacterium tumefaciens*
360 C58 genomic DNA. The amplicons and plasmid pNTPS139 were digested overnight and
361 ligated overnight at 4°C using NEB T4 DNA ligase.
362

363 ***Construction of deletion constructs and deletion strains****.* Gene deletions were achieved
364 by allelic exchange and vectors were constructed as previously described (86). Briefly, 500
365 base pairs (bp) fragments upstream and downstream of the gene of interest were amplified
366 using primer pairs P1/P2; which amplify 500 bp upstream of the gene of interest, and P3/P4;
367 which amplify the 500 bp downstream of the gene of interest. Overlapping PCR was used to
368 merge and amplify the amplicons generated by P1/P2 and P3/P4, using primer pair P1/P4.

369 The 1,000 bp amplicon was digested and ligated into the deletion plasmid, pNTPS139. The
370 deletion plasmids were introduced into *A. tumefaciens* by mating using an *E. coli* S17
371 conjugation strain to create kanamycin (KAN) resistant, sucrose sensitive primary
372 exconjugants. Deletion strains were constructed as described previously (74). Briefly, primary
373 exconjugants were grown overnight at 28°C in minimal medium ATGN with no selection, and
374 plated in ATGN KAN 300 for 48 h at 28°C. Colonies were screened by patching for KAN
375 resistance and sucrose sensitivity. Colony PCR using primers P5/P4 were used to confirm
376 that recombination took place and at the region of interest. Next, positive colonies are grown
377 in ATGN at 28°C overnight and plated in ATSN 5% sucrose. Secondary recombinants were
378 screened by patching for sucrose resistance and KAN sensitivity. Colony PCR with primers
379 P5/P6 for the respective gene target was used to confirm deletion. PCR products from P5/P6
380 primer sets were sequenced to further confirm deletions.
381
382 ***Cell viability spot assays***. The cell viability assay was performed as described (87). For cell
383 viability spot assays, cultures were grown overnight and diluted to an OD_{600} of 0.05 and
384 serially diluted in LB up to 10^{-6}. 4 µl of each dilution was spotted onto LB plates and incubated
385 at 28°C for 36-40 h before imaging. To determine ampicillin antibiotic resistance, *A.*
386 *tumefaciens* cultures were grown overnight and diluted to an optical density at 600 nm (OD_{600})
387 of 0.05 and serially diluted and spotted onto LB plates containing AMP 25, AMP 80 and/or
388 AMP 160 using the cell viability assay protocol.
389
390 ***Phase-contrast microscopy.*** For phase-contrast microscopy, 0.8 µl of exponentially-grown
391 cultures were spotted onto a 1.25% agarose pad as previously described (87) using a Nikon

392 Eclipse Ti inverted microscope through a Nikon Plan 60X oil Ph3 objective. Cell length
393 analysis was performed using the MicrobeJ plug-in for Fiji (88). A One-Way analysis of
394 variance (ANOVA) Kruskal-Wallis test with Dunn's post-test was used to compare the
395 indicated strains. Images were prepared using Adobe Photoshop, Adobe Illustrator, and
396 Prism.
397
398 ***Growth curves***. For growth curves, exponentially-growing cultures were diluted to an OD_{600} =
399 0.2 and submitted to a plate reader at 28°C with shaking every 5-10 min. Ampicillin was
400 added to a final concentration of AMP 25 or AMP 100. OD_{600} represents two technical
401 replicates for each culture measured every 5 minutes for 24-48 h in 96-well plates.
402
403 ***Disc diffusion method***. The disc-diffusion assay was used to determine the resistance of
404 *Agrobacterium* to various antibiotics. Cells were growth on LB agar plates. Sterile discs (6.5
405 mm in diameter) were placed on the surface of LB agar plates seeded with overnight culture
406 of indicated strains. We used three different discs: Blank (sterile disc containing no antibiotic),
407 AMP 10 (ampicillin 10µg/ml), and AMP 10/SUL 10 (ampicillin 10µg/ml sulbactam 10µg/ml).
408 The LB agar assay plates used for testing *A. tumefaciens* susceptibility were incubated at
409 28°C for 24-36 h. The assessment of antibacterial activity was based on the measurement of
410 diameter of the zone of inhibition formed around the disc minus the size of the disc. Three
411 independent trials were conducted for each concentration of each antibiotic. A Brown-
412 Forsythe and Welch analysis of variance (ANOVA) was used to compare the means within
413 each indicated group.
414

415 ***Nitrocefin Assay***. Nitrocefin is a chromogenic substrate for measuring β-lactamase activity.
416 Nitrocefin has an absorbance maximum of 390 nm. Upon hydrolysis of the β-lactam ring by a
417 β-lactamase, the absorbance shifts from 390 nm to 486 nm. By monitoring absorbance at 486
418 nm over time and using Beer's law (A=εlc), we directly measured the rate of β-lactamase
419 hydrolytic activity. In two separate experiments, indicated strains were grown in LB until
420 desired optical densities (OD600) of 0.6 were reached. The cells were then pelleted by
421 centrifugation at 25,900 x g (rotor: Fiberlite F14-6x250y) for 5 minutes and the supernatant
422 collected and washed three times with PBS. Cell lysates were generated by adding the cell
423 lysis buffer BugBuster and sonication. No lysozyme or protase inhibitors were added. Cell
424 lysates were normalized based on total protein content (7.5 µg/ml) and volume before
425 incubation in 100 µM nitrocefin solution in a 200 µl reaction volume at room temperature in 20
426 mM HEPES, 300 mM NaCl, pH 7.5. BugBuster lysis buffer was used as blank. *ΔampD* lysates
427 were normalized based on total protein content (7.5 µg/ml) and subsequently diluted to 1:5 as
428 the rate of nitrocefin hydrolysis was significantly faster than the controls (WT, WT AMP 25).
429 Absorbance was immediately measured at 486 nm in 5 second intervals for 300 seconds (5
430 minutes). The change in absorbance (A) was converted to change in concentration (c) of
431 hydrolyzed product by using Beer's law (A=εlc) where the molar extinction coefficient (ε) =
432 20500 M-1 cm-1 and path length (l) = 1 cm. Data represents one biological replicate.
433
434 ***Construction of Agrobacterium strain for plant transformation experiments.*** *mltB3* was
435 deleted from the genome of WT *Agrobacterium tumefaciens* GV3101 using the same plasmid
436 and technique used to delete the gene in the strain C58. The resulting strain,
437 GV3101 *ΔmltB3*, was next transformed by introduction of the empty binary vector, pUBQ10-

438 GW (89) via electroporation. This plasmid confers KAN resistance to
439 the *Agrobacterium* strain, and also contains T-DNA repeat blocks which allow the transfer of
440 BASTA resistance to transformed into *Arabidopsis thaliana*. pUBQ10-GW was also
441 electroporated into wildtype GV3101 as a control.
442
443 ***Arabidopsis thaliana* transformation efficiency**. Plants with a bolt height between 2 and 7
444 centimeters were transformed via the floral dip method (85). 5 plants were transformed on
445 separate days from independent colonies for each C58 and GV3101. Plants were grown in
446 Promix BX (Premier Tech Horticulture) at 23°C, 16 h light/8 h dark, 100–150 μE m^{-2} s^{-1}, and
447 50–70% humidity until seeds were fully developed. Seeds were collected from fully matured
448 plants and store at 4°C at low humidity for one week. Seeds were then surface sterilized in
449 15% bleach containing 0.1% Triton X-100 with gentle rocking for 5 minutes. Seeds were then
450 washed 3 times using sterile water. Seeds were then imbibed in sterile water at 4°C for 3
451 days. Seeds were then sown into soil using conditions listed above. Once seeds developed
452 true leaves BASTA (glufosinate ammonium) was applied via spray at a concentration of
453 10mg/L. After 4 days BASTA was re-applied ensuring only transformed plants survived.
454
455 **Determining bacterial load of transformed *Arabidopsis thaliana* seeds**. Seeds were
456 collected from fully matured transformed plants and stored at 4°C for 1 week with low
457 humidity. Seeds were then surface sterilized in 15% bleach containing 0.1% Triton X-100 with
458 gentle rocking for 5 minutes. Seeds were then washed 3 times using sterile water. 10mg of
459 seeds for each experimental condition were ground with a sterile mortar and pestle. Ground
460 seeds were then suspended in 1mL of sterile water, and serially diluted. 200μl of 10^{-1} dilution

461 was plated on minimal medium ATGN No AMP and AMP 25. Plates were then incubated at
462 28°C before colonies were counted.
463

464 **ACKNOWLEDGEMENTS**

465 We thank the Brown lab for helpful discussions. Research in the Brown lab on *A. tumefaciens*
466 cell growth is supported by the National Science Foundation (IOS1557806). WFC was
467 supported by a Gus T. Ridgel Fellowship.
468

469 **AUTHOR CONTRIBUTIONS**

470 WFC and PJBB conceptualized this work; WFC and CR developed methods; WFC, MH,
471 CR, AMR and AKY performed the experiments and completed data analysis; WFC, AMR, and
472 PJB wrote the manuscript; WFC, AMR, AKY, FC, and PJBB edited the manuscript; PJBB
473 acquired funding for this work.
474

475 **REFERENCES**

476 1. Valentine L, Crops GM. 2003. *Agrobacterium tumefaciens* and the plant: The David and
477 Goliath of modern genetics. Plant Physiol 133:948–955.
478 2. Escobar MA, Dandekar AM. 2003. *Agrobacterium tumefaciens* as an agent of disease.
479 Trends Plant Sci 8:380–386.
480 3. Pulawska J. 2010. Crown gall of stone fruits and nuts, economic significance and
481 diversity of its causal agents: Tumorigenic *Agrobacterium* spp. J Plant Pathol 92:S87–
482 S98.
483 4. Otten L, Burr T, Szegedi E. 2008. *Agrobacterium*: A disease-causing bacterium. Tzfira

484 T, Citovsky V (eds) *Agrobacterium* from Biol to Biotechnol Springer 1–46.
485 5. Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the
486 "gene-jockeying" tool. Microbiol Mol Biol Rev 67:16–37.
487 6. Nester EW. 2015. *Agrobacterium*: Nature's genetic engineer. Front Plant Sci 5:1–16.
488 7. Dessaux Y, Faure D. 2018. Niche construction and exploitation by *Agrobacterium*: How
489 to survive and face competition in soil and plant habitats. Curr Top Microbiol Immunol
490 Springer 418.
491 8. Singer K. 2018. The mechanism of T-DNA integration: Some major unresolved
492 questions. Curr Top Microbiol Immunol 418:287–317.
493 9. Tzfira T, Citovsky V. 2000. From host recognition to T-DNA integration: the function of
494 bacterial and plant genes in the *Agrobacterium*-plant cell interaction. Mol Plant Pathol
495 1:201–212.
496 10. Gelvin S. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and
497 integration. Annu Rev Plant Biol 51:223–256.
498 11. Tzfira T, Citovsky V. 2006. *Agrobacterium*-mediated genetic transformation of plants:
499 biology and biotechnology. Curr Opin Biotechnol 17:147–154.
500 12. Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the
501 "gene-jockeying" tool. Society 67:16–37.
502 13. De La Riva GA, González-Cabrera J, Vázquez-Padrón R, Ayra-Pardo C. 1998.
503 *Agrobacterium tumefaciens*: A natural tool for plant transformation. Electron J
504 Biotechnol 1:25–48.
505 14. Leifert C, Cassells AC. 2000. Microbial hazards in plant tissue and cell cultures. Vitr Cell
506 Dev Biol 37:133–138.

507 15. Mogilner N, Zutra D, Gafny R, Bar-Joseph M. 1993. The persistence of engineered
508 *Agrobacterium tumefaciens* in agroinfected plants. Mol plant-microbe Interact MPMI.
509 16. Matzk A, Mantell S, Schiemann J. 1996. Localization of persisting agrobacteria in
510 transgenic tobacco plants. Mol Plant-Microbe Interact.
511 17. Hammerschlag FA, Zimmerman RH, Yadava UL, Hunsucker S, Gercheva P. 1997.
512 Effect of antibiotics and exposure to an acidified medium on the elimination of
513 *Agrobacterium tumefaciens* from apple leaf explants and on shoot regeneration. J Am
514 Soc Hortic Sci.
515 18. Mohana Priya A, Karutha Pandian S, Manikandan R. 2012. The effect of different
516 antibiotics on the elimination of *Agrobacterium* and high frequency *Agrobacterium*-
517 mediated transformation of indica rice (Oryza sativa L.). Czech J Genet Plant Breed
518 48:120–130.
519 19. Demain AL, Blander RP. 1999. The β-lactam antibiotics: Past, present, and future.
520 Antonie van Leeuwenhoek, Int J Gen Mol Microbiol 75:5–19.
521 20. Okkels FT, Pedersen MG. 1988. The toxicity to plant tissue and to *Agrobacterium*
522 *tumefaciens* of some antibiotics. Acta Hortic 225:199–208.
523 21. Shackelford NJ, Chlan CA. 1996. Identification of antibiotics that are effective in
524 eliminating *Agrobacterium tumefaciens*. Plant Mol Biol Report 14:50–57.
525 22. Estopà M, Marfà V, Melé E, Messeguer J. 2001. Study of different antibiotic
526 combinations for use in the elimination of *Agrobacterium* with kanamycin selection in
527 carnation. Plant Cell Tissue Organ Cult 65:211–220.
528 23. Holford P, Newbury HJ. 1992. The effects of antibiotics and their breakdown products
529 on the *in vitro* growth of *Antirrhinum majus*. Plant Cell Rep 11:93–96.

530 24. Minden V, Deloy A, Volkert AM, Leonhardt SD, Pufal G. 2017. Antibiotics impact plant
531 traits, even at small concentrations. AoB Plants 9.
532 25. Jiang C, Brown PJB, Ducret A, Brun Y V. 2014. Sequential evolution of bacterial
533 morphology by co-option of a developmental regulator. Nature 506:489–493.
534 26. Wiebke B, Ferreira F, Pasquali G, Bodanese-Zanettini MH, Droste A. 2006. Influence of
535 antibiotics on embryogenic tissue and *Agrobacterium tumefaciens* suppression in
536 soybean genetic transformation. Bragantia 65:543–551.
537 27. Obembe O, Adesoye A, Machuka J. 2005. Evaluation of the Effectiveness of Selected
538 Antibiotics in the Suppression of *Agrobacterium* from Cowpea (Vigna Unguiculata L.
539 Walp.) Embryo Explants and as Potential Selective Agents in *Agrobacterium*-Mediated
540 Transformation. J Trop Microbiol Biotechnol 1:27–33.
541 28. Haddadi F, Aziz MA, Abdullah SNA, Tan SG, Kamaladini H. 2015. An efficient
542 *Agrobacterium*-mediated transformation of Strawberry cv. Camarosa by a dual plasmid
543 system. Molecules 20:3647–3666.
544 29. Tang H, Ren Z, Krczal G, Glimelius K. 2000. An evaluation of antibiotics for the
545 elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for the
546 effects on the proliferation of somatic embryos and regeneration of transgenic plants.
547 Plant Cell Rep 19:881–887.
548 30. Waxman DJ, Strominger JL. 1983. Penicillin-binding proteins and the mechanism of
549 action of β-lactam antibiotics. Annu Rev Biochem 52:825–869.
550 31. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. 2008. The Penicillin-binding
551 proteins: Structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32:234–
552 258.

553 32. Spratt BG. 2006. Distinct Penicillin-binding proteins involved in the division, elongation,
554 and shape of *Escherichia coli* K12. Proc Natl Acad Sci 72:2999–3003.
555 33. Rolinson GN. 1980. Effect of β-lactam antibiotics on bacterial cell growth rate.
556 Microbiology 120:317–323.
557 34. Jacoby GA. 2009. AmpC β-lactamases. Clin Microbiol Rev 22:161–182.
558 35. Egan AJF, Cleverley RM, Peters K, Lewis RJ, Vollmer W. 2017. Regulation of bacterial
559 cell wall growth. FEBS J 284:851–867.
560 36. Den Blaauwen T, Andreu JM, Monasterio O. 2014. Bacterial cell division proteins as
561 antibiotic targets. Bioorg Chem 55:27–38.
562 37. Egan AJF, Biboy J, van't Veer I, Breukink E, Vollmer W. 2015. Activities and regulation
563 of peptidoglycan synthases. Philos Trans R Soc B Biol Sci 370.
564 38. Typas A, Banzhaf M, Gross CA, Vollmer W. 2012. From the regulation of peptidoglycan
565 synthesis to bacterial growth and morphology. Nat Rev Microbiol 10:123–136.
566 39. Vollmer W, Blanot D, De Pedro MA. 2008. Peptidoglycan structure and architecture.
567 FEMS Microbiol Rev 32:149–167.
568 40. de Pedro MA, Cava F. 2015. Structural constraints and dynamics of bacterial cell wall
569 architecture. Front Microbiol.
570 41. Margolin W. 2009. Sculpting the Bacterial Cell. Curr Biol 19:812–822.
571 42. Park JT, Uehara T. 2008. How bacteria consume their own exoskeletons (turnover and
572 recycling of cell wall peptidoglycan). Microbiol Mol Biol Rev 72:211–227.
573 43. Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harb
574 Perspect Biol 2:1–16.
575 44. Scheffers D, Pinho MG. 2005. Bacterial cell wall synthesis: New insights from

576 localization studies. Microbiol Mol Bio Rev 69:585–607.
577 45. Vermassen A, Leroy S, Talon R, Provot C, Popowska M, Desvaux M. 2019. Cell wall
578 hydrolases in bacteria: Insight on the diversity of cell wall amidases, glycosidases and
579 peptidases toward peptidoglycan. Front Microbiol 10:331.
580 46. Frirdich E, Gaynor EC. 2013. Peptidoglycan hydrolases, bacterial shape, and
581 pathogenesis. Curr Opin Microbiol 16:767–778.
582 47. Vollmer W, Joris B, Charlier P, Foster S. 2008. Bacterial peptidoglycan (murein)
583 hydrolases. FEMS Microbiol Rev 32:259–286.
584 48. van Heijenoort J. 2011. Peptidoglycan hydrolases of *Escherichia coli*. Microbiol Mol Biol
585 Rev 75:636–663.
586 49. Dhar S, Kumari H, Balasubramanian D, Mathee K. 2018. Cell-wall recycling and
587 synthesis in *Escherichia coli* and *Pseudomonas aeruginosa* – their role in the
588 development of resistance. J Med Microbiol 67:1–21.
589 50. Dik DA, Fisher JF, Mobashery S. 2018. Cell-wall recycling of the Gram-negative
590 bacteria and the nexus to antibiotic resistance. Chem Rev. review-article, American
591 Chemical Society.
592 51. Lee TK, Huang KC. 2013. The role of hydrolases in bacterial cell-wall growth. Curr Opin
593 Microbiol 16:760–766.
594 52. Vollmer W. 2012. Bacterial growth does require peptidoglycan hydrolases. Mol
595 Microbiol 86:1031–1035.
596 53. Fisher JF, Mobashery S. 2014. The sentinel role of peptidoglycan recycling in the β-
597 lactam resistance of the Gram-negative Enterobacteriaceae and *Pseudomonas*
598 *aeruginosa*. Bioorg Chem 56:41–48.

599 54. Jacobs C, Joris B, Jamin M, Klarsov K, van Beeumen J, Mengin-Lecreulx D, van
600 Heijenoort J, Park JT, Normark S, Frère J-M. 1995. AmpD, essential for both β-
601 lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-
602 alanine amidase. Mol Microbiol 15:553–559.
603 55. Langaee TY, Gagnon L, Huletsky A. 2000. Inactivation of the *ampD* gene in
604 *Pseudomonas aeruginosa* leads to moderate-basal-level and hyperinducible AmpC β-
605 lactamase expression. Antimicrob Agents Chemother 44:583–589.
606 56. Schmidtke AJ, Hanson ND. 2008. Role of *ampD* homologs in overproduction of AmpC
607 in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother
608 52:3922–3927.
609 57. Jacobs C, Fre J. 1997. Cytosolic intermediates for cell wall biosynthesis and
610 degradation control inducible β-lactam resistance in Gram-negative bacteria 88:823–
611 832.
612 58. Dik DA, Domínguez-Gil T, Lee M, Hesek D, Byun B, Fishovitz J, Boggess B, Hellman
613 LM, Fisher JF, Hermoso JA, Mobashery S. 2017. Muropeptide binding and the X-ray
614 structure of the effector domain of the transcriptional regulator AmpR of *Pseudomonas*
615 *aeruginosa*. J Am Chem Soc 139:1448–1451.
616 59. Johnson JW, Fisher JF, Mobashery S. 2013. Bacterial cell-wall recycling. Ann N Y Acad
617 Sci 1277:54–75.
618 60. Balasubramanian D, Kumari H, Mathee K. 2014. *Pseudomonas aeruginosa* AmpR: An
619 acute-chronic switch regulator. Pathog Dis 73:1–14.
620 61. Vadlamani G, Thomas MD, Patel TR, Donald LJ, Reeve TM, Stetefeld J, Standing KG,
621 Vocadlo DJ, Mark BL. 2015. The β-lactamase gene regulator AmpR is a tetramer that

622 recognizes and binds the D-Ala-D-Ala motif of its repressor UDP-N-acetylmuramic acid
623 (MurNAc)-pentapeptide. J Biol Chem 290:2630–2643.
624 62. Lindberg F, Normark S. 1987. Common mechanism of ampC β-lactamase induction in
625 enterobacteria: Regulation of the cloned *Enterobacter cloacae* P99 β-lactamase gene. J
626 Bacteriol 169:758–763.
627 63. Torrens, G, Hernández S, Alfonso Ayala J, Moya B, Juan C, Cava F, Oliver A. 2019.
628 Regulation of AmpC-driven β-lactam resistance in *Pseudomonas aeruginosa*: Different
629 pathways, different signaling 4:1–14.
630 64. Juan C, Torrens G, González-Nicolau M, Oliver A. 2017. Diversity and regulation of
631 intrinsic β-lactamases from non-fermenting and other Gram-negative opportunistic
632 pathogens. FEMS Microbiol Rev 41:781–815.
633 65. Bajaj P, Singh NS, Virdi JS. 2016. *Escherichia coli* β-lactamases: What really matters.
634 Front Microbiol 7:1–14.
635 66. Jaurin BA, Normark S. 1979. *In vivo* regulation of chromosomal β-lactamase in
636 *Escherichia coli*. J Bacteriol 138:896–902.
637 67. Jaurin B, Grundström T, Edlund T, Normark S. 1981. The *E. coli* β-lactamase attenuator
638 mediates growth rate-dependent regulation. Nature 290:221–225.
639 68. Honoré N, Nicolas MH, Cole ST. 1986. Inducible cephalosporinase production in clinical
640 isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been
641 deleted from *Escherichia coli*. EMBO J 5:3709–3714.
642 69. Jacobs C, Huang LJ, Bartowsky E, Normark S, Park JT. 1994. Bacterial cell wall
643 recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO
644 J 13:4684–4694.

645 70. Ogawa Y, Mii M. 2004. Screening for highly active β-lactam antibiotics against
646 *Agrobacterium tumefaciens*. Arch Microbiol 181:331–336.
647 71. Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao
648 Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Epp A,
649 Liu F, Wollam C, Allinger M, Doughty D, Scott C, Lappas C, Markelz B, Flanagan C,
650 Crowell C, Gurson J, Lomo C, Sear C, Strub G, Cielo C, Slater S. 2001. Genome
651 Sequence of the Plant Pathogen and Biotechnology Agent *Agrobacterium tumefaciens*
652 C58. Science (80-) 294:2323 LP – 2328.
653 72. CAO Y, Niimi Y, HU S lian. 2006. Meropenem as an alternative antibiotic agent for
654 suppression of *Agrobacterium* in genetic transformation of orchid. Agric Sci China
655 5:839–846.
656 73. O'Callaghan CH, Morris A, Kirby SM, Shingler AH. 1972. Novel method for detection of
657 β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob Agents
658 Chemother 1:283–288.
659 74. Morton ER, Fuqua C. 2012. Genetic manipulation of *Agrobacterium*. Curr Protoc
660 Microbiol Chapter 3:Unit-3D.2.
661 75. Uehara T, Park JT. 2007. An anhydro-N-acetylmuramyl-L-alanine amidase with broad
662 specificity tethered to the outer membrane of *Escherichia coli*. J Bacteriol 189:5634–
663 5641.
664 76. Pennartz A, Généreux C, Parquet C, Mengin-Lecreulx D, Joris B. 2009. Substrate-
665 induced inactivation of the *Escherichia coli* AmiD N-acetylmuramoyl-L-alanine amidase
666 highlights a new strategy to inhibit this class of enzyme. Antimicrob Agents Chemother
667 53:2991–2997.

668 77. Langaee TY, Gagnon L, Huletsky A. 2000. Inactivation of the *ampD* gene in
669 P*seudomonas aeruginosa* leads to moderate-basal-level and hyperinducible AmpC β-
670 lactamase expression. Antimicrob Agents Chemother 44:583–589.
671 78. Mercier F, Zervosen A, Teller N, Frre JM, Herman R, Pennartz A, Joris B, Luxen A.
672 2010. 1,6-AnhMurNAc derivatives for assay development of amidase AmiD. Bioorganic
673 Med Chem 18:7422–7431.
674 79. Juan C, Moyá B, Pérez JL, Oliver A. 2006. Stepwise upregulation of the *Pseudomonas*
675 *aeruginosa* chromosomal cephalosporinase conferring high-level β-lactam resistance
676 involves three AmpD homologues. Antimicrob Agents Chemother 50:1780–1787.
677 80. Wu C, Huang Y, Lin Y, Yang T. 2016. Inactivation of lytic transglycosylases increases
678 susceptibility to aminoglycosides and macrolides by altering the outer membrane
679 permeability of *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother 60:3236–
680 3239.
681 81. Yin J, Sun Y, Sun Y, Yu Z, Qiu J, Gao H, Oliver A. 2018. Deletion of lytic
682 transglycosylases increases β-lactam resistance in *Shewanella oneidensis*. Front
683 Microbiol 9:1–10.
684 82. Zielińska A, Billini M, Möll A, Kremer K, Briegel A, Izquierdo Martinez A, Jensen GJ,
685 Thanbichler M. 2017. LytM factors affect the recruitment of autolysins to the cell division
686 site in *Caulobacter crescentus*. Mol Microbiol 106:419–438.
687 83. Lamers RP, Nguyen UT, Nguyen Y, Buensuceso R, Burrows LL. 2015. Loss of
688 membrane-bound lytic transglycosylases increases outer membrane permeability and
689 β-lactam sensitivity in *Pseudomonas aeruginosa*. Microbiology 6:879–895.
690 84. Cho H, Uehara T, Bernhardt TG. 2014. Beta-lactam antibiotics induce a lethal

691 malfunctioning of the bacterial cell wall synthesis machinery. Cell 159:1300–1311.
692 85. Clough SJ, Bent AF. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated
693 transformation of *Arabidopsis thaliana*. Plant J 16:735–743.
694 86. Howell M, Aliashkevich A, Sundararajan K, Daniel JJ, Lariviere PJ, Goley ED, Cava F,
695 Brown PJB. 2019. *Agrobacterium tumefaciens* divisome proteins regulate the transition
696 from polar growth to cell division. Mol Microbiol 111:1074–1092.
697 87. Howell M, Daniel JJ, Brown PJB. 2017. Live cell fluorescence microscopy to observe
698 essential processes during microbial cell growth. JoVE e56497.
699 88. Ducret A, Quardokus EM, Brun Y V. 2016. MicrobeJ, a tool for high throughput bacterial
700 cell detection and quantitative analysis. Nat Microbiol 1:16077.
701 89. Michniewicz M, Frick EM, Strader LC. 2015. Gateway-compatible tissue-specific vectors
702 for plant transformation Plant Biology. BMC Res Notes 8:6–13.
703 90. Figueroa-Cuilan W, Daniel JJ, Howell M, Sulaiman A, Brown PJB. 2016. Mini-Tn7
704 insertion in an artificial attTn7 site enables depletion of the essential master regulator
705 ctrA in the phytopathogen *Agrobacterium tumefaciens*. Appl Environ Microbiol 82.
706 91. Ikeda RA, An Chi Lin, Clarke J. 1992. Initiation of transcription by T7 RNA polymerase
707 at its natural promoters. J Biol Chem 267:2640–2649.
708 92. Garcia PS, Simorre JP, Brochier-Armanet C, Grangeasse C. 2016. Cell division of
709 Streptococcus pneumoniae: think positive! Curr Opin Microbiol 34:18–23.
710

711 **TABLES**

712 **Table 1.** Strains and plasmids used in this study.
713

Strain or plasmid	Relevant characteristics	Reference/Source
Source Plasmids		
pSRKKm-Plac-sf*gfp*	pSRKKm vector containing lacI^q and *lac* promoter with sfGFP	(90)
pSRKKm-Pcym-sfGfp	pSRKKm vector containing lac promoter flanked by *cuO* operator site along with the *cymR* repressor gene	This study
pSRKKm-T7-sf*gfp*	pSRKKm vector containing T7 and *lac* promoters with sfGFP	(91)
pNTPS139	Km^r; Suicide vector containing *oriT* and *sacB*	D. Alley
Plasmids		
Deletion plasmids		
pNTPS139 *Δatu3077*	Km^r Suc^s; deletion plasmid for *atu3077*	This study
pNTPS139 *Δatu3078*	Km^r Suc^s; deletion plasmid for *atu3078*	This study
pNTPS139 *Δatu0092*	Km^r Suc^s; deletion plasmid for a*tu0092*	This study
pNTPS139 *Δatu2122*	Km^r Suc^s; deletion plasmid for a*tu2122*	This study
pNTPS139 *Δatu3779*	Km^r Suc^s; deletion plasmid for a*tu3779*	This study

pNTPS139 Δ*atu2113*	Km^r Suc^s; deletion plasmid for *ampD*	This study
***E. coli* strains**		
DH5α	Cloning strain	Life Technologies
S17-1	Sm^r; RP4-2, Tc::Mu,Km-Tn7, for plasmid mobilization	(92)
***A. tumefaciens* strains**		
C58	Nopaline type strain; pTiC58; pAtC58	
GV3101	C58-derived; pTiC58DT-DNA; strain for *Agrobacterium* mediated transformation of dicots	John Walker Lab
FC2452	Deletion strain for *ΔmltA (atu0009)* in C58	This study
FC2444	Deletion strain for *ΔmltB1 (atu0092)* in C58	This study
FC2465	Deletion strain for *ΔmltB2 (atu2122)* in C58	This study
FC2487	Deletion strain for *ΔmltB3 (atu3779)* in C58	This study
FC2446	Deletion strain for *Δslt1 (atu1022)* in C58	This study
FC2448	Deletion strain for *Δslt2 (atu2112)* in C58	This study
FC2450	Deletion strain for *Δslt3 (atu2117)* in C58	This study
C58 *ΔtetRA*::a-*att*Tn7	Replacement of the *ΔtetRA* locus with an artificial *att*Tn7 site	(90)
C58 *ΔtetRA*:: a-*att*Tn7 *Δatu3077*	Deletion strain for *ΔampC*	This study

C58 *ΔtetRA*:: a-*att*Tn7 *Δatu3078*	Deletion strain for *ΔampR*	This study
C58 Δ*tetRA*::a-*att*Tn7 *ΔampD*	Deletion strain for *ΔampD*	This study
C58 Δ*tetRA*::a-*att*Tn7 *ΔampD ΔampC*	Deletion strain for *ΔampDΔampC*	This study
C58 Δ*tetRA*::a-*att*Tn7 *ΔampD ΔampR*	Deletion strain for *ΔampDΔampR*	This study
C58 GV3101 *Δmltb3*	Deletion strain for *ΔmltB3*	This study

714

715 **Table 2**. Synthesized DNA primers used in this study.
716

Synthesized DNA	Sequence
atu3077 P1	5'-GCGGCGACTAGTAAACGGATGCCGCTTTTGAAATGC-3'
atu3077 P2	5'-AAGCTTGGTACCGAATTCGCGATTAAATTTCATCTTTCGTGT-3'
atu3077 P3	5'-GAATTCGGTACCAAGCTTGCGCTCGAAAAGGCGCAATAA-3'

atu3077 P4	5’-GTCGTCGGATCCAGATAACTCGGCACACGCCCA-3'
atu3077 P5	5’-CTGCGCCGCCGGTGAAACGCCCGC-3'
atu3077 P6	5’-TGTGCCGGAGGCGCTTGCGATCGC-3'
atu3078 P1	5’-GTCGTCACTAGTGGGTTTTCCCTTCATGGACGG-3'
atu3078 P2	5’-AAGCTTGGTACCGAATTCAAATTGCCGAACCATTCAAGACCT-3'
atu3078 P3	5’-GAATTCGGTACCAAGCTTGAGACCATCGGAACGGCGTGA-3'
atu3078 P4	5’-GTCGTCGGATCCCCTGTTTGATGCTTTTTATCGCGC-3'
atu3078 P5	5’-CCAAGCGCCGGGAAAAGCGTGTCC-3'
atu3078 P6	5’-GGCATCGTCTGCGTGGTGTTCGTC-3'
atu2113 P1	5’-GTCGTCACTAGTCAGATTTTCGATTTCCCGGACGAA-3’
atu2113 P2	5’-AAGCTTGGTACCGAATTCCGAACATTCTTTCATGCGACG-3’
atu2113 P3	5’-GAATTCGGTACCAAGCTTCTGCCGAGATTTTCGGCCGCCTGA-3’
atu2113 P4	5’-GTCGTCGGTACCCCACCGAAACCACGGCATGCGCCA-3’
atu2113 P5	5’-GCGCAATCGGCGACGCGG-3'

atu2113 P6	5’-GTCGTCTGCTGCACACTTCGCCGC-3’
atu0009 P1	5’-AAAAAGCTTAACGCATCTTCTAGCCTTGCG-3’
atu0009 P2	5’-TATTCATTGCTCGGATTCGG-3’
atu0009 P3	5’-GCAATGAATATATCGGCGATGAAAGGC-3’
atu0009 P4	5’-AAAGGATCCGAAAGAACAATTCCTCCGC -3’
atu0009 P5	5’-TTTCGGCGACCTATGACAAGGACGG-3’
atu0009 P6	5’-TTACCAGTTTGCGGAACGCTGGG-3’
atu0092 P1	5’-AAACTGCAGATTCTTGCCCTGATGCCCATTGTCGC-3’
atu0092 P2	5’-AGACCGAATATCGTCTTTTAATGCTGGTCGG-3’
atu0092 P3	5’-ATTCGGTCTCCTCTTGGATGG-3’
atu0092 P4	5’-AAAGGATCCAAGTCGAGATCGACTGAGCCC-3’
atu0092 P5	5’-AATATGTCCGCCACAACCATCGTCGC-3’
atu0092 P6	5’-ATTACATCACAGACCGCCTCTCCG-3’
atu2122 P1	5’-AAAGAATTCAATCATCAGGGTTCCAATGCGG-3’
atu2122 P2	5’-TAGTGCGATTTTCCTCGATAGGTTGTTGGC-3’
atu2122 P3	5’-ATCGCACTACCGGGCCTTTAATCTATCGG-3’

atu2122 P4	5'-AAAAAGCTTATAATGACGTCTTTGAACGC-3'
atu2122 P5	5'-TATACCGCAACCGGCGTCGTACCCG-3'
atu2122 P6	5'-TTTCTGTGATGCGGTGCAGCACGG-3'
atu3779 P1	5'-AAACTGCAGTAGAAATTCGACGGCGCCG-3'
atu3779 P2	5'-TTTCGATTGCGAAAACGCATCGGGCG-3'
atu3779 P3	5'-GCAATCGAAATAATGTGCCGGCGAATTCGG-3'
atu3779 P4	5'-AAAGGATCCTTGGCCGTTCATGTCGTAGCC-3'
atu3779 P5	5'-TTTCGGAACTGCCTTGGTGGCGG-3'
atu3779 P6	5'-ATTCCCGGCCGGAACTACCATCGC-3'
atu2112 P1	5'- AAAAAGCTTTATTTCGTCTTCGAGGATGGG-3'
atu2112 P2	5'- GATGATCGATATGACAGAAACAGTGAAATGGC-3'
atu2112 P3	5'-ATCGATCATCTGCGAAATTGCG-3'
atu2112 P4	5'-AAAGAATTCATATCGTCCTCGGTTTCCGC-3'
atu2112 P5	5'- AAAAAGCTTTATTTCGTCTTCGAGGATGGG-3'
atu2112 P6	5'- TTTGCACCGAAAGATGCCGCG-3'
atu1022 P1	5'-AAAAAGCTTTTATGCGCTTTGACCAGCGCACCC-3'

atu1022 P2	5’-AATCCCCAGACTGTCTTTTTCATGCCG-3’
atu1022 P3	5’-TGGGGATTATTCAGGCACGGGCTAGCC-3’
atu1022 P4	5’-AAAGAATTCAATACGCTCTTCAACTCCATCCG-3’
atu1022 P5	5’- AAAAGCGACGTCGCTCGCG-3’
atu1022 P6	5’-AAACTACTACGACGAAGACGGTCAGG-3’
atu2117 P1	5’-AAAGAATTCAAGAGAATGTCTGGACAGGCGTGGC-3’
atu2117 P2	5’-ATTTTCAATCTAGAGTTGCCGCCTTCGTTATGCC-3’
atu2117 P3	5’- TTGAAAATGCTGCGGCTCCC-3’
atu2117 P4	5’-AAAAAGCTTTAGATGTTCCTGTCGAACACCG-3’
atu2117 P5	5’-ATACAGGCGCATCGCGGCC-3’
atu2117 P6	5’-AAGGCGAGGTGGTCACTGACC -3’
atu3093 P1	5’-GCTGCAACTAGTGCGCCACAGCCCATCTCG-3’
atu3093 P2	5’-AAGCATGGTACCGAATTCGCGGCTTGTTGATATTCCT-3’
atu3093 P3	5’-GAATTCGGTACCATGCTTTAGCAGCGGCGGGCGCCGGA-3’
atu3093 P4	5’-GCACGTAAGCTTGCGGTGATCTTGATGAT-3’
atu3093 P5	5’-GAGCTTCTCGGGCAATTCCG-3’

atu3093 P6	5'-GCCTCACGAAGCCGCACGATC-3'

717

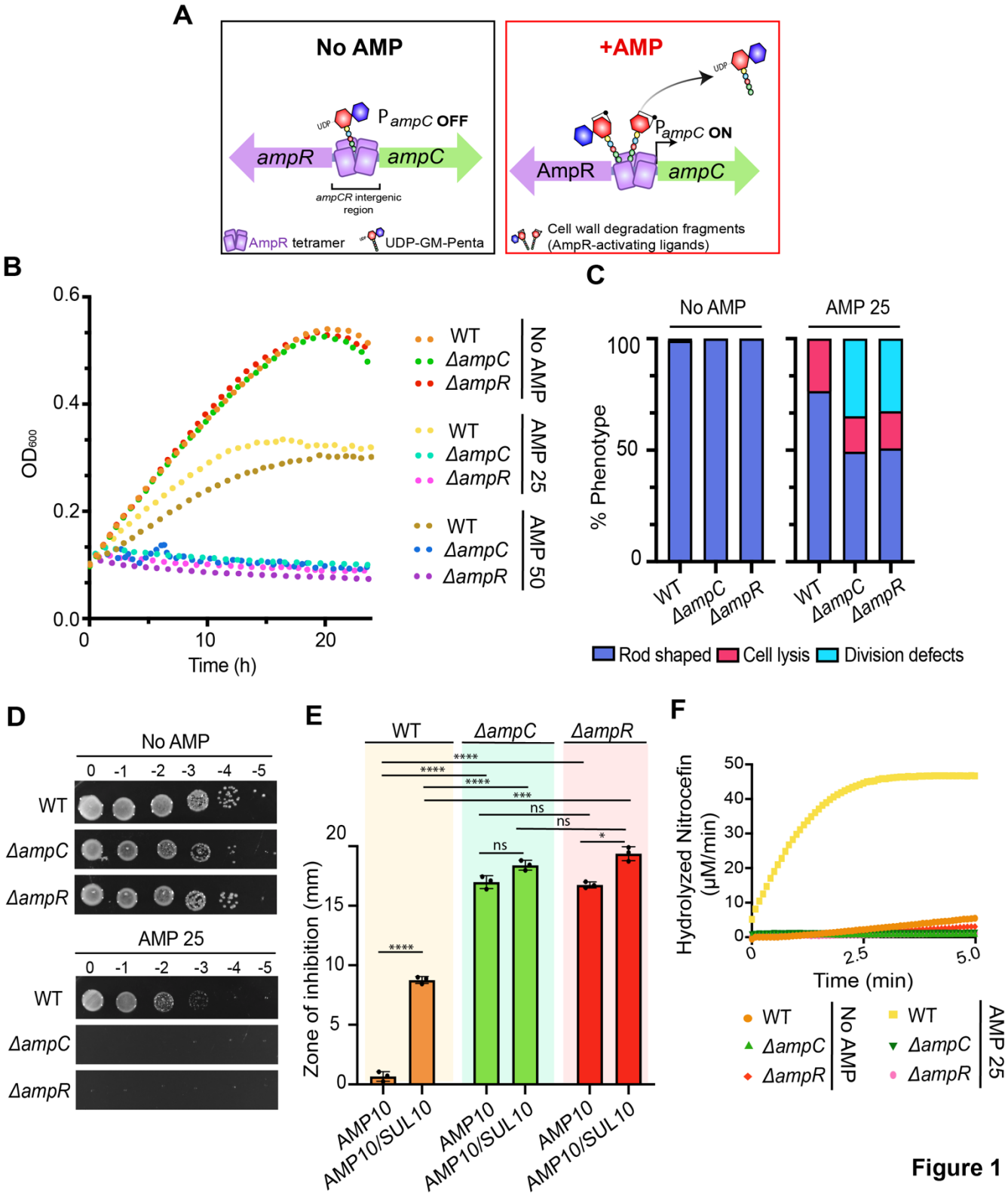
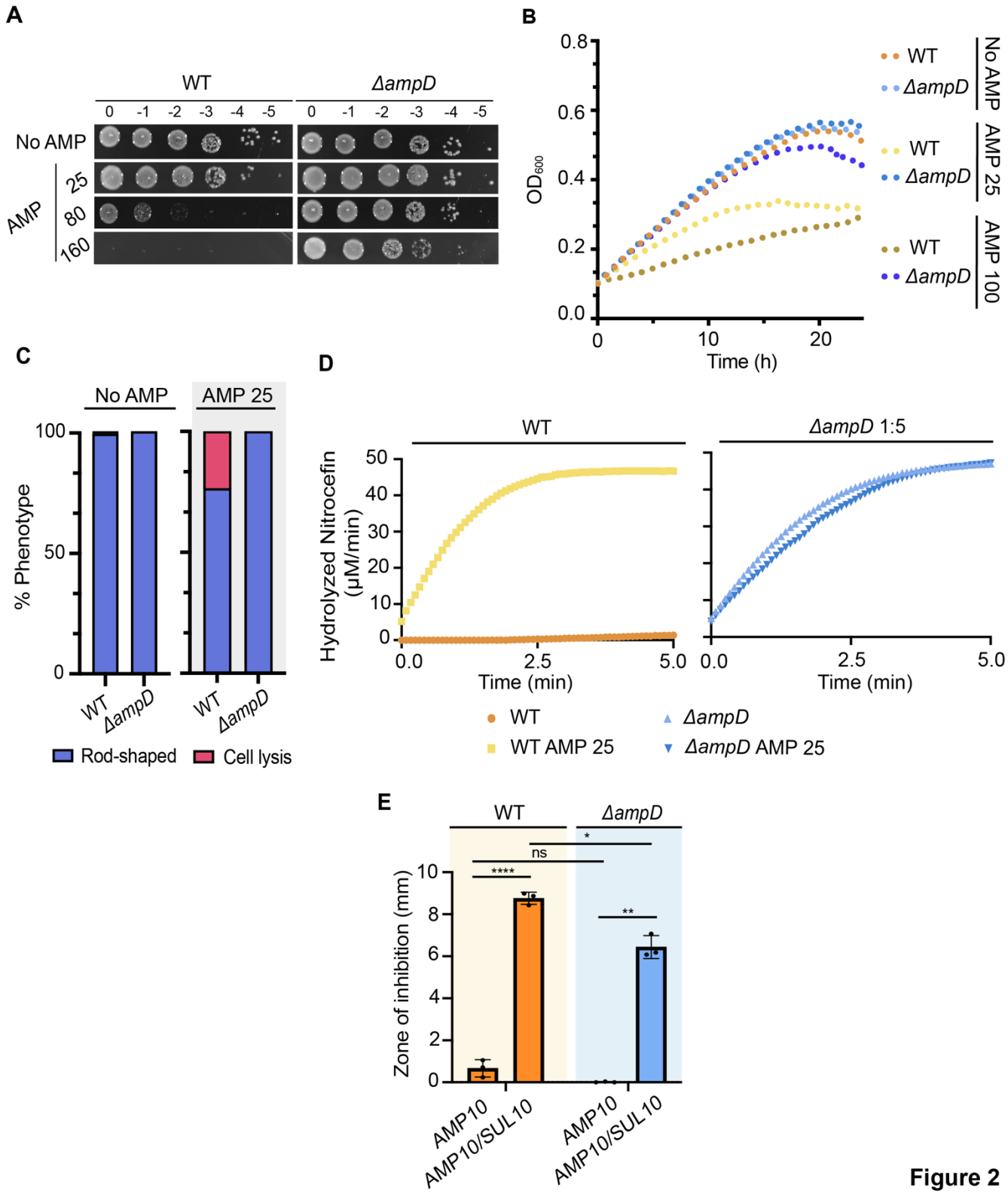


FIG 1. The AmpC-AmpR operon is responsible for induced ampicillin resistance in

720 ***Agrobacterium tumefaciens* C58. (A)** Operon organization and proposed ampicillin
721 resistance mechanism (53). Briefly, in the absence of β-lactams such as ampicillin (AMP),
722 *ampC* expression is repressed by AmpR. AmpR-mediated repression is maintained as long
723 as the AmpR-inactivating ligand, UDP-GM-Pentapeptide, is bound to AmpR ("P*ampC* OFF").
724 In contrast, the presence of ampicillin (AMP) increases the pools of AmpR-activating ligands,
725 or the cell wall degradation fragments (anhydro modification is depicted by ring), which are
726 known to displace AmpR-inactivating ligands. As a result, the increase of AmpR-activating
727 ligands activate AmpR and *ampC* is transcribed ("P*ampC* ON"). **(B)** Growth of WT *A.*
728 *tumefaciens, ΔampC,* and *ΔampR* in the absence (No AMP) and presence of ampicillin 25 or
729 50 µg/ml (AMP 25, AMP 50) for 24 h (n = 1, 2 replicates). **(C)** Quantitative analysis of phase-
730 contrast microscopy of exponentially-growing strains in absence (No AMP) or presence of
731 ampicillin 25 ug/ml (AMP 25). % Phenotype was calculated by counting the number of cells
732 displaying one of the phenotypes indicated (1 cell = 1 phenotype) and dividing it by the total
733 number of cells per strain. **(D)** Ampicillin susceptibility assay was performed via spotting
734 dilutions. Briefly, indicated strains were cultured overnight (ON) on LB medium, serially
735 diluted, spotted on LB solid medium containing no ampicillin (No AMP) or ampicillin 25 µg/ml
736 (AMP 25), and incubated at 28°C for 36 h before imaging. **(E)** Disc susceptibility was
737 performed on lawn of indicated strains grown on LB plates for 24 h at 28°C (n = 2). AMP 10 =
738 disc containing 10 µg/ml ampicillin, AMP 10/SUL 10 = disc containing 10 µg/ml ampicillin and
739 10 µg/ml sulbactam, a broad-spectrum β-lactamase inhibitor. Data represent the mean (±
740 standard deviation, SD) of three independent experiments. **** = $P < 0.0001$; *** = $P < 0.001$;
741 ** = $P < 0.01$; * = $P < 0.1$; ns = not significant. **(F)** Determination of β-lactamase production
742 was performed via a nitrocefin assay using cell lysates. "No AMP" or "AMP 25" indicates cells

743 untreated or treated, respectively, with ampicillin 25 μg/ml for 2 h before the generation of cell

744 lysates. Data represents one out of two biological replicates (see **Supplemental Fig. 1D**).



745 **Figure 2**

746 **FIG 2. Loss of AmpD results in constitutive β-lactamase activity and elevated ampicillin**

747 **resistance. (A)** Ampicillin susceptibility assay was performed via spotting dilutions. Briefly,
748 indicated strains were cultured ON at 28°C, serially diluted, spotted on solid medium
749 containing no ampicillin (No AMP) or ampicillin 25, 80, or 160 µg/ml (AMP 25, 80, 160), and
750 incubated at 28°C for ~40 h before imaging. **(B)** Growth of WT *A. tumefaciens* and *ΔampD* in
751 the absence (No AMP) and presence (AMP) of various concentrations of ampicillin 25, 100
752 µg/ml (AMP 25, AMP 100) for 24 h (n=1, 2 replicates). **(C)** Quantitative analysis of phase-
753 contrast microscopy of exponentially-growing strains treated with ampicillin 25 µg/ml (AMP
754 25). % Phenotype was calculated by counting the number of cells displaying one of the
755 indicated phenotypes (1 cell = 1 phenotype). **(D)** Determination of β-lactamase production
756 was performed via a nitrocefin assay using cell lysates. "No AMP" or "AMP 25" indicates cells
757 untreated or treated, respectively, with ampicillin 25 µg/ml for 2 h before the generation of cell
758 lysates. *ΔampD* lysates were normalized based on total protein content (7.5 µg/ml) and
759 subsequently diluted to 1:5 as the rate of nitrocefin hydrolysis was significantly faster than the
760 controls (WT, WT AMP 25). Finally, data represents one out of two biological replicates (see
761 **Supplemental Fig. 2**). **(E)** Disc susceptibility was performed on lawn of indicated strains
762 grown on LB plates for 24 h at 28°C. Blank = no antibiotic disc, AMP 10 = disc containing 10
763 µg/ml ampicillin, AMP 10/SUL 10 = disc containing 10 µg/ml ampicillin and 10 µg/ml
764 sulbactam, a broad-spectrum β-lactamase inhibitor. Data represent the mean (± standard
765 deviation, SD) of three independent experiments. **** = $P < 0.0001$; *** = $P < 0.001$; ** = $P <$
766 0.01; * = $P < 0.1$; ns = not significant.

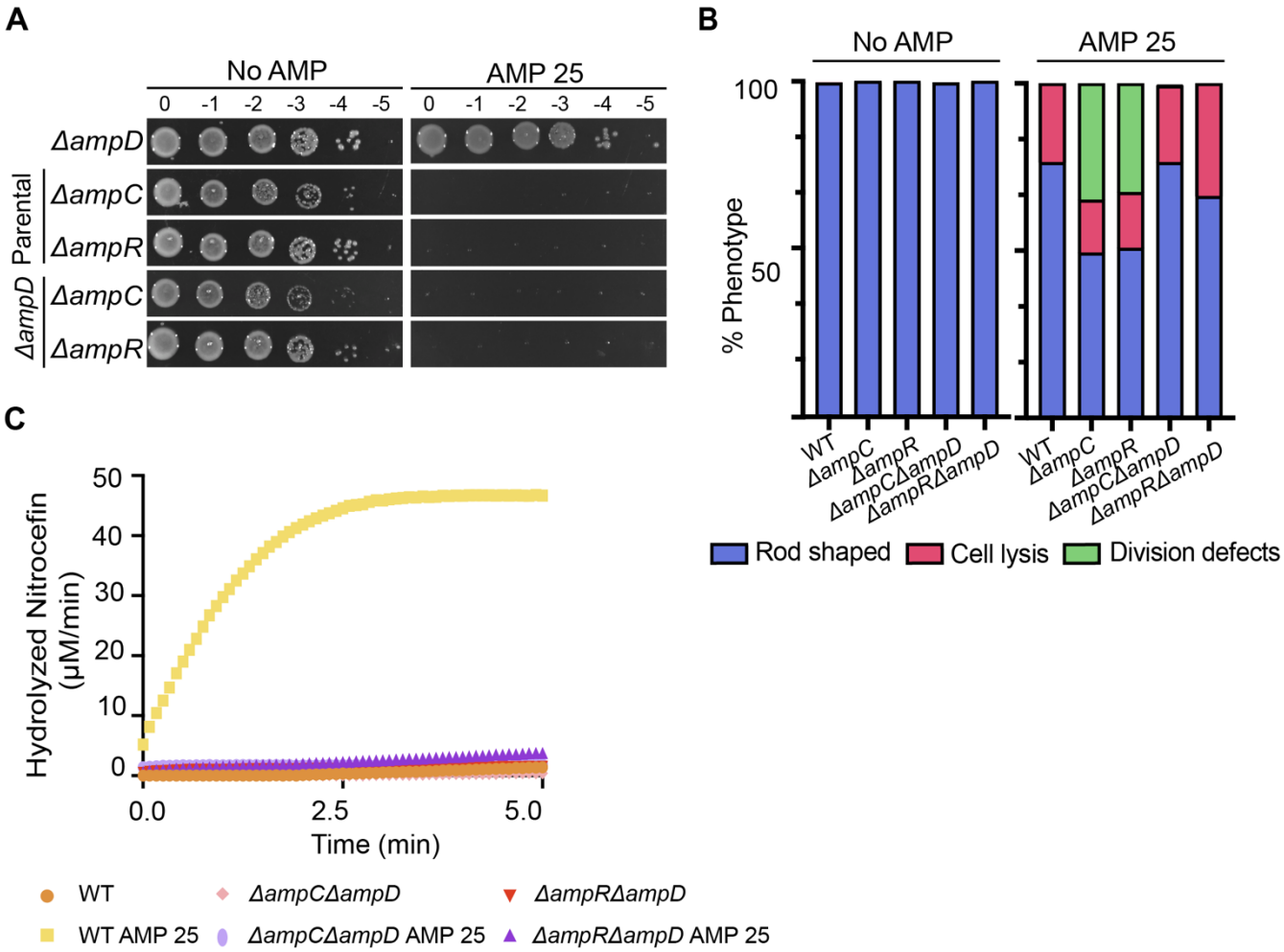


Figure 3

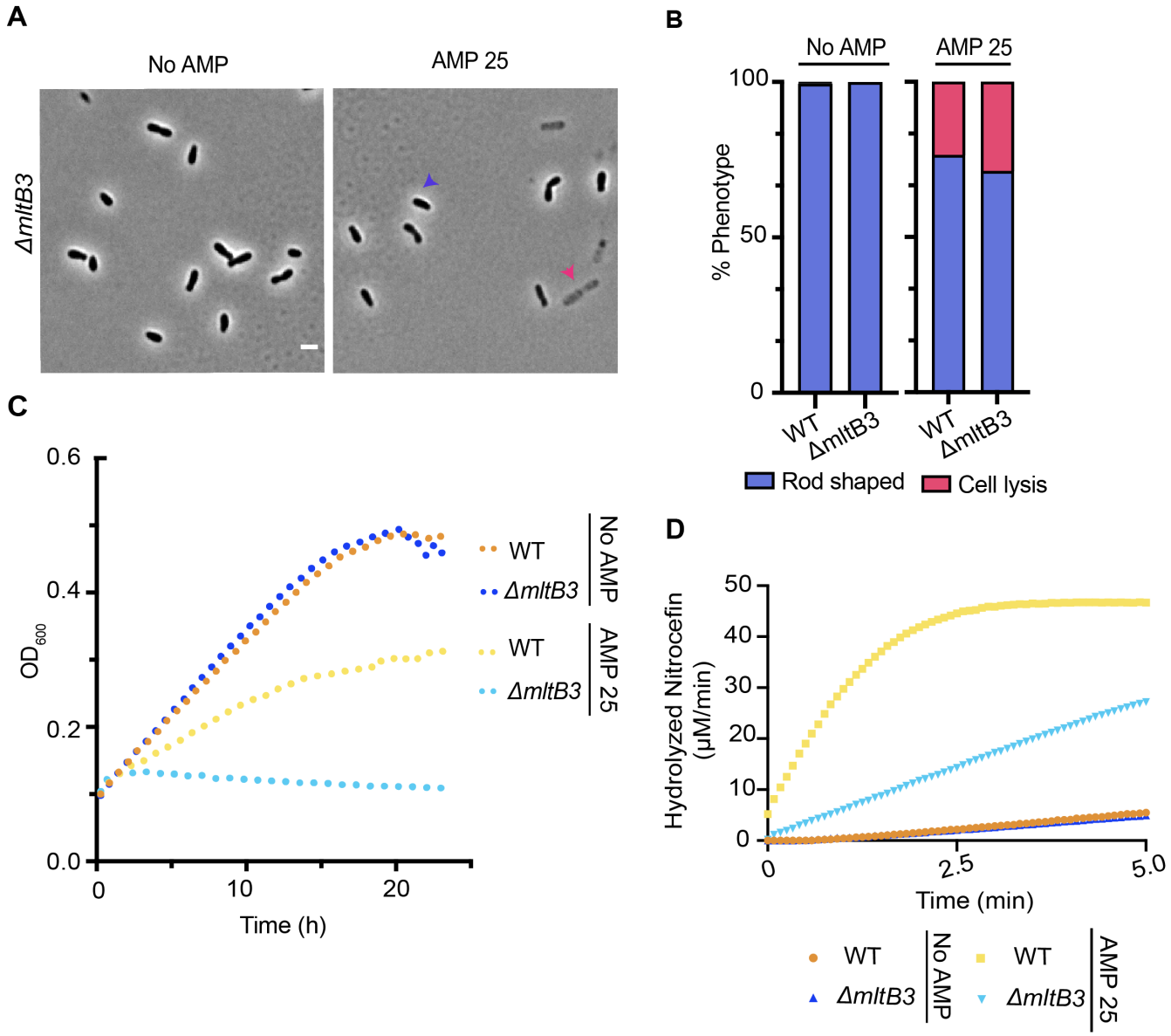
FIG 3. AmpC is the β-lactamase that confers *ΔampD* elevated ampicillin resistance. **(A)** Ampicillin susceptibility assay was performed via spotting dilutions. Briefly, indicated strains were cultured ON at 28°C, serially diluted, spotted on solid medium containing no ampicillin (No AMP) or ampicillin 25 µg/ml (AMP 25) and incubated at 28°C for ~40 h before imaging. **(B)** Quantitative analysis of phase-contrast microscopy of exponentially-growing strains untreated (No AMP) or treated with ampicillin 25 µg/ml (AMP 25). % Phenotype was calculated by counting the number of cells displaying one of the indicated phenotypes (1 cell = 1 phenotype). **(C)** Determination of β-lactamase production was performed via a nitrocefin assay using cell lysates. “No AMP” or “AMP 25” indicates cells untreated or treated,

777 respectively, with ampicillin 25 μg/ml for 2 h before the generation of cell lysates. Data

778 represents one out of two biological replicates (see **Supplemental Fig. 3**).

779

780



781 **Figure 4**

782 **FIG 4. MtlB3 is required for ampicillin resistance in *A. tumefaciens*. (A)** Phase-contrast

783 microscopy of exponentially-growing strains treated with ampicillin 25 μg/ml (AMP 25) for 2 h.

784 **(B)** Quantitative analysis of phase-contrast microscopy of exponentially-growing strains

785 treated with ampicillin 25 μg/ml (AMP 25) for 2 h. % Phenotype was calculated by counting

786 the number of cells displaying one of the phenotypes indicated (1 cell = 1 phenotype) and
787 diving it by the total number of cells per strain. **(C)** Growth of WT *A. tumefaciens* and *ΔmltB3*
788 in the absence (No AMP) and presence of ampicillin 25 µg/ml (AMP 25) for 24 h (n = 1, 2
789 replicates). **(D)** Determination of β-lactamase production was performed via a nitrocefin assay
790 using cell lysates. “No AMP” or “AMP 25” indicates cells untreated or treated, respectively,
791 with ampicillin 25 µg/ml for 2 h before the generation of cell lysates. Data represents one out
792 of two biological replicates (see **Supplemental Fig. 4**).
793

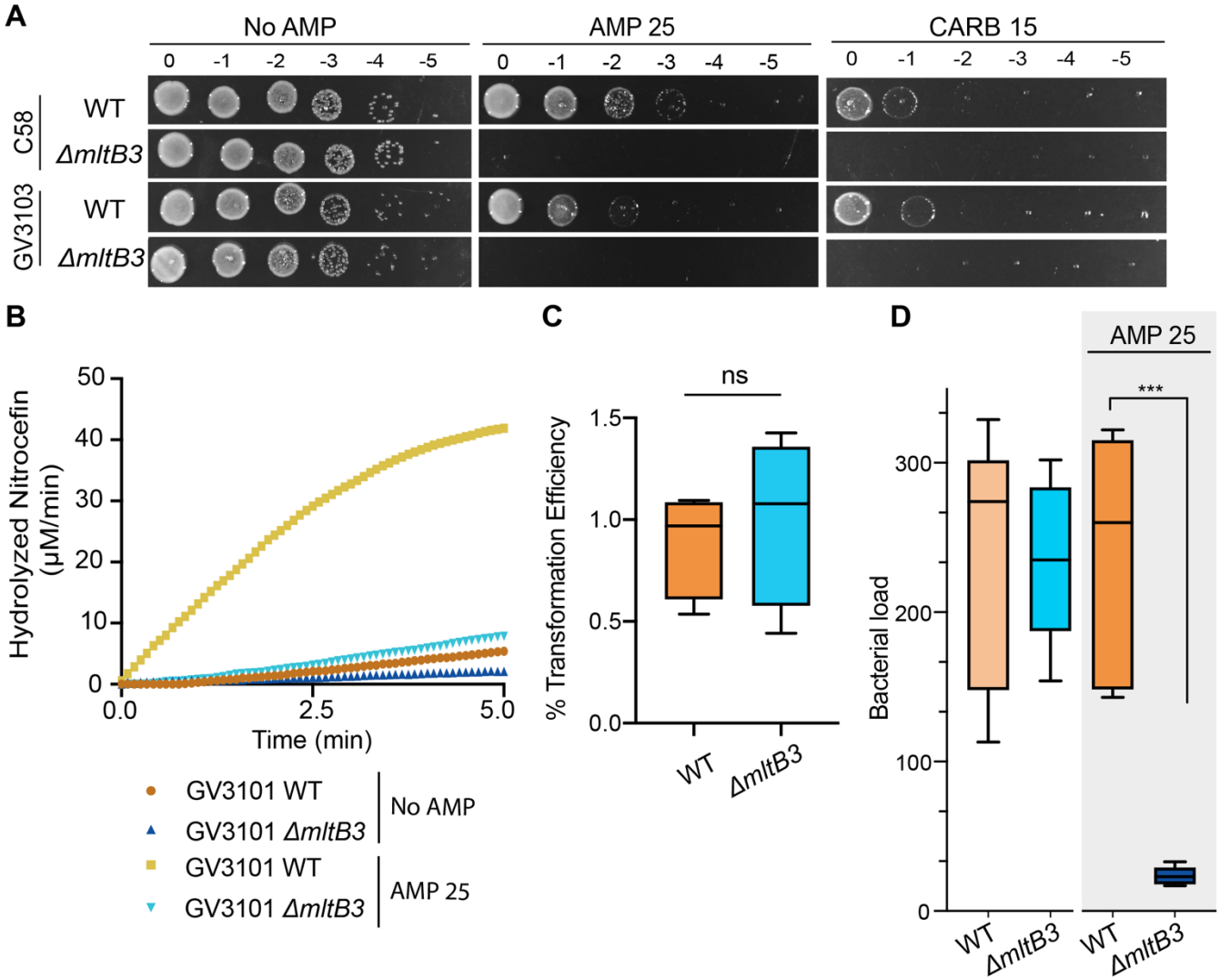
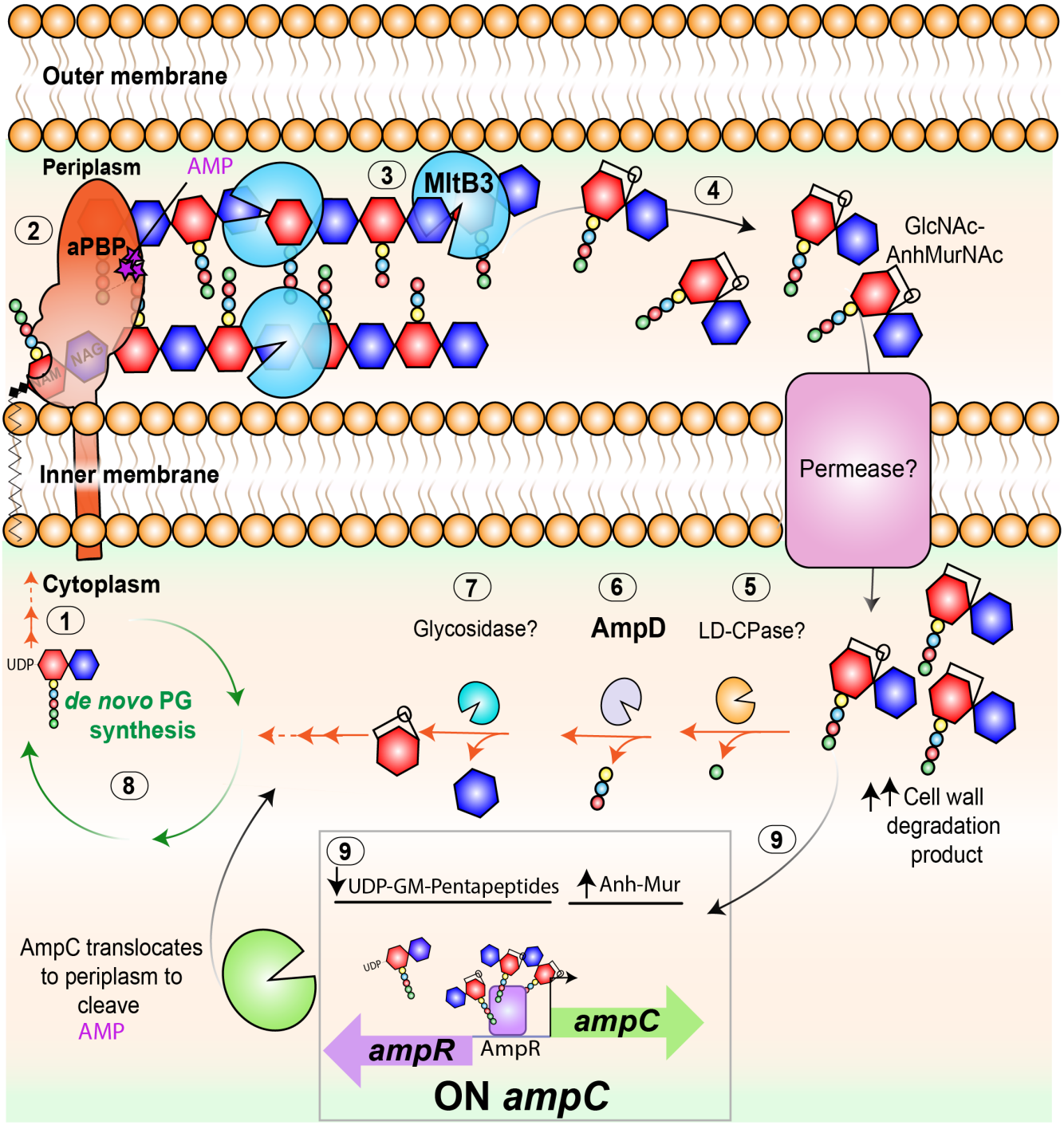


Figure 5

FIG 5. *A. tumefaciens* GV3103 *ΔmltB3* can be used to transform plants efficiently and bacteria can be removed using low concentrations of ampicillin. (A) Ampicillin susceptibility assay was performed via spotting dilutions. Briefly, indicated strains were cultured ON at 28°C, serially diluted, spotted on solid medium containing no ampicillin (No AMP), ampicillin 25 µg/ml (AMP 25), or carbenicillin 15 µg/ml (CARB 15), and incubated at 28°C for ~40 h before imaging. **(B)** Determination of β-lactamase production was performed

802 via a nitrocefin assay using cell lysates. Data represents one out of two biological replicates
803 (see **Supplemental Fig. 5**). “No AMP” or “AMP 25” indicates cells untreated or treated,
804 respectively, with ampicillin 25 µg/ml for 2 h before the generation of cell lysates. **(C)**
805 Transformation efficiency and **(D)** bacterial loads of seeds transformed with WT *A.*
806 *tumefaciens* GV3101 and GV3101 *ΔmltB3* using the floral dip assay technique (85).



807 **Figure 6**

808 **FIG 6. Working model for *A. tumefaciens* ampicillin resistance**. Bifunctional PBPs extend
809 the cell wall through the transglycosylation (linking of carbohydrates) and transpeptidation
810 reactions (linking of peptide stems). β-lactams such as ampicillin (purple stars) target the

811 transpeptidase domain of Penicillin-Binding Proteins (PBPs) (**Fig S6**, Step 1-2), leading to a
812 block in bacterial cell growth. In *A. tumefaciens*, treatment with ampicillin leads to induction of
813 β-lactamases. Inactivation of the lytic transglycosylase MltB3 results inhibition of β-lactamase
814 derepression and lysis suggesting that MltB3 is likely required for the generation of cell wall
815 degradation products (**Fig S6**, Step 3). Similarly to treatment with β-lactams, where a block in
816 cell growth leads to an increase in cell wall degradation products (**Fig S6**, Step 5), inactivation
817 of anhydro amidases such as AmpD increases the poll of this molecules leading to β-lactam
818 resistance. In *A. tumefaciens* inactivation of AmpD leads to derepression of β-lactamases and
819 ampicillin resistance (**Fig S6**, Step 9). Both AmpC, an inducible β-lactamase that is under the
820 transcriptional control of AmpR, and AmpR seem to be responsible for the derepression
821 observed in *ΔampD.* Thus, our working model suggests that upon ampicillin exposure, a block
822 in growth leads to increased activity of MltB3. An increase in cell wall degradation products
823 leads to induction of AmpC expression by AmpR and the presumed translocation of AmpC to
824 the periplasm resulting in ampicillin resistance.
825