

1 **Characterization of a membrane enzymatic complex for**
2 **heterologous production of poly- γ -glutamate in *E. coli***

3
4 Bruno Motta Nascimento, Nikhil U. Nair*

5
6 Department of Chemical and Biological Engineering, Tufts University, Medford, MA, 02155,
7 USA

8 *Corresponding author: Nikhil.Nair@tufts.edu, @nair_lab

9 Present/permanent address: Science and Technology Center #276, 4 Colby St., Medford, MA
10 02155.

11

12 **HIGHLIGHTS**

- 13 • Successfully expressed active poly- γ -glutamate synthetase (PGS) in *E. coli*.
- 14 • Confirmed PGS localization at inner membrane of *E. coli*.
- 15 • Elucidated topology of PGS components in *E. coli* membrane.
- 16 • Culture and expression in microplates might allow future screening of a high number of
17 samples.
- 18 • Faster production of poly- γ -glutamate in *E. coli* supernatant compared to *B. subtilis*.

19

20 **ABSTRACT**

21

22 Poly- γ -glutamic acid (PGA) produced by many *Bacillus* species is a polymer with many
23 distinct and desirable characteristics. However, the multi-subunit enzymatic complex responsible
24 for its synthesis, PGA Synthetase (PGS), has not been well characterized yet, in native nor in
25 recombinant contexts. Elucidating structural and functional properties are crucial for future
26 engineering efforts aimed at altering the catalytic properties of this enzyme. This study focuses
27 on expressing the enzyme heterologously in the *Escherichia coli* membrane and characterizing
28 localization, orientation, and activity of this heterooligomeric enzyme complex. In *E. coli*, we
29 were able to produce high molecular weight PGA polymers with minimal degradation at titers of
30 approximately 13 mg/L in deep-well microtiter batch cultures. Using fusion proteins, we
31 observed, for the first time, the association and orientation of the different subunits with the inner
32 cell membrane. These results elucidate provide fundamental structural information on this poorly
33 studied enzyme complex and will aid future fundamental studies and engineering efforts.

34

35 **Keywords:** heterologous, poly-gamma-glutamate, synthetase, biopolymer, localization,
36 membrane

37

38 **Abbreviations:**

39 PGA – poly- γ -glutamic acid

40 PGS – poly- γ -glutamic acid synthetase

41 MG $\Delta\Delta$ – *E. coli* MG1655 ^{Δ recA Δ endA}

42 RFU – relative fluorescence unit

43 OD – optical density

44 MW – molecular weight

45 EV – empty vector

46

47 **1. INTRODUCTION**

48

49 Poly- γ -glutamate synthetase (PGS) is a multimeric enzyme present in some *Bacillus* species
50 and a few other organisms. While some other amide ligases are able to synthesize poly-glutamate
51 with α -amide linkages between the amino and α -carboxyl groups of L-glutamate (Hamano et al.,
52 2013; Kino et al., 2011), PGS produces an unusual anionic polymer with γ -amide linkages,
53 where the amino group reacts with the side chain carboxyl group of glutamate. In its natural cell
54 environment, this poly- γ -glutamate (PGA) polymer functions as a glutamate storage, a
55 cryoprotective material, and a protection against protease attacks and pH changes near the cell
56 surface (Ashiuchi and Misono, 2003, 2002). *Bacillus* cultures that produce PGA usually have a
57 highly viscous appearance and they have been used in Japan for a long time to produce
58 fermented food products such as natto (Ashiuchi and Misono, 2002). Each species/strain presents
59 a different preference for glutamate enantiomer utilization and molecular size distribution of the
60 final polymer. *Bacillus anthracis* has a PGA composed of only D-glutamate, while *Bacillus*
61 *halodurans* uses only L-glutamate for polymer production.

62 For industrial applications, most studies have relied on biosynthesizing PGA in *B. subtilis*
63 (Ashiuchi, 2013; Halmschlag et al., 2019; Park et al., 2005; Wang et al., 2017) and other *Bacillus*
64 species (Feng et al., 2017, 2015; Ogunleye et al., 2015; Tian et al., 2014; Xavier et al., 2019;
65 Yoon et al., 2000), although there have been significant efforts in moving the complex to
66 recombinant hosts such as *E. coli* (Ashiuchi et al., 1999; Cao et al., 2013, 2011; Jiang et al.,
67 2006; Liu et al., 2019; Wang et al., 2011), *Corynebacterium glutamicum* (Xu et al., 2019), and
68 even tobacco plants (Tarui et al., 2005). A major advantage of recombinant hosts is that PGA
69 synthesis can be decoupled from native cellular regulatory processes and engineered for higher
70 productivity, yield, stereochemical composition, and molecular weight. Further, the absence of
71 any endogenous PGA hydrolytic enzyme (e.g. PgsD and GGT (Ojima et al., 2019; Scoffone et
72 al., 2013)) ensures higher product stability during culture. Despite significant molecular and
73 bioprocess engineering efforts, there have been few efforts focused on characterizing the
74 structure, assembly, and function of this enzyme complex. Current research findings support the
75 hypothesis that PGS is a membrane-associated protein comprised of four subunits – PgsB, PgsC,
76 PgsA, and PgsE, as proposed previously (Ashiuchi, 2013) – and that polymerization of glutamate
77 occurs concurrently with secretion of the growing chain. However, the role and membrane
78 localization of the different subunits have not been well-characterized. Based on all these
79 previous insights, our study is focused on producing the PgsBCAE enzymatic complex from *B.*
80 *subtilis* in *E. coli* and analyzing if it was enzymatically active and localized correctly to the
81 membrane in this non-native host. We characterize PGS by co-expressing different combinations
82 of its four subunits and fusing different reporters to assess membrane (co-)localization and
83 orientation. We determine the orientation of the different subunits within the cytoplasmic and

84 periplasmic space. Results from this work shed light into the assembly of PGS and will aid in
85 future engineering efforts.

86

87 **2. MATERIALS AND METHODS**

88

89 *2.1. Strains and culture media*

90 Bacterial strains used for this project are listed in Table S1. *Escherichia coli* NEB5 α
91 competent strain was used during the cloning steps. *E. coli* MG1655(DE3) ^{Δ recA, Δ endA} was used for
92 recombinant gene expression. Chemically competent cells were prepared by the calcium
93 chloride/MES method. Luria-Bertani (LB) media (VWR Life Science, #97064-110) was used for
94 propagation, preservation of bacterial cells and expression experiments. In the case of solid
95 medium, 18 g/L of bacteriological agar was added, and whenever the cells were transformed
96 with plasmids, the appropriate antibiotic was added to the cooled medium at the recommended
97 final concentration (25 μ g/mL chloramphenicol or 100 μ g/mL ampicillin). Isopropyl β -D-1-
98 thiogalactopyranoside (IPTG) was added for induction, and its concentration varied among
99 experiments. Growth in liquid media was done in orbital shaker at 250 rpm and 37 °C, unless
100 noted otherwise. “Magic” medium was used in initial expression trials, and constituted of 15 g/L
101 LB broth (VWR Life Science, #97064-110), 5 g/L glucose, 10 g/L yeast extract, 2 g/L Tris, 4
102 mL/L glycerol, 55 mM K₂HPO₄, 15 mM KH₂PO₄, 10 mM MgSO₄, and 10 mM (NH₄)₂SO₄
103 prepared with tap water.

104

105 2.2. Cloning and expression

106 Plasmid extraction was performed using the E.Z.N.A.[®] Plasmid DNA Mini Kit I (OMEGA
107 Bio-tek, #D6943-02). Horizontal DNA electrophoresis in agarose gel was performed in 1× TAE
108 buffer according to (Sambrook and Russell, 2001). Purification of DNA fragments from agarose
109 gels used the MicroElute[®] Gel Extraction Kit (OMEGA Bio-tek, #D6294-02). DNA
110 concentration was quantified by UV absorbance with the SpectraMax M3 microplate reader
111 using a SpectraDrop Micro-Volume Microplate (Molecular Devices). DNA amplification with
112 Taq DNA polymerase or Phusion[®] High-Fidelity DNA polymerase was performed according to
113 manufacturer recommendations (New England Biolabs, Thermo Fisher Scientific). DNA
114 digestion with restriction enzymes, DNA ligation with T4 DNA ligase, and DNA assembly with
115 NEBuilder kit were done according to the manufacturer recommendations (New England
116 Biolabs). DNA sequences of constructs were confirmed by sequencing with the appropriate
117 primers, which was performed by GENEWIZ (Boston Lab, Cambridge, MA). The *pgsBCAE*
118 operon was amplified from *B. subtilis* 168 genome and used as template for all the constructs. In
119 general, the PGS subunits were inserted in a pACYC-Duet1 plasmid under control of a T7
120 promoter. Different combinations of the subunits in a operon inducible by IPTG, generating the
121 plasmids described on Table S2. Later, tagged versions of each subunit were created at the C
122 terminus of each protein a 12 amino acid linker and different reporters (6xHis tag, sfGFP,
123 mCherry, GFPuv, PhoA).

124 For construction of *E. coli* strain MG $\Delta\Delta$ - Δ phoA, the lambda red recombineering method was
125 used (Datsenko and Wanner, 2000). In brief, MG $\Delta\Delta$ cells were first transformed with pKD46 for
126 lambda red recombinase expression under an arabinose-induced promoter. A deletion cassette

127 was constructed by amplifying part of pKD3 containing a *cat* gene flanked by FRT sites.
128 Flanking both sides of the cassette it was added a 40 bp sequence homologous to the *phoA* gene.
129 This cassette was electroporated in the MG $\Delta\Delta$ pKD46 cells for recombination at the locus site to
130 occur. Selected colonies were finally transformed with pCP20 for removal of the
131 chloramphenicol resistance by action of the FLP recombinase at the FRT sites. Deletion of *phoA*
132 was confirmed by both sequencing of this genomic region and the absence of PhoA activity in
133 the resulting cells.

134

135 2.3. PGA production

136 *E. coli* cells were grown in 10 mL culture tubes containing 5 mL of LB or rich media broth
137 supplemented with 30 g/L L-glutamate, 10 μ M IPTG, and 25 μ g/mL chloramphenicol. Cultures
138 were incubated at 30 °C, 250 rpm, for 24 h. *B. subtilis* strains were also grown in similar
139 conditions, except without the addition of IPTG and antibiotic. The PGA produced in the
140 supernatant was either measured directly or purified by a modified version of the copper
141 precipitation method described by (Manocha and Margaritis, 2010; Yuan et al., 2019). In brief,
142 0.5 M CuSO₄ was added to the supernatant and the solution was mixed by inversion and let stand
143 for 1 h at room temperature. Tubes were centrifuged at 5,000 \times g for 30 min and the pellets were
144 later resuspended in phosphate buffered saline (PBS) pH 7.4 with 50 mM EDTA. The solution
145 was dialyzed with SnakeSkin® Dialysis Tubing 3.5 kDa molecular weight cut-off (MWCO)
146 (Thermo Fisher Scientific, #68035) to remove salts, followed by drying in a vacuum centrifuge
147 (Eppendorf Vacufuge Concentrator 5301).

148

149 2.4. Analytical methods

150

151 2.4.1. Polymer and amino acids detection

152 The concentration of dialyzed samples of PGA was performed by adapting the cetrimonium
153 bromide (CTAB) turbidity method described by (Halmschlag et al., 2019). To every 100 μ L of
154 sample it was added 50 μ L of CTAB solution (CTAB 0.1 M, NaCl 1 M) and incubated for 5 min
155 without agitation. Turbidity of the resulting solution was measured at 400 nm. Based on a
156 calibration curve using standards of pure PGA (Sigma-Aldrich, #G1049-100MG) in the
157 concentration range of 0.25 – 0.025 g/mL, the concentration of γ -PGA from the culture
158 supernatant was determined. PGA detection by dot blot with nylon membrane also uses a similar
159 staining procedure. The membrane was briefly dried at 37 °C and 2.5 μ L each sample was
160 applied to the membrane. The loaded membrane was dried again at 37 °C for 30 min and fixed
161 with 60 % ethanol for 20 min. Ethanol was evaporated at 37 °C and the dry membrane was
162 stained with 0.04% methylene blue in methanol for 5 – 10 min. Final de-staining was done with
163 25 % ethanol for 30 min. PAGE separation of PGA was done in a NuPAGE Bis-Tris 4 – 12 %
164 gel (Thermo Fisher Scientific, # NP0322BOX) and ran in 1 \times MOPS buffer (50 mM MOPS, 50
165 mM Tris, 0.1 % SDS, 1 mM EDTA, pH 7.7) at 120 V for 2 h. The gel was washed twice with
166 distilled wash for 5 min and stained with 0.03 % methylene blue in 300 mM sodium acetate pH
167 5.2 for 15 min with a gentle rocker agitation. The gel was de-stained by multiple washes with
168 deionized water until the background and clear against the PGA bands (Soto and Draper, 2012).

169

170 2.4.2. PGA hydrolysis and HPLC

171 Purified PGA samples from *E. coli* were hydrolyzed 6 M HCl at 100 °C for 4 h in a vacuum
172 sealed tube (Chemglass, #CG-4025-01). Acid was immediately removed with a vacuum
173 centrifuge and pellet was resuspended in deionized water. The presence of glutamate in the
174 hydrolyzed samples was analyzed on an Agilent 1100 Series HPLC System, equipped with a
175 Poroshell 120 HILIC-Z column (guard: 2.5 × 5 mm, 2.7 μm; main: 2.1 × 150 mm, 2.7 μm) and
176 a diode array detector (Agilent, G1315B) measuring a signal at 338 nm wavelength (bandwidth =
177 10 nm) using reference wavelength 390 nm (bandwidth = 20 nm). Amino acid samples were
178 derivatized with Fluoraldehyde *o*-phthaldialdehyde reagent (Thermo Fisher Scientific, #26025)
179 prior injection in the system. The flow rate was constant and set at 0.3 mL/min. Solvents were
180 run in a gradient condition with mobile phase A consisting of 10 mM ammonium acetate at pH
181 9.0 in water and mobile phase B consisting of 10 mM ammonium acetate at pH 9.0 in
182 water:acetonitrile 1:9. After sample injection, mobile phases were run according to the following
183 conditions: 0 – 2 min: 0 % A – 100 % B; 2 – 15 min: linear gradient to 30 % A – 70 % B; 15 –
184 16 min: linear gradient to 45 % A – 55 % B; 16 – 20 min: linear gradient to 0 % A – 100 % B; 20
185 – 25 min: 0 % A – 100 % B.

186

187 2.4.3. Immunodetection of subunits

188 Subunits of PGS with 6xHis tags were detected by western blot. Expressing cells were
189 recovered from culture media and resuspended in 2500 μL phosphate buffered saline (PBS) with
190 50 μL lysozyme 5 mg/mL, 5 μL DNaseI 5 mg/mL, and 2 μL 1M phenylmethylsulfonyl fluoride
191 (PMSF). The cell suspension was lysed by sonication in a BRANSON Sonifier 150 (10 s ON, 1
192 min OFF, 5 min total ON, Amplitude 40 % with microtip). Following sonication, the tubes were

193 centrifuged 3,000 ×g for 15 min at 4 °C to remove debris and un-lysed cells. Proteins from this
194 lysate was quantified by BCA method (Abelson and Simon, 2009) and diluted to same
195 concentration to load similar amounts of protein in each gel lane. These samples were separated
196 in a 4 – 12 % NuPAGE Bis-Tris gel and ran in 1× MES buffer at 120 V until loading dye
197 reached the bottom of the gel. Protein staining was done with SimplyBlue SafeStain (Thermo
198 Fisher Scientific, #LC6060) according to manufacturer instructions. Transference to a PVDF
199 membrane for western blot was done with the Invitrogen Xcell II blot module. Membrane was
200 blocked with 5 % skim milk and antibody labeling used a primary mouse monoclonal anti-6xHis
201 (Thermo Fisher, #MA1-21315) and a secondary rabbit anti-mouse IgG with HRP (Abcam,
202 #ab6728). Chemiluminescence was detected with SuperSignal West Dura Extended Duration
203 Substrate (Thermo Fisher Scientific, #34075).

204

205 *2.4.4. Microscopy of labeled subunits*

206 Cells were pelleted and washed with 1× PBS solution for preparation of microscope slides.
207 A thin 2 % (w/v) agarose pad was prepared on a glass slide and 1 µL of cell suspension was
208 added on top and air dried for about 5 min before placing a glass cover slip (Ke et al.,
209 2016). Imaging was performed with a DMi8 automated inverted microscope (Leica
210 Microsystems, #11889113) equipped with a CCD camera (Leica Microsystems, #DFC300 G),
211 and a TXR (Leica Microsystems, #11525310) and YFP (Leica Microsystems, #11525306) filter
212 cube. The Z-stack of captured images was processed in a blind deconvolution to remove out of
213 focus fluorescence with the LAS X software version 3.3.3.16958 (Leica Microsystems,
214 #11640612) with 3D deconvolution package (Leica Microsystems, #11640865).

215

216 *2.4.5. Detection of membrane orientation*

217 Fusions to PhoA (alkaline phosphatase) are the most widespread periplasmic reporter
218 fusions. Colonies expressing periplasmic PhoA fusions can be visually screened by
219 supplementation of the agar medium with a PhoA-specific substrate 5-bromo-4-chloro-3-indolyl
220 phosphate (X-Pho), yielding blue colonies when enzyme is active in the periplasm (Karimova
221 and Ladant, 2017). Cells were streaked in a LB plate supplemented with 100 µg/mL X-Pho and
222 grown at 30 °C for 4 h. After this period, a filter disc containing 5 nmol IPTG was plated at the
223 center and the cells continued to grow at 30 °C for 24 h. Plates were then photographed to check
224 development of a blue color due to PhoA activity. Conversely, GFPuv folds efficiently in the
225 cytoplasm but does not form stable structure when targeted to the periplasm by a Sec-type signal
226 peptide. GFPuv does fold properly, however, when attached to cytoplasmic domains of inner-
227 membrane proteins (Drew et al., 2002). Cells were inoculated in 5mL LB-IPTG 10 µM media
228 and induced at 30 °C for 4 h with shaking at 250 rpm. After this period, OD₆₀₀ and fluorescence
229 (excitation: 395 nm, emission: 509 nm) were measured with black microplates plates with clear
230 bottom in a SpectraMax M3 microplate reader.

231

232 *2.5. Bioinformatic analysis*

233 An initial prediction of each protein interaction with the cell membrane was performed with
234 the Constrained Consensus TOPology prediction server (CCTOP) (Dobson et al., 2015). It uses a
235 combination of prediction methods (HMMTOP, Memsat, Octopus, Philius, Phobius, Pro, Prodiv,
236 Scampi-single, Scampi-msa, TMHMM, SignalP) and generates a consensus topology with

237 increased prediction accuracy. Homology model were also constructed with the SWISS-MODEL
238 server (Schwede et al., 2003).

239

240 **3. RESULTS AND DISCUSSION**

241

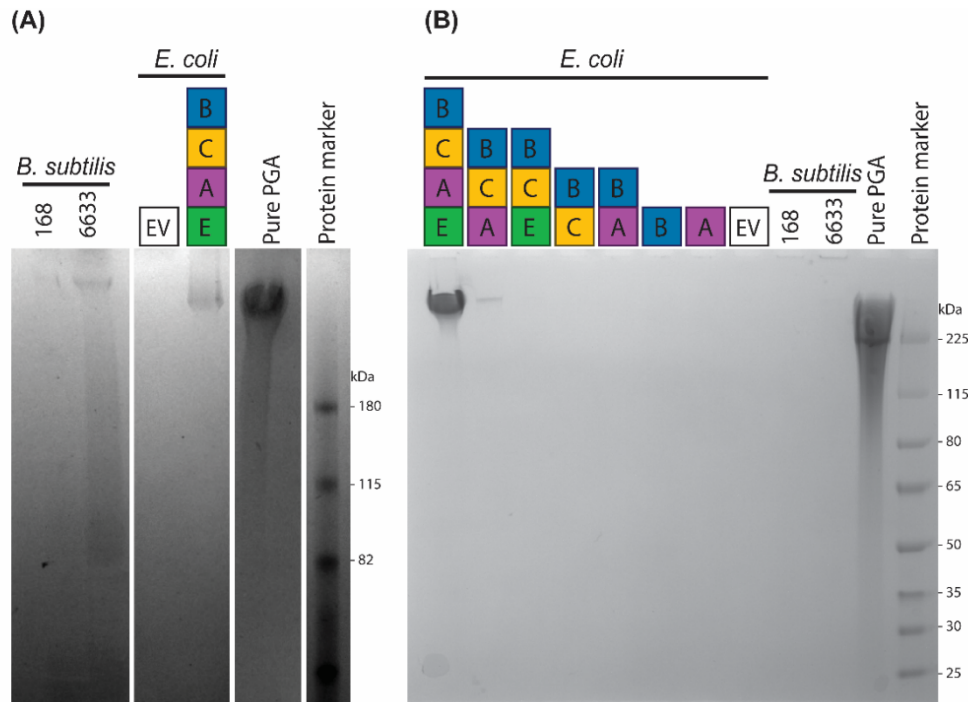
242 *3.1. Functional expression of recombinant PGA synthetase in E. coli*

243 Initially, *E. coli* MGΔΔ cells expressing all PGS subunits (PgsBCAE) and wild type *B.*
244 *subtilis* strains (168 and 6633) were cultured in “Magic” medium supplemented with 30 g/L L-
245 glutamate. Cultures were grown at 30 °C (*E. coli*) and 37 °C (*B. subtilis*) – a lower incubation
246 temperature was chosen for the former because burden of expression resulted in poor growth,
247 probably due to membrane destabilization by the enzyme complex and the polymer produced.
248 The crude fermented broth was recovered and run on a PAGE and the PGA was detected using
249 methylene blue staining (Fig 1A). We found that the polymer produced by these bacteria have a
250 high molecular weight (HMW), comparable to the pure commercial HMW PGA (>750 kDa). To
251 reduce burden of expression, we moved the operon to a low-copy vector, which significantly
252 improved growth of the *E. coli* transformants, allowing us to switch from “Magic” medium to
253 glutamate-supplemented LB. We then tested which of the components are essential for PGA
254 synthesis using strains expressing PgsBCAE, PgsBCA, PgsBCE, PgsBC, PgsBA, PgsB, or PgsA.
255 Cultivation conditions were scaled down to a 24-deepwell plate containing 5 mL of media,
256 sealed with aluminum film, and agitated at 700 rpm in a microplate shaker. After 24 h, a fraction
257 of the supernatant was collected and precipitated with a copper solution as described in materials

258 and methods to obtain a clearer PAGE staining result (Figure 1B). With purified PGA, we could
259 also quantify the polymer by the CTAB method and estimate a concentration of 13 mg/L and <
260 1.6 mg/L by PgsBCAE and PgsBCA expressing *E. coli*, respectively (Figure S1). None of the
261 other constructs tested in *E. coli*, nor wildtype *B. subtilis* 168, produced any detectable amounts
262 of PGA under these conditions. These results also indicate that while PgsE is not essential for
263 activity or processivity (Ashiuchi et al., 2013; Yamashiro et al., 2011a, 2011b), its presence
264 enhances PGS activity. The non-essentiality of PgsE corroborates with other published studies
265 where only the PgsBCA subunits were used for expression (Ashiuchi and Misono, 2002; Cao et
266 al., 2011, 2010; Jiang et al., 2006).

267 To confirm that the band detected in the PAGE corresponded to PGA, the purified samples
268 from a PgsBCAE expressing *E. coli* culture and a commercial PGA (Sigma-Aldrich, #G1049-
269 100MG) were hydrolyzed with HCl and analyzed by HPLC and compared to pure L-glutamate.
270 The hydrolysate presented a single peak with a elution time similar to pure L-glutamate (Figure
271 S2), suggesting that the polymer we purified is indeed PGA and not any other negatively-
272 charged polymer that may be interacting with the methylene blue stain.

273



274
275 Figure 1 – PGA produced by bacterial strains detected in cell-free culture supernatants by PAGE.
276 (A) 48 h (“magic” medium) culture supernatants from *B. subtilis* (strain 168 and 6633) and *E.*
277 *coli* MGΔΔ expressing PgsBCAE on high-copy vector. Purchased pure PGA with molecular
278 weight > 750 kDa was used as a positive control for methylene blue staining. Rightmost lane
279 contains a protein MW marker for reference. (B) Precipitated PGA from 24 h culture supernatant
280 (LB medium) from *B. subtilis* and *E. coli* MGΔΔ with PgsBCAE construct on a low-copy vector.
281 EV = empty vector negative control.
282

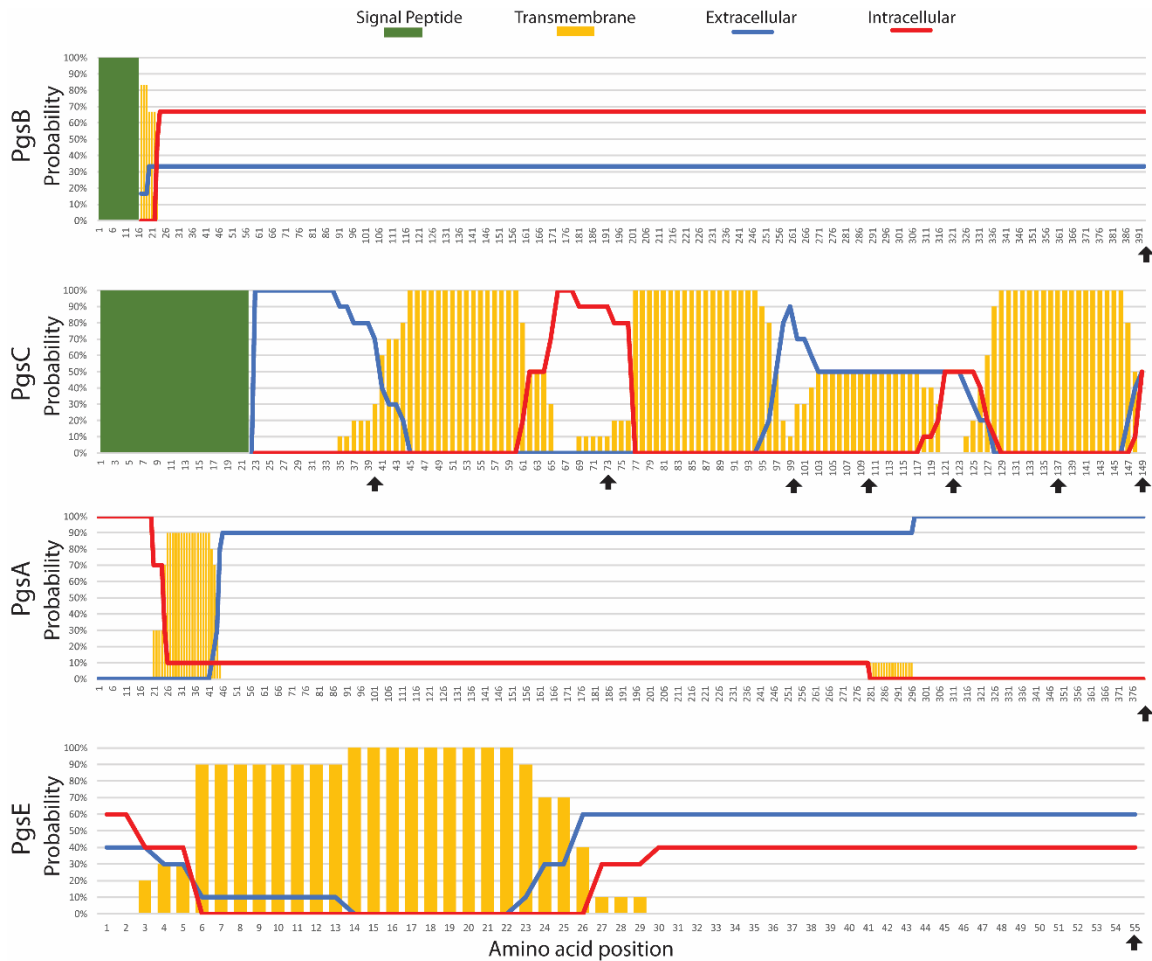
283 3.2. Topological prediction and expression of individual PGA synthetase subunits

284 The amino acid sequence of each PGS subunit was analyzed by different prediction tools in
285 CCTOP to attest the presence of possible signal peptides and transmembrane regions. Due to the
286 nature of each prediction algorithm and the training set that each of them uses, CCTOP compiles
287 all results and outputs a probable topology for the sequence (Figure 2). The algorithm predicted a
288 signal peptide for PgsB and PgsC but only presence of a transmembrane domain for PgsA and
289 PgsE. Overall, this indicates that there is a high probability of co-localization of these proteins at
290 cell membrane.

291 We also performed a created a homology model for each subunit (Figure S4). Sequence
292 alignment by ClustalO (Sievers et al., 2011) of PgsB with *E. coli* Mur family and FolC proteins
293 shows some conserved motifs related the ATP binding site. Construction of PgsB homology
294 structure model with SWISS-MODEL server (Schwede et al., 2003) presented highest similarity
295 of ~29% and sequence coverage ~80% to proteins in the Mur ligase family, which also catalyze
296 peptide bonds (Smith, 2006). These analyses are consistent with previous studies that PgsB is
297 homologous to other amide ligases and is the subunit responsible for catalysis (Ashiuchi et al.,
298 2001).

299 To confirm the correct localization of these proteins in *E. coli*, individual PGS subunits were
300 linked to a 6xHis tag for detection. Cells expressing tagged subunits were lysed and the soluble
301 fractions (containing the soluble cytoplasmic proteins and small membrane fractions) and
302 analyzed by SDS-PAGE and Western Blotting. All proteins have the expected size (PgsB: 44
303 kDa, PgsA: 43 kDa, PgsE: 7 kDa), except for PgsC (16 kDa), which was not detected (Figure 3).
304 From the sequence analysis (Figure 2), we expect PgsC to have a lot of interactions with the
305 membrane, which likely hinders proper immunodetection within the soluble fraction.
306 Interestingly, even though PgsA and PgsE are expected to have significant transmembrane
307 interactions, we were able to detect them in the soluble fraction, suggesting relatively weak
308 membrane anchoring.

309



310

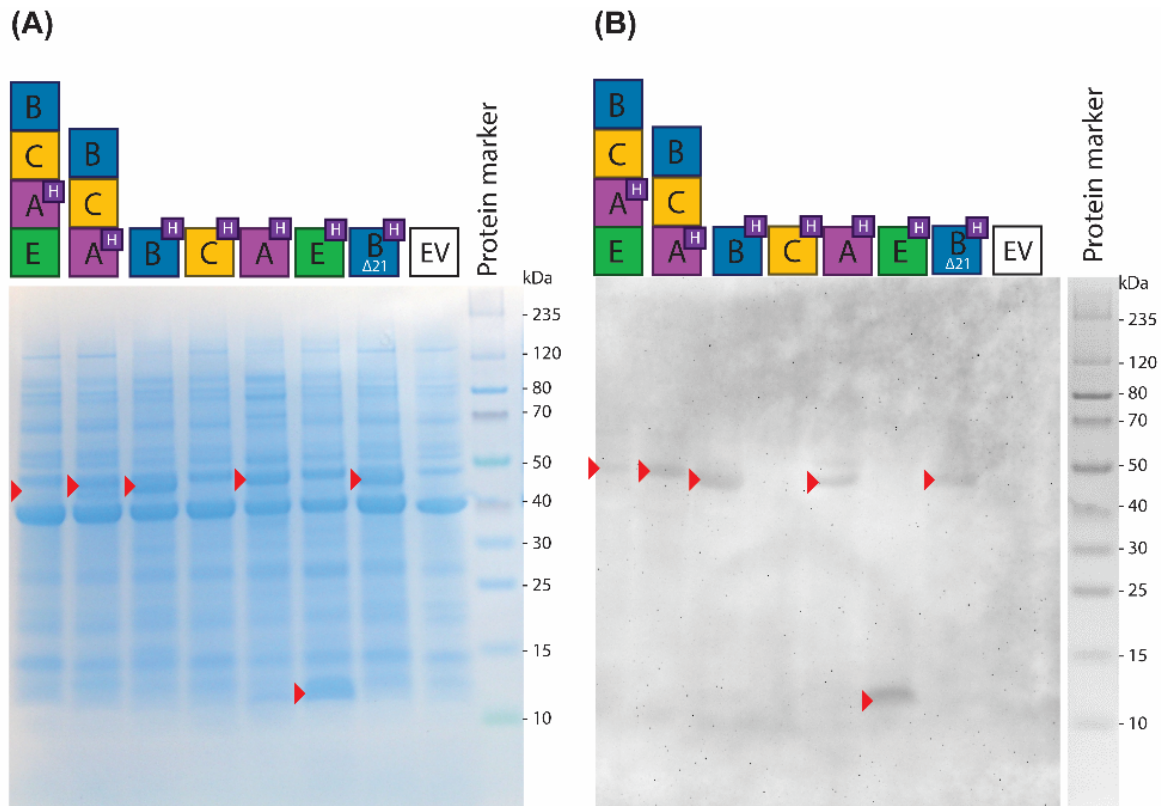
311 Figure 2 – Topology prediction by CCTOP (Dobson et al., 2015). Each PGS subunit was
 312 analyzed individually by multiple prediction algorithms, and CCTOP combined the probability
 313 of each amino acid being intracellular, transmembrane, extracellular, or part of a signal peptide.
 314 Black arrows indicate residues where fusions are constructed (*vide infra*).
 315

316 We also prepared an additional construct of PgsB designated PgsB Δ 21, which does not
 317 contain the predicted 21 amino acid N-terminal signal peptide. We expect that this variant of the
 318 protein to not be directed to the membrane and remain in the cytoplasm. We were also able to
 319 detect C-terminally tagged PgsA as part of operon structure (*pgsBCAE* and *pgsBCA*), indicating
 320 that presence of other subunits does not occlude the tag. The presence of these proteins in the

321 soluble fraction of the lysate indicate that they are not forming inclusion bodies inside the cell,

322 which is important to note when interpreting that the subsequent microscopy studies.

323



324

325 Figure 3 – (A) SDS-PAGE of *E. coli* cell lysate expressing PgsBCAE subunits with a 6xHis-tag
326 at the C-terminal (labeled with ‘H’ at legend). Red arrows indicate proteins present in the
327 recombinant strains when compared to *E. coli* with empty vector control (EV). (B) Western blot
328 of same samples using anti-6xHis antibodies.

329

330

331 3.3. Localization of PGA synthetase subunits at the membrane

332 Fusing each subunit with a fluorescent protein (sfGFP) enables us to visualize how the

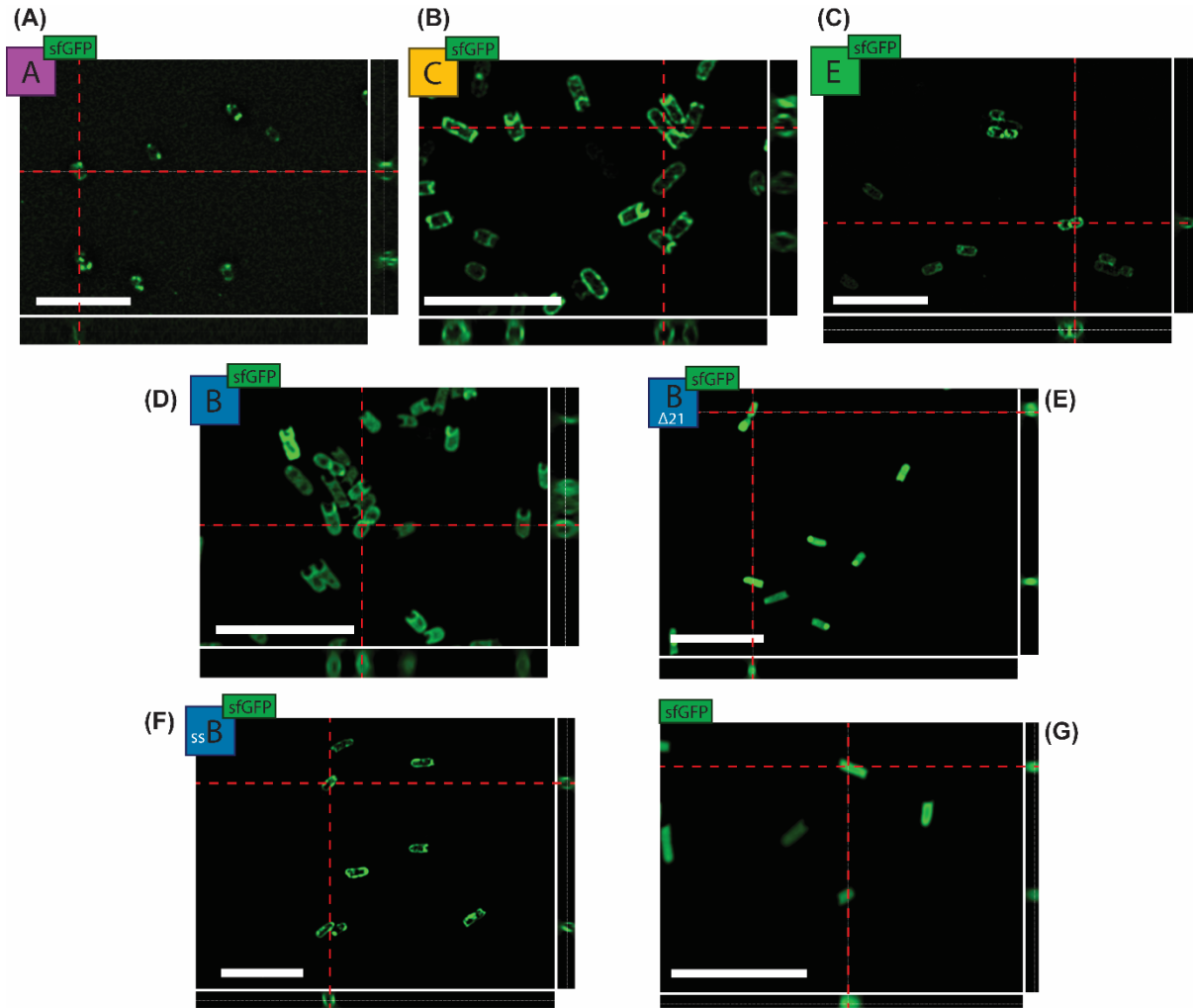
333 different subunits localize in the cell. Superfolder GFP (sfGFP) is a highly efficient and stable

334 folding variant, forming a folding intermediate that prevents the cysteine residues to form

335 disulfide bonds in the periplasm (Dammeyer and Tinnfeld, 2012). Therefore, it fluoresces
336 regardless of whether the C-terminal is exposed to the cytoplasm or periplasm. We fused each of
337 the four subunits at their C-terminal to sfGFP and also created two variants of PgsB – one
338 lacking the initial signal peptide (PgsB Δ 21-sfGFP) and another with just the signal sequence
339 (ssPgsB-sfGFP). The localization of full-length PgsB fusion as well as the signal peptide only
340 fusion is evident at the membrane (Figure 4A, C), especially when compared to the N-terminally
341 truncated (PgsB Δ 21) variant (Figure 4B), where fluorescence is diffuse throughout the
342 cytoplasm. For PgsC, PgsA, and PgsE we also note membrane localization, albeit with lower
343 fluorescence intensities compared to PgsB (Figure 4D–F).

344 To determine that all components of PGS are directed and co-localized to the *E. coli* cell
345 membrane, we created two-fluorophore *pgs* operon constructs. All express PgsC fused to sfGFP
346 and a second component (PgsB, A, or E) tagged with mCherry – giving PgsBrCgAE,
347 PgsBCgArE, and PgsBCgAEr (where r = red/mCherry and g = green/sfGFP). For the
348 PgsBCgAEr, it is evident that both proteins colocalize since we found high correlation between
349 the green and red fluorescence signals (Figure 5). This is indicative that the proteins are correctly
350 expressed in this heterologous host. However, for the other constructs (BrCgAE and BCgAr) we
351 were unable to detect both fluorescent signals simultaneously, even after extensive attempts to
352 optimize induction. We found that insertion of a fluorophore in the operon structure had a strong
353 polarity effect on downstream genes.

354

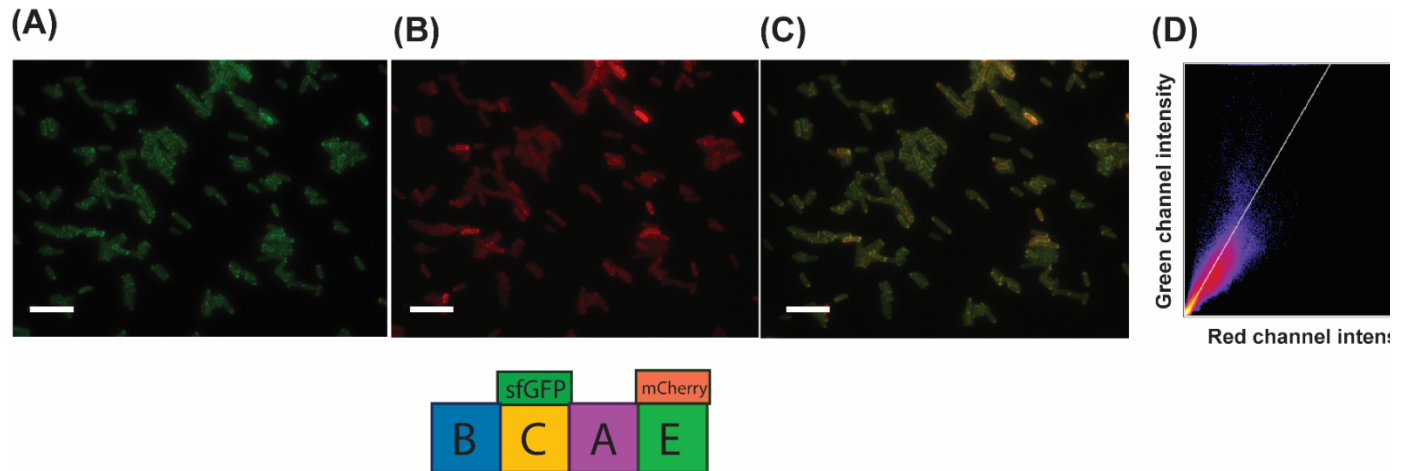


355

356 Figure 4 – (A-D) Fluorescence microscopy of cells expressing individual subunits PgsA, C, E,
357 and B, respectively, each with sfGFP at the C-terminus. Variants of PgsB are (E) without signal
358 peptide (PgsB Δ 21) and (F) and with only the signal peptide (ssPgsB). (G) sfGFP alone as
359 cytoplasmic expression control. Each image represents the blind deconvolution of each captured
360 z-stack. Side images represent orthogonal sections of the z-stack. Scale bar = 10 μ m.

361

362



363

364 Figure 5 - Fluorescence microscopy of cells expressing *pgsBCgAEr* operon, where the PgsC
365 subunit is linked to sfGFP/green and PgsE to mCherry/red, both at the C-terminal. (A) GFP
366 channel. (B) Red channel. (C) Overlay of both channels. (D) Fluorescence intensity of
367 colocalized pixels from the green and red channels. Scale bar = 10 μ m.
368

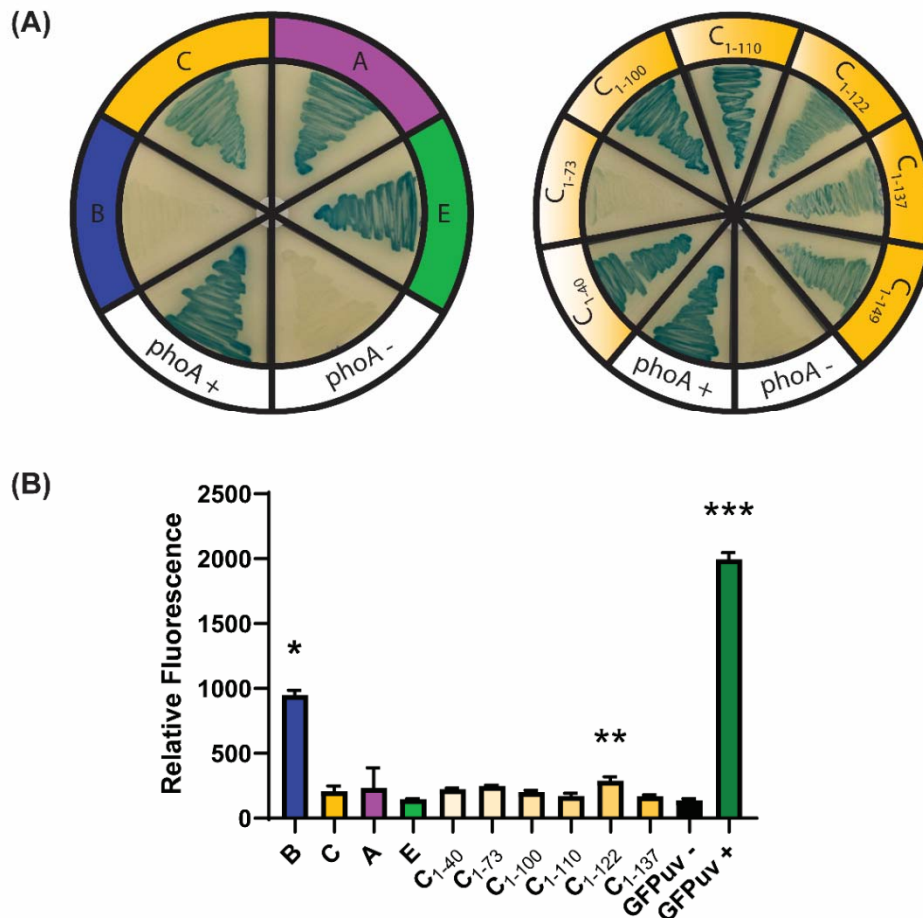
369 3.4. Topology of PGA synthetase subunits

370 Having confirmed that all four components of PGS localize to the membrane and that at least
371 some of them co-localize, we wanted to determine the topology of each the polypeptide. The
372 bioinformatic predictions summarized in Figure 2 (and Figure S3) indicate no clear consensus,
373 likely due to the fact that each method uses a different training set. Therefore, we want to
374 experimentally determine the orientation of the different subunits in the inner membrane. For
375 this, our reporters of choice were alkaline phosphatase (PhoA) and GFPuv as C-terminal fusions.
376 PhoA is an enzyme that only folds correctly and presents activity if exported to the periplasm of
377 *E. coli*, where disulfide bonds can be formed. In the presence of a specific substrate such as 5-
378 bromo-4-chloro-3-indolyl phosphate (X-Pho), colonies expression PhoA in the periplasm
379 develop a blue color, while colonies whose enzyme is expressed in the cytoplasm remains white
380 (Jiménez-Guerrero et al., 2013). Conversely, GFPuv only fluoresces in the cytoplasm since the

381 oxidizing environment in the periplasm promotes the formation of disulfide bonds between C49
382 and C71, which causes misfolding, impeding chromophore maturation (Dammeyer and
383 Tinnefeld, 2012).

384 Based on the predictions summarized in Figure 2, we expect only PgsC to have multiple
385 transmembrane domains, with both cytoplasmic and periplasmic segments. Each of the other
386 subunits are expected to wholly localized to either the cytoplasmic or periplasmic side of the
387 inner membrane. We created PhoA fusions to each full-length PGS component and found that
388 only PgsB did not develop a blue color, indicating that its C-terminus is in the cytoplasm (Figure
389 6A). All other components (PgsA, C, and E) developed a strong blue color signal suggesting
390 their C-termini are periplasmically localized. For further analysis of PgsC, we created specific
391 truncation (indicated by black arrows in Figure 2 and numbered subscripts in Figure 6) and fused
392 them to PhoA. Figure 6A shows that only peptide 1-73 has a negative X-Pho signal, indicating
393 that segments between 40 and 100 of PgsC are within the cytoplasm. All other truncations
394 present as PhoA⁺, indicating periplasmic localization. To further confirm localization, we used
395 the GFPuv fusions. Specifically, constructs that are positive for PhoA should be negative for
396 fluorescence. In Figure 6B, PgsB presented a high signal, corroborating with the previous result
397 that its C-terminus is in the cytoplasm. Since GFPuv fusions to full-length PgsC, PgsA, and PgsE
398 subunits emit fluorescence that is not statistically different from the negative control (GFPuv⁻),
399 we conclude that all these components terminate in the periplasmic space. While fully-folded and
400 active GFPuv can be translocated to the periplasm by the Tat pathway (Drew et al., 2002), it is
401 most likely that these subunits use the Sec-pathway, in which translocation is concurrent with
402 translation. We expected, based on PhoA activity data, that PgsC fragment 1-73 would present a

403 high fluorescence signal. However, only fragment 1-122 had fluorescence statistically different
404 from the negative control, albeit much lower when compared the positive control (GFPuv+).
405 Thus, the GFPuv data are not very conclusive about the topology of PgsC.
406



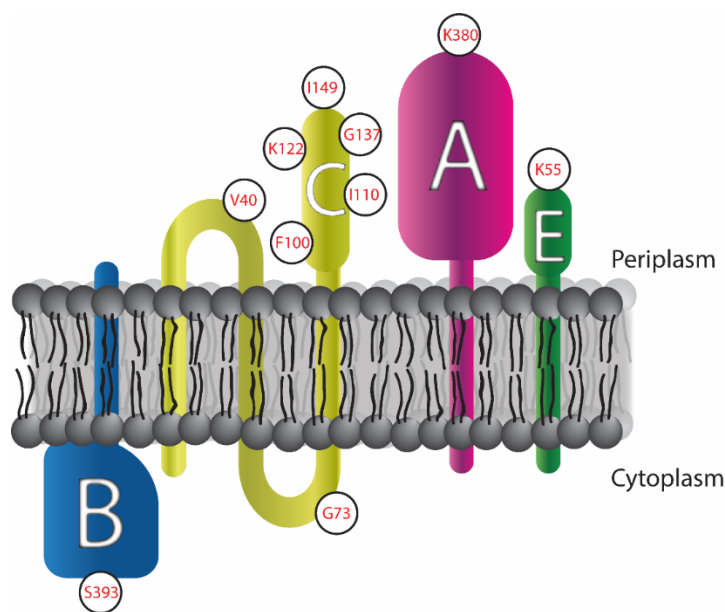
407

408 Figure 6 – (A) LB+X-Pho plates after induction with IPTG. Each section is an *E. coli* MGΔΔ
409 strain expressing a full-length PGS subunit with a PhoA fusion at its C-terminus. At the bottom
410 there is a positive and negative control for PhoA activity. Truncated versions of PgsC were also
411 assayed by the same method. (B) Relative fluorescence (RFU/OD₆₀₀) of *E. coli* MGΔΔ strains
412 expressing each full-length subunit with GFPuv fused at its C-terminus. Negative and positive
413 controls constituted of cells expressing GFPuv in the periplasm and cytoplasm, respectively.

414 Error bars indicate standard deviation of triplicate experiments. Asterisks indicate samples
415 statistically different from negative control ($p < 0.05$).

416 Taking the PhoA and GFPuv results together, we can construct a consensus schematic of
417 these proteins in the inner membrane of *E. coli* (Figure 7). The results for PgsB are the most
418 conclusive, having a very distinct signal for its presence in the cytoplasm. The predicted signal
419 peptide from amino acids 1-21 does not direct the protein to the periplasm but rather to the
420 cytoplasmic side of the inner membrane. Based on homology predictions (Figure S4) and the
421 proposed reaction mechanism (Ashiuchi and Misono, 2002), this subunit has access to the
422 cytoplasmic glutamate and nucleotide pools necessary for reaction. Similarly, results were quite
423 conclusive for PgsA and PgsE, both of which are present largely in the periplasm. Discounting
424 the GFPuv results for PgsC, where the signals are very weak, and using only results from PhoA
425 activity, we conclude that it has at least two (and at most three) transmembrane regions with the
426 bulk of the protein being in the periplasm.

427



428

429 Figure 7 – Schematic representation of PgsBCAE localization at cell membrane. White circles
430 indicate the amino acid location where reporter fusions were added.
431

432 **4. CONCLUSIONS**

433
434 In this study, we successfully expressed the PgsBCAE membrane enzyme in a heterologous
435 host, *E. coli*. The enzymatic complex was fully function and PGA was recovered from the
436 supernatant of culture media. Even though production was low due to the small scale of
437 experiments, achieving 13 mg/L of PGA, the polymer presented a high molecular weight and
438 minimal degradation due to the absence of hydrolytic enzymes in *E. coli*. The difference in
439 membrane structure between *B. subtilis* and *E. coli* motivated us to further investigate if the
440 enzyme had the correct localization in the heterologous host. Not only did we observe that the
441 tagged enzyme subunits are correctly directed to the inner cell membrane by microscopy, we
442 could also report for the first time the orientation of the N- and C- termini of the different
443 components across the membrane. These results will help us further understand the role of each
444 subunit in the complex and aid in future engineering efforts.

445

446

447 **5. CRediT AUTHOR CONTRIBUTION STATEMENT**

448

449 **Bruno Motta Nascimento:** Data curation; Formal analysis; Funding acquisition; Investigation;
450 Methodology; Visualization; Writing - original draft & editing. **Nikhil U. Nair:**

451 Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project
452 administration; Resources; Supervision; Visualization; Writing - review & editing.

453

454 **6. DECLARATION OF COMPETING INTEREST**

455

456 We confirm that there are no conflicts of interest associated with this publication.

457

458 **7. ACKNOWLEDGEMENTS**

459

460 The authors would like to thank the funding provided by NIH grant # DP2HD91798 and Tufts
461 University (to N.U.N.), and CAPES Science without Borders grant # 13015-13-3 (to B.M.N.).

462

463 **8. REFERENCES**

464

465 Abelson, J.N., Simon, M.I., 2009. METHODS IN ENZYMOLOGY - Guide to Protein
466 Purification, 2nd Editio. ed. Academic Press - Elsevier. [https://doi.org/10.1016/S0076-](https://doi.org/10.1016/S0076-6879(09)63045-7)
467 [6879\(09\)63045-7](https://doi.org/10.1016/S0076-6879(09)63045-7)

468 Ashiuchi, M., 2013. Microbial production and chemical transformation of poly- γ -glutamate.
469 Microb. Biotechnol. 6, 664–674. <https://doi.org/10.1111/1751-7915.12072>

470 Ashiuchi, M., Misono, H., 2003. Poly- γ -glutamic acid, in: Fahnestock, S.R., Steinbüchel, A.

- 471 (Eds.), *Biopolymers: Volume 7 - Polyamides and Complex Proteinaceous Materials I*.
472 WILEY-VCH, pp. 123–173.
- 473 Ashiuchi, M., Misono, H., 2002. Biochemistry and molecular genetics of poly- γ -glutamate
474 synthesis. *Appl. Microbiol. Biotechnol.* 59, 9–14. [https://doi.org/10.1007/s00253-002-0984-](https://doi.org/10.1007/s00253-002-0984-x)
475 x
- 476 Ashiuchi, M., Nawa, C., Kamei, T., Song, J.-J., Hong, S.-P., Sung, M.-H., Soda, K., Yagi, T.,
477 Misono, H., 2001. Physiological and biochemical characteristics of poly γ -glutamate
478 synthetase complex of *Bacillus subtilis*. *Eur. J. Biochem.* 268, 5321–5328.
479 <https://doi.org/10.1046/j.0014-2956.2001.02475.x>
- 480 Ashiuchi, M., Soda, K., Misono, H., 1999. A Poly- γ -glutamate Synthetic System of *Bacillus*
481 *subtilis* IFO 3336: Gene Cloning and Biochemical Analysis of Poly- γ -glutamate Produced
482 by *Escherichia coli* Clone Cells. *Biochem. Biophys. Res. Commun.* 263, 6–12.
- 483 Ashiuchi, M., Yamashiro, D., Yamamoto, K., 2013. *Bacillus subtilis* EdmS (formerly PgsE)
484 participates in the maintenance of episomes. *Plasmid* 70, 209–215.
485 <https://doi.org/10.1016/j.plasmid.2013.03.008>
- 486 Cao, M., Geng, W., Liu, L., Song, C., Xie, H., Guo, W., Jin, Y., Wang, S., 2011. Glutamic acid
487 independent production of poly- γ -glutamic acid by *Bacillus amyloliquefaciens* LL3 and
488 cloning of *pgsBCA* genes. *Bioresour. Technol.* 102, 4251–4257.
489 <https://doi.org/10.1016/j.biortech.2010.12.065>
- 490 Cao, M., Geng, W., Zhang, W., Sun, J., Wang, S., Feng, J., Zheng, P., Jiang, A., Song, C., 2013.
491 Engineering of recombinant *Escherichia coli* cells co-expressing poly- γ -glutamic acid (γ -
492 PGA) synthetase and glutamate racemase for differential yielding of γ -PGA. *Microb.*

- 493 Biotechnol. 6, 675–684. <https://doi.org/10.1111/1751-7915.12075>
- 494 Cao, M., Song, C., Jin, Y., Liu, L., Liu, J., Xie, H., Guo, W., Wang, S., 2010. Synthesis of poly
495 (γ -glutamic acid) and heterologous expression of *pgsBCA* genes. J. Mol. Catal. B Enzym.
496 67, 111–116. <https://doi.org/10.1016/j.molcatb.2010.07.014>
- 497 Dammeyer, T., Tinnefeld, P., 2012. Engineered Fluorescent Proteins Illuminate the Bacterial
498 Periplasm. Comput. Struct. Biotechnol. J. 3, e201210013.
499 <https://doi.org/10.5936/csbj.201210013>
- 500 Datsenko, K. a, Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia*
501 *coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645.
502 <https://doi.org/10.1073/pnas.120163297>
- 503 Dobson, L., Reményi, I., Tusnády, G.E., 2015. CCTOP: A Consensus Constrained TOPology
504 prediction web server. Nucleic Acids Res. 43, W408–W412.
505 <https://doi.org/10.1093/nar/gkv451>
- 506 Drew, D., Sjöstrand, D., Nilsson, J., Urbig, T., Chin, C.N., De Gier, J.W., Von Heijne, G., 2002.
507 Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and
508 PhoA/GFP fusion analysis. Proc. Natl. Acad. Sci. U. S. A. 99, 2690–2695.
509 <https://doi.org/10.1073/pnas.052018199>
- 510 Feng, J., Gu, Y., Quan, Y., Cao, M., Gao, W., Zhang, W., Wang, S., Yang, C., Song, C., 2015.
511 Improved poly- γ -glutamic acid production in *Bacillus amyloliquefaciens* by modular
512 pathway engineering. Metab. Eng. 32, 106–115.
513 <https://doi.org/10.1016/j.ymben.2015.09.011>
- 514 Feng, J., Quan, Y., Gu, Y., Liu, F., Huang, X., Shen, H., Dang, Y., Cao, M., Gao, W., Lu, X.,

- 515 Wang, Y., Song, C., Wang, S., 2017. Enhancing poly- γ -glutamic acid production in *Bacillus*
516 *amyloliquefaciens* by introducing the glutamate synthesis features from *Corynebacterium*
517 *glutamicum*. *Microb. Cell Fact.* 16, 1–12. <https://doi.org/10.1186/s12934-017-0704-y>
- 518 Halmschlag, B., Steurer, X., Putri, S.P., Fukusaki, E., Blank, L.M., 2019. Tailor-made poly- γ -
519 glutamic acid production. *Metab. Eng.* 55, 239–248.
520 <https://doi.org/10.1016/j.ymben.2019.07.009>
- 521 Hamano, Y., Arai, T., Ashiuchi, M., Kino, K., 2013. NRPSs and amide ligases producing
522 homopoly(amino acid)s and homooligo(amino acid)s. *Nat. Prod. Rep.* 30, 1087–97.
523 <https://doi.org/10.1039/c3np70025a>
- 524 Jiang, H., Shang, L., Yoon, S.H., Lee, S.Y., Yu, Z., 2006. Optimal production of poly- γ -
525 glutamic acid by metabolically engineered *Escherichia coli*. *Biotechnol. Lett.* 28, 1241–
526 1246. <https://doi.org/10.1007/s10529-006-9080-0>
- 527 Jiménez-Guerrero, I., Cubo, M., Pérez-Montaña, F., López-Baena, F., Guash-Vidal, B., Ollero,
528 F., Bellogín, R., Espuny, M., 2013. Bacterial Protein Secretion Systems, Beneficial Plant-
529 microbial Interactions. <https://doi.org/10.1201/b15251-10>
- 530 Karimova, G., Ladant, D., 2017. Defining Membrane Protein Topology Using *pho-lac* Reporter
531 Fusions, in: Journet, L., Cascales, E. (Eds.), *Bacterial Protein Secretion Systems. Methods*
532 *in Molecular Biology*, Vol 1615. Humana Press, New York, NY, pp. 129–142.
- 533 Ke, N., Landgraf, D., Paulsson, J., Berkmen, M., 2016. Visualization of periplasmic and
534 cytoplasmic proteins with a self-labeling protein tag. *J. Bacteriol.* 198, 1035–1043.
535 <https://doi.org/10.1128/JB.00864-15>
- 536 Kino, K., Arai, T., Arimura, Y., 2011. Poly- α -glutamic acid synthesis using a novel catalytic

- 537 activity of RimK from *Escherichia coli* K-12. *Appl. Environ. Microbiol.* 77, 2019–2025.
538 <https://doi.org/10.1128/AEM.02043-10>
- 539 Liu, C.L., Dong, H.G., Xue, K., Yang, W., Liu, P., Cai, D., Liu, X., Yang, Y., Bai, Z., 2019.
540 Biosynthesis of poly- γ -glutamic acid in *Escherichia coli* by heterologous expression of
541 pgsBCAE operon from *Bacillus*. *J. Appl. Microbiol.* 128, 1390–1399.
542 <https://doi.org/10.1111/jam.14552>
- 543 Manocha, B., Margaritis, A., 2010. A novel method for the selective recovery and purification of
544 γ -polyglutamic acid from *Bacillus licheniformis* fermentation broth. *Biotechnol. Prog.* 26,
545 734–742. <https://doi.org/10.1002/btpr.370>
- 546 Ogunleye, A., Bhat, A., Irorere, V.U., Hill, D., Williams, C., Radecka, I., 2015. Poly- γ -glutamic
547 acid: production, properties and applications. *Microbiology* 161, 1–17.
548 <https://doi.org/10.1099/mic.0.081448-0>
- 549 Ojima, Y., Kobayashi, J., Doi, T., Azuma, M., 2019. Knockout of *pgdS* and *ggt* gene changes
550 poly- γ -glutamic acid production in *Bacillus licheniformis* RK14-46. *J. Biotechnol.* 304, 57–
551 62. <https://doi.org/10.1016/j.jbiotec.2019.08.003>
- 552 Park, C., Choi, J.C., Choi, Y.H., Nakamura, H., Shimanouchi, K., Horiuchi, T., Misono, H.,
553 Sewaki, T., Soda, K., Ashiuchi, M., Sung, M.H., 2005. Synthesis of super-high-molecular-
554 weight poly- γ -glutamic acid by *Bacillus subtilis* subsp. *chungkookjang*. *J. Mol. Catal. B*
555 *Enzym.* 35, 128–133. <https://doi.org/10.1016/j.molcatb.2005.06.007>
- 556 Sambrook, J., Russell, D.W., 2001. *Molecular Cloning - A Laboratory Manual - Vol. 1, 2 and 3*,
557 Third Edit. ed. Cold Spring Harbor Laboratory Press, New York.
- 558 Schwede, T., Kopp, J., Guex, N., Peitsch, M.C., 2003. SWISS-MODEL: An automated protein

- 559 homology-modeling server. *Nucleic Acids Res.* 31, 3381–3385.
560 <https://doi.org/10.1093/nar/gkg520>
- 561 Scoffone, V., Dondi, D., Biino, G., Borghese, G., Pasini, D., Galizzi, A., Calvio, C., 2013.
562 Knockout of *pgdS* and *ggt* genes improves γ -PGA yield in *B. subtilis*. *Biotechnol. Bioeng.*
563 110, 2006–2012. <https://doi.org/10.1002/bit.24846>
- 564 Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H.,
565 Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of
566 high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7.
567 <https://doi.org/10.1038/msb.2011.75>
- 568 Smith, C.A., 2006. Structure, Function and Dynamics in the mur Family of Bacterial Cell Wall
569 Ligases. *J. Mol. Biol.* 362, 640–655. <https://doi.org/10.1016/j.jmb.2006.07.066>
- 570 Soto, A.M., Draper, D., 2012. White gels: An easy way to preserve methylene blue stained gels.
571 *Anal. Biochem.* 421, 345–346. <https://doi.org/10.1016/j.ab.2011.10.048>
- 572 Tarui, Y., Iida, H., Ono, E., Miki, W., Hirasawa, E., Fujita, K.I., Tanaka, T., Taniguchi, M.,
573 2005. Biosynthesis of poly- γ -glutamic acid in plants: Transient expression of poly- γ -
574 glutamate synthetase complex in tobacco leaves. *J. Biosci. Bioeng.* 100, 443–448.
575 <https://doi.org/10.1263/jbb.100.443>
- 576 Tian, G., Fu, J., Wei, X., Ji, Z., Ma, X., Qi, G., Chen, S., 2014. Enhanced expression of *pgdS*
577 gene for high production of poly- γ -glutamic acid with lower molecular weight in *Bacillus*
578 *licheniformis* WX-02. *J. Chem. Technol. Biotechnol.* 89, 1825–1832.
579 <https://doi.org/10.1002/jctb.4261>
- 580 Wang, N., Yang, G., Che, C., Liu, Y., 2011. Heterogenous expression of poly-gamma-glutamic

- 581 acid synthetase complex gene of *Bacillus licheniformis* WBL-3. Appl. Biochem. Microbiol.
582 47, 381–385. <https://doi.org/10.1134/s0003683811040193>
- 583 Wang, Q., Wei, X., Chen, S., 2017. Production and Application of Poly- γ -glutamic Acid, Current
584 Developments in Biotechnology and Bioengineering. Elsevier B.V.
585 <https://doi.org/10.1016/B978-0-444-63662-1.00030-0>
- 586 Xavier, J.R., Madhan Kumarr, M.M., Natarajan, G., Ramana, K.V., Semwal, A.D., 2019.
587 Optimized production of poly (γ -glutamic acid) (γ -PGA) using *Bacillus licheniformis* and
588 its application as cryoprotectant for probiotics. Biotechnol. Appl. Biochem. 1–12.
589 <https://doi.org/10.1002/bab.1879>
- 590 Xu, G., Zha, J., Cheng, H., Ibrahim, M.H.A., Yang, F., Dalton, H., Cao, R., Zhu, Y., Fang, J.,
591 Chi, K., Zheng, P., Zhang, X., Shi, J., Xu, Z., Gross, R.A., Koffas, M.A.G., 2019.
592 Engineering *Corynebacterium glutamicum* for the de novo biosynthesis of tailored poly- γ -
593 glutamic acid. Metab. Eng. 56, 39–49. <https://doi.org/10.1016/j.ymben.2019.08.011>
- 594 Yamashiro, D., Minouchi, Y., Ashiuchi, M., 2011a. Moonlighting role of a poly- γ -glutamate
595 synthetase component from *Bacillus subtilis*: insight into novel extrachromosomal DNA
596 maintenance. Appl. Environ. Microbiol. 77, 2796–2798.
597 <https://doi.org/10.1128/AEM.02649-10>
- 598 Yamashiro, D., Yoshioka, M., Ashiuchi, M., 2011b. *Bacillus subtilis pgsE* (formerly *ywtC*)
599 stimulates poly- γ -glutamate production in the presence of zinc. Biotechnol. Bioeng. 108,
600 226–230. <https://doi.org/10.1002/bit.22913>
- 601 Yoon, S.H., Do, J.H., Lee, S.Y., Chang, H.N., 2000. Production of poly- γ -glutamic acid by fed-
602 batch culture of *Bacillus licheniformis*. Time 585–588.

603 Yuan, Z., Ran, Q., Chang, Z., Gao, H., Jia, C., 2019. Recovery of low-molecular-weight γ -PGA
604 by metal cation from the fermentation broth. *Process Biochem.* 82, 215–221.
605 <https://doi.org/10.1016/j.procbio.2019.04.001>
606