C-di-GMP is Required for Swarming in *E. coli*: a Role for DgcO and Colanic Acid

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Abstract

Many bacteria use flagella to swim individually through bulk liquid, or swarm collectively over a semi-solid surface. In *E. coli*, c-di-GMP inhibits swimming via the effector protein YcgR. We show in this study that contrary to its effect on swimming, a certain threshold level of c-di-GMP is required for swarming. Gene expression profiles first indicated that several c-di-GMP synthases – *dgcJ, dgcM, dgcO* - were upregulated during swarming. Of these, we found DgcO to play a critical role. DgcO is reported to affect PGA (poly-β-1,6-N-acetylglucosamine) synthesis in *E. coli*. We show that DgcO also promotes production of colanic acid - one of the three major exopolysaccharides in *E. coli*, and that colanic acid has hitherto-unknown surfactant properties that are expected to aid swarming.

10 ## Importance

11 It is well-established that in bacteria, c-di-GMP inhibits flagella-driven motility at various points
12 in the pathway. Concomitantly, elevated c-di-GMP levels induce the expression and synthesis
13 of a variety of exopolysaccharides that enmesh the bacteria in a biofilm, thereby also
14 interfering with flagella function. This study reports the surprising finding that in *E. coli*, the
15 exopolysaccharide colanic acid is required to enable surface navigation and that the
16 diguanylate cyclase DgcO is employed for this purpose. For surface navigation, there appears
17 to be a sweet spot where c-di-GMP levels are just right to produce polysaccharides that can
18 serve as surfactants and wetting agents rather than promote formation of biofilms.

Introduction

c-di-GMP signaling offers bacteria lifestyle choices, the most prevalent being a choice between moving freely or settling in a biofilm [1-3]. *E. coli* has multiple diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) that synthesize and degrade c-di-GMP, respectively [4]. In free-swimming or planktonic *E. coli*, DgcN, DgcO, DgcQ and DgcE are reported to be some of the most active DGCs, and PdeH the most active PDE [5-7]. Elevated c-di-GMP levels resulting from the inactivation of PdeH inhibit swimming but have only a moderate effect on swarming [8]. The inhibitory effect of c-di-GMP on swimming is well-studied and is orchestrated via interaction of the effector protein YcgR with both the flagellar motor and rotor [5, 8-13]. High levels of c-di-GMP promote biofilm formation [1, 3, 14]. *E. coli* biofilms consist of various extracellular polymers and polysaccharides [15], including colanic acid (CA), cellulose, curli fimbriae, and poly-β-1,6-N-acetyl-d-glucosamine (PGA) [2].

Swarming presents unique challenges not encountered while swimming [16, 17]. These include limited water availability necessary for flagella to work, and formidable physical forces such as surface tension, friction, capillary and viscous forces. Bacteria overcome these challenges by secreting surfactants to reduce surface tension and osmolytes/wetting agents to draw water to the surface, altering cell shape to improve side-by-side alignment to lower viscous drag, and increasing flagella numbers or recruiting special stator-associated proteins to enhance motor power [17-20]. In the laboratory, swarming is observed on media solidified with agar, whose consistency can range from semi-solid to solid depending on the agar concentration (0.5%-0.7% or 1-2% w/v), presenting differing levels of navigational challenge. Bacteria that only swarm on the lower range of agar concentration have been classified as 'temperate', and those that swarm on the higher range as 'robust' swarmers [17]. *E. coli* and *Salmonella* swarmers belong to the former category, while *Proteus mirabilis* and *Vibrio parahaemolyticus* fall under the robust category; the latter set of bacteria increase flagella

44 production to enhance motor power. Some temperate swarmers like *Serratia marcescens* and
45 *Bacillus subtilis* secrete copious amounts of surfactants and/or increase flagella numbers [19].

46 *E. coli* and *Salmonella* neither increase flagella numbers nor are reported to secrete
47 specific surfactants [21-24]. These bacteria require nutrient-rich media for swarming, with *E.*
48 *coli* being particularly fastidious, needing in addition 0.5% glucose and a specific type of agar
49 (Eiken agar) [24, 25]. Several studies suggested that lipopolysaccharide (LPS) might serve to
50 attract water to the surface in these bacteria and implicated an additional role for flagella
51 rotation in enabling a wet surface [26-29]. One of these studies identified non-swarming
52 mutants in the LPS biosynthetic pathway of *Salmonella* and suggested that the hydrophilic
53 nature of LPS may serve as a wetting agent [25], while another detected high osmotic pressure
54 at the leading edge of an *E. coli* swarm using fluorescent liposomes, and suggested that a
55 secreted high molecular weight substance (perhaps LPS) extracts water from the underlying
56 agar to enable motility [26]. Microarray data showed that LPS synthesis was upregulated
57 specifically in *Salmonella* swarms [30]. Non-swarming mutants of *Salmonella* also mapped to
58 the CA biosynthesis pathway [26]. CA is a polyanionic heteropolysaccharide composed of
59 repeating hexo-saccharides and could also serve as a potential secreted wetting agent. We
60 note that in the surfactant literature, the terms ‘surfactant’ and ‘wetting agent’ have been used
61 interchangeably; here we make a distinction and use wetting agent to mean ‘ability to attract
62 water’.

63 The present study was initiated to understand the significance of the differential
64 expression of several DGCs and PDEs observed in RNAseq data collected during *E. coli*
65 swarming. Of the upregulated DGCs, DgcJ is reported to associate with and activate NfrB
66 glycosyl transferase to produce an exopolysaccharide that is a receptor for phage N4 [31, 32],
67 DgcM is reported to regulate the production of curli fibers through the *csgABC* operon [33-35],
68 and DgcO is recognized for its ability to bind oxygen via its heme domain [36] and to enhance
69 PGA synthesis [37]. Deletion analysis of the upregulated genes indicated that only absence
70 of DgcM and DgcO had a negative effect on swarming, the absence of DgcO being critical.

71 We therefore investigated the role of DgcO and found that its swarming defect is caused
72 primarily by a lack of CA production. We demonstrate that CA has surfactant properties that,
73 along with its expected wetting properties, assist swarming.

74

75 # Results

76 ## DgcO plays a critical role in swarming

77 Mutations in *dgcJ* were recovered during a screen for motile antibiotic-resistant mutants
78 emerging from the edge of an *E. coli* swarm, suggesting that DgcJ is active in the swarm [38].
79 That c-di-GMP enzymes might be differentially regulated during swimming vs swarming was
80 inferred by the differential effect of the absence of PdeH on the two types of motilities [8]
81 (Figure S1A). Estimation of c-di-GMP levels in WT under the two conditions using a riboswitch-
82 based c-di-GMP sensor [39], showed these to be similar (Table 1). However, there was a
83 much larger increase of c-di-GMP in the Δ*pdeH* mutant in swim compared to swarm cells (Fig.
84 S1B and Table 1). These observations motivated us to examine RNAseq data (generated as
85 part of a different project; see Methods) for expression of DGCs and PDEs during a 2- 20 h
86 the time course of swarming. Compared to planktonic or swim cells, we observed that the
87 expression of *dgcJ, dgcM, dgcO* was elevated ~3-8 fold during this time course (Fig. 1A).
88 Among the PDEs, *pdeH* was drastically downregulated during swarming, with a reduction of
89 over 100-fold in the measured raw values (Fig. S1C), while *pdeO* and *pdeR* were significantly
90 upregulated (Fig. S1C). The contribution of the upregulated DGCs and PDEs to both
91 swimming swarming was investigated next by deleting them individually (Fig. 1B,C and Fig.
92 S1D). Deletion of neither *pdeO* nor *pdeR* had any effect on either motility (Fig. S1D). Deletion
93 of *dgcJ* also had no observable effect, deletion of *dgcM* decreased swarming (an effect more
94 evident if the plates were allowed to dry longer prior to inoculation), while deletion of *dgcO* had
95 a consistently worse outcome for swarming (Fig. 1C). When Δ*dgcO* cells were observed under

96 the microscope at 4h, a time point when active motion is observable in WT, they were seen to
97 be moving as well as WT (Movies S1 & S2), showing that the defect was not in motility, but in
98 their ability to advance across the surface.

99 We conclude that both DgcM and DgcO play a positive role specifically during
100 swarming, with DgcO being the dominant player. Going forward, we focused on the
101 contribution of DgcO.

102

103 **c-di-GMP is required for swarming**

104 Complementation of Δ*dgcO* with a plasmid encoding *dgcO* driven from an arabinose-inducible
105 promoter (pDgcO) partially rescued the swarming defect without added inducer (i.e. leaky
106 expression; Fig. 2A); addition of arabinose inhibited rescue, suggesting that a narrow window
107 of c-di-GMP levels contributed by DgcO supported swarming. To test whether the Δ*dgcO*
108 phenotype is related solely to alterations in c-di-GMP levels, we introduced into the mutant a
109 plasmid encoding *dgcN* ($_p$DgcN). Leaky expression of *dgcN* was sufficient to rescue swarming,
110 while addition of inducer was inhibitory (Fig. 2A and Table 1). The swarming inhibition
111 observed by induction of pDgcN in the Δ*dgcO* mutant (Fig. 2A) was relieved by deletion of
112 *ycgR*, showing that the inhibition at the higher levels of c-di-GMP is due to interference with
113 flagellar function as established in studies on swimming (Fig. S2). To confirm the assessment
114 that a certain threshold level of c-di-GMP is required for swarming, we took an opposite
115 approach, reducing c-di-GMP levels in the WT by introducing a plasmid expressing *pdeH*
116 (pPdeH) under the control of an IPTG-inducible promoter. Even leaky expression had an
117 inhibitory effect. Addition of as low a concentration as 10 μM IPTG reduced c-di-GMP levels
118 below WT (Table 1), inhibiting swarming (Fig. 2B and 2C, left), but not swimming (Fig. 2C,
119 right). c-di-GMP levels in all of these genetic backgrounds, in cells taken directly from the
120 swarm and swim plates, are plotted in Fig. 2B,C and tabulated in Table 1. There was a

121 correspondence between the measured c-di-GMP levels (Fig. 2D, left) and the extent of
122 swarming (Fig. 2C, left), a correspondence that did not hold for swimming (compare 2C right
123 with 2D right), suggesting that within the range tested, swarming is sensitive to c-di-GMP
124 levels while swimming is not.

125 We conclude that under our experimental conditions, c-di-GMP is necessary and
126 supports optimal swarming within a narrow concentration range.

127

128 **Colanic acid is important for swarming**

129 To investigate how c-di-GMP positively regulates swarming, we initially looked for suppressors
130 that would restore swarming in a *ΔdgcO* strain, but no useful leads emerged. Given that the
131 mutant strain could 'move in place' but not venture out (Movie S2), we wondered if the mutant
132 might be defective in the production of polysaccharides that could contribute to surface wetting.
133 We therefore examined the RNAseq data for changes in all exopolymers – LPS, CA, PGA,
134 cellulose. Of these, several genes in the CA (especially *wcaF* and *wcaJ)* and cellulose
135 biosynthetic pathways were seen to be upregulated (Fig. 3A). However, our WT MG1655
136 strain has a defect in cellulose production [40], so we focused on examining the contribution
137 of CA. Using qRT-PCR, we confirmed that the expression levels of *wcaJ* were significantly
138 lower in the *ΔdgcO* strain compared to the WT (Fig. 3B), suggesting a possible involvement
139 of this DGC in CA synthesis.

140 To test the role of CA in swarming, we constructed two mutant strains: *ΔwcaJ* and
141 *ΔwaaF*. *wcaJ* is part of a multi-gene operon that controls CA biosynthesis [41]; deletion of this
142 gene interferes with CA synthesis but does not affect LPS synthesis. *waaF* belongs to a multi-
143 gene operon controlling LPS biosynthesis [42]; deletion of this gene not only produces a defect
144 in the LPS core, but also overproduces CA, possibly via the RcsCDB system [42]. *ΔwcaJ* was
145 swarming defective (Fig. 4A). *ΔwaaF* could not be tested for swarming because flagella

146 biosynthesis is inhibited in this strain [42]; however, we exploited its CA-overproduction
147 phenotype to extract CA by established protocols (see Methods), adding it to the non-
148 swarming *ΔwcaJ* directly on the swarm plate to test if external supplementation would rescue
149 the defect (Fig. 4A). Through trial and error, we identified an amount of the extract that fully
150 complemented the *ΔwcaJ* defect (Fig. 4A, +CA). It's important to note that adding the same
151 volume of water as a control was not effective, nor was a similar extract made from the *ΔwcaJ*
152 strain (Fig. 4A).

153 To ensure that our CA extracts were not contaminated with LPS [41, 43], we
154 compared them with LPS extracts from the WT strain [50] using a standard protocol [44].
155 Bands corresponding to LPS in WT extracts were absent in the CA extracts of *wcaJ* and *waaF*
156 mutants (Fig. 4B). We conclude that CA and not LPS complements the *wcaJ* mutant of *E. coli*
157 for swarming.

158 To determine if the swarming defect of *ΔdgcO* is due to lack of CA production, we first
159 measured their levels in this strain (see Methods) (Fig. 4C). The *ΔdgcO* strain showed a
160 significant decrease in CA production compared to the WT strain. Additionally, leaky
161 expression from DgcN was sufficient to rescue CA levels in *ΔdgcO,* showing that c-di-GMP is
162 required for maintaining CA production (Fig. 4C). To test if external addition of CA would
163 rescue the *dgcO* defect, we added to it the CA extract from the *ΔwaaF* strain. We observed a
164 positive correlation of swarming with the amount of CA added (Fig. 4D). Taken together, the
165 data in Figure 4A-D allow us to conclude that DgcO is important for regulating the production
166 of CA, which is a critical component for surface movement in *E. coli*. We have not investigated
167 the nature of this regulation.

168

169 **Addition of glucose rescues *ΔdgcO* swarm defect**

170 The availability of glucose has been reported to stimulate the Rcs regulon, which controls CA
171 synthesis [41]. *E. coli* requires 0.5% glucose for optimal swarming, this dependency
172 traditionally attributed to the need for maintaining the energy-intensive nature of swarming
173 [45, 46]. However, examination of WT *E. coli* inoculated on swarm plates with either 0%
174 glucose (no swarming) or 0.5% glucose, revealed that cells were similarly motile at the site of
175 inoculation on both plates (Movie S3 and S4). Considering that addition of CA can rescue
176 *ΔdgcO* cells from the swarm defect (Fig. 4D) and that glucose enhances CA production, we
177 tested whether the addition of glucose could bypass the requirement for c-di-GMP signaling
178 and enable the *ΔdgcO* strain to swarm. Swarming was monitored in four different strains - WT,
179 *ΔdgcO, ΔwaaF*, and *ΔwcaJ* - on plates supplemented with either 0.5% or 1% glucose (w/v).
180 *ΔdgcO* exhibited a full restoration of swarming at 1% glucose. In contrast, the *ΔwcaJ* strain
181 showed only a slight increase in swarm diameter, likely due to the absence of a key component
182 of the CA pathway in this mutant (Fig. 5A). (The lop-sided growth evident in the Δ*waaF* strain
183 is due to the highly mucoid nature of excess CA.) These results were further verified by
184 measuring relative CA levels across these plates (Fig. 5B), where addition of glucose
185 increased the CA levels in both WT and *ΔdgcO,* but not in the *ΔwcaJ* strain.

186 We conclude that CA is important for *E. coli* swarming.

187

188 **PGA is semi-essential for swarming, cellulose is not**

189 In *E. coli*, both PGA and cellulose are regulated by c-di-GMP signaling [33, 37, 47-49], DgcO
190 being implicated in PGA regulation [37]. To test the role of PGA in swarming, we constructed
191 two mutant strains, *ΔpgaA* and *ΔpgaB*, that are crucial for PGA production. Neither strain was
192 defective in swimming (Fig. S3A). Under standard conditions for preparing swarm plates,
193 where we dry freshly poured plates for 1 h in a hood under laminar flow, these mutants were
194 not significantly impaired for swarming compared to WT (Fig. S3B, top row). However, when

195 the plates were dried for an extra 30 min (1.5h), a noticeable decrease in swarming was
196 observed in the mutants compared to the WT. In *ΔpgaA*, this defect was overcome upon
197 complementing *pgaA* from a plasmid (pPgaA), suggesting that PGA plays a niche role for *E.*
198 *coli* motility on drier surfaces.

199 MG1655, the *E. coli* strain used in our study contains a mutation in the *bcsQ* gene in
200 the cellulose synthesis pathway, that introduces an early stop codon, similar to many strains
201 derived from the K-12 lineage [50]. This mutation results in reduced cellulose production
202 compared to other *E. coli* strains, such as enteroaggregative (EAEC) that are known to form
203 a robust biofilm [40]. To assess the role of cellulose in swarming motility, we reversed the
204 premature stop codon by introducing a single mutation in codon 6 (TAG→TTG, A17T) of *bcsQ*
205 to restore cellulose production as previously reported [40]. Indirect measurement of cellulose
206 using crystal violet staining indicated a significant increase in staining (Fig. S3C). However,
207 the reverted strain did no better than the original in swarming, even when plates were dried
208 for an extra 30 minutes (Fig. S3D). From these experiments, we conclude that among the
209 three polymers *E. coli* secretes, CA plays a major role, PGA a secondary role, and cellulose
210 no role in swarming motility under our experimental conditions.

211

212 **Colanic acid acts as a surfactant**

213 To understand how CA assists swarming motility, we had a choice between a wetting or a
214 surfactant function. CA satisfies the former criterion, but we decided to evaluate the latter as
215 well. To do so, we conducted a drop collapse test, a method commonly used to assay
216 surfactants, where the curvature angle of the droplet is inversely correlated with its surfactant
217 capacity [22]. CA extracts from WT, *ΔwcaJ*, and *ΔwaaF* strains propagated on swarm media
218 were placed on a clean plastic surface, along with water as control. The measured curvature
219 angle (see Methods) of the *ΔwcaJ* extract was comparable to that of water, whereas that of
220 the *ΔwaaF* extract showed a significant decrease (Fig. 6A, B), demonstrating that CA indeed

221 has surfactant properties. Next, we compared the curvature angle of these extracts
222 immediately after being added to the surface of swarm media set with either Eiken or Fisher
223 agar (outline marked with red-dotted lines) by capturing a close-up, top-view image of the drop
224 (Fig. 6C, left). The larger the diameter of the drop base, the smaller the angle of contact [22]
225 (Fig. 5C, right). Eiken agar showed a significantly lower contact angle compared to Fischer
226 agar, indicating that the added water drop experiences lower surface tension on the former as
227 surmised earlier ([24, 51]). The CA extract from Δ*waaF* placed on Eiken agar had the lowest
228 contact angle of all. These results suggest that the addition of CA effectively lowers surface
229 tension not only on plastic (Fig. 6A,B) but also on swarm agar plates. In summary, these data
230 support our conclusion that CA has surfactant properties.

231

232 Discussion

233 Studies on flagellar motility have thus far primarily focused on swimming populations to
234 understand the role of c-di-GMP. This study explores how c-di-GMP influences swarming in
235 *E. coli* and reports the following discoveries: 1. DgcJ/O/M are all upregulated during swarming
236 (Fig. 1A). 2. DgcO was observed to play a major role, and DgcM a secondary role, in facilitating
237 swarming, while no phenotype was evident for DgcJ under our experimental conditions (Fig.
238 1B,C). 3. c-di-GMP is required for swarming but enables this motility within a narrow range of
239 c-di-GMP levels (Fig. 2 and Table 1). 4. DgcO promotes CA synthesis (Fig. 3). 5. External
240 addition of CA to the swarming-defective *dgcO* mutant restores swarming (Fig. 4D). 6. CA has
241 surfactant properties that are expected to aid swarming (Fig. 6A,B). 7. PGA, also known to be
242 controlled by DgcO ([37]), contributes to swarming on drier surfaces (Fig. S3B). These findings
243 are summarized in Figure 7.

244 A particularly gratifying aspect of this study is understanding why the *E. coli* strain we
245 work with needs the special Eiken agar and addition of glucose, a requirement identified 30
246 years ago [24]. We show that plates set with Eiken agar have a lower surface tension

247 compared to those with Fischer agar (not conducive to *E. coli* swarming) (Fig. 6C). We surmise
248 that the role of added glucose is related to its known role in stimulating CA synthesis [41], thus
249 additionally contributing to lowering surface tension.

250 Negatively charged polysaccharides have been hypothesized to act as wetting
251 agents . That CA would also play such a role in *E. coli* is supported by a study that used
252 osmolarity-sensing fluorescent liposomes to calculate that the wetting agent had to be a high
253 molecular weight secreted substance [26]. While LPS would fit the bill, our study demonstrated
254 that it is not a major player in our *E. coli* strain. We note that the robust swarmers *P. mirabilis*
255 and *V. parahaemolyticus* both produce abundant capsular polysaccharides; for *P. mirabilis*,
256 these are required for swarming [52, 53]. That such polysaccharides could also serve as
257 surfactants, as shown here for CA, is a new realization.

258 While this study identified DgcO as specifically contributing to the regulation of CA
259 synthesis, the ability of a heterologous DGC (DgcN) to complement the *ΔdgcO* defect (Fig. 2)
260 does not exclude the possibility of a dedicated role for DgcO in the CA pathway, as observed
261 for several other DGCs [54-57]. For instance in *P. aeruginosa*, the DGC GcbC directly interacts
262 with its partner protein LapD, to achieve both specificity and maximal signaling output [57].
263 However, heterologous expression of DGC PA1107 can also induce biofilm formation through
264 LapD [58]. Similarly, DGC LchD in *Lysobacter enzymogenes* forms a complex with its partner
265 protein LchP to synthesize antibiotics; however, this can also be achieved by overexpression
266 of another DGC [59]. In *E. coli*, a prior study showed an interaction between DgcO and DgcM,
267 but that study only queried the interaction between their GGDEF domains [60]. Thus, more
268 experiments are needed to address how DgcO influences CA synthesis.

269 When planktonic bacteria are transferred to a surface, they use a myriad surface and
270 nutrient cues to change their physiology and adapt, as discussed extensively in a recent
271 review [61]. c-di-GMP plays an important role in this process as demonstrated in several
272 bacteria, particularly *P. aeruginosa* and *C. crescentus* [1, 3, 61]. A known nutrient cue for *E.*

273 *coli* swarming is low intracellular iron [62]. In *P. aeruginosa* c-di-GMP levels are regulated by
274 the amount of iron present [63]. The connection between iron and c-di-GMP remains to be
275 elucidated in *E. coli*. We note that DgcO possesses a heme domain that interacts with oxygen
276 [36, 64]. However, mutations in DgcO reported to interfere with heme binding did not
277 reproduce the Δ*dgcO* swarming phenotype (data not shown).

278 Finally, while we have established conditions to observe *E. coli* swarming in the
279 laboratory, the composition and texture of surfaces *E. coli* encounters in the environment must
280 vary vastly. The role of DGCs or other enzymes contributing to facilitating collective motion
281 may vary in importance accordingly.

282

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287 # Methods

288 **Strains, growth conditions, genetic manipulations and motility assays**

289 Strains and plasmids used in this study are listed in **Table S1**. The WT parent strain for *E. coli*
290 was MG1655. Growth media and genetic manipulations have been described earlier [13].
291 Swarm plates were dried at room temperature (RT) for 1h inside Mystaire® MY-PCR prep
292 station laminar flow, prior to inoculation. CA or LPS extracts added were distributed across the
293 surface by gentle shaking with beads (3mm) (CoilRollers Plating Beads, Novagen Co.).

294

295 **Determination of c-di-GMP levels by RFI**

296 This method employs a riboswitch that specifically binds to c-di-GMP, causes a conformational
297 change in the RNA structure that impacts downstream gene expression of RFP (Red
298 Fluorescent Protein). A divergent constitutively active promoter controls CFP (Cyan
299 Fluorescent Protein) expression and normalizes the data for cell number. Relative
300 fluorescence intensity (RFI) of the two readouts at 574 nm (TurboRFP) and 489 nm (AmCyan)
301 is a measure of relative c-GMP levels [65].

302 All c-di-GMP reporter strains were cultured overnight in gentamicin-containing medium
303 at 37 °C with shaking at 200 rpm before being inoculated onto swim or swarm plates. The
304 plates were incubated for 12 h (swim) or 20 h (swarm) and then stored at 4 °C for subsequent
305 experiments. For swarm plate samples, cells were washed off the plates using PBS and
306 resuspended to a final OD_{600} of 0.1. For swim plate samples, the central portion of the agar
307 plate (diameter = 3 cm) was excised, transferred to a 50 mL tube, and washed with PBS. The
308 tube was centrifuged at 1,000 × g for 10 minutes, the supernatant was discarded, and cells
309 from the top of the agar were carefully collected using PBS and adjusted to a final OD_{600} of
310 0.1. Fluorescence spectra were measured using an RF-5301PC fluorescence
311 spectrophotometer (Shimadzu, Kyoto, Japan). Samples were diluted with water to an OD_{600}

312 of 0.1 before fluorescence measurement.

313

314 **Imaging phase contrast view of the edge of a swarm**

315 A phase-contrast microscope (Olympus BX53) equipped with a 40× phase-contrast PH1
316 objective was used to observe the swarm front. A micro cover glass (18 × 18 mm, VWR) was
317 carefully placed on top of the front and cell movement were captured using cellSens Imaging
318 Software (Olympus Co.) at a rate of 10 frames per second, with a spatial resolution of 1004 ×
319 997 pixels and a field of view measuring 120 × 120 μm^2 for up to 30s.

320

321 **RNA sequencing analysis of swarms**

322 Analysis of *E. coli* swarm cells collected at 2, 4 and 20 h was performed by Marta Perez as
323 part of another project. Swarm cells were harvested at 2, 4, and 20 h after inoculation. Cells
324 were rinsed from the agar plates using a 2:1 mixture of RNAprotect Bacteria Reagent (Qiagen)
325 and PBS and collected in 1.5 mL test tubes. A total volume of 1 mL was used to rinse all the
326 cells present on each plate. Once collected, the bacterial suspension was vortexed for 5
327 seconds to mix thoroughly and then incubated at room temperature for 5 minutes. Cells were
328 pelleted by centrifugation at 5000 x g for 10 minutes, and the supernatant was discarded. Total
329 RNA was isolated from the cell samples treated with RNAprotect Bacteria Reagent using the
330 RNeasy Mini Kit (Qiagen), following the enzymatic lysis and Proteinase K digestion protocol.
331 A maximum of 6×10^8 cells were processed per column. To prevent DNA contamination, on-
332 column DNA digestion was performed using the RNase-free DNase Set (Qiagen), following
333 the manufacturer's instructions. RNA was eluted in 40 µL of RNase-free water and immediately
334 kept cold.

335 RNA sequencing was outsourced to Novogene. RNA quality was assessed via
336 visualization on agarose gel and determination of RNA concentration and RNA Integrity
337 Number (RIN) using a Bioanalyzer (Agilent). The RNA quantity used for sequencing was at

338 least 0.5 µg, with a minimum concentration of 10 ng/µL. The RIN values were ≥6 with a smooth
339 baseline. $OD_{260/280}$ and $OD_{260/230}$ ratios were 2.0 or higher. Sequencing was performed on the
340 Illumina NovaSeq 6000 system with a sequencing depth of 6.7 million reads per sample and
341 a read length of 150 bp (paired-end).

342

343 **Quantitative reverse transcriptase PCR**

344 *E. coli* cells grown on swarm plates (at 20h) were collected with PBS and resuspended in a
345 final concentration of OD_{600}=1. RNA was then purified using Qiagen RNeasy Mini kit (Qiagen
346 co.) according to the manufacturer protocol. RNA concentration was determined using
347 NanoDrop™ One (Thermo Scientific). RNA (1 µg) from each sample was subjected to 1-step
348 qPCR using SuperScript III One-Step (Thermo Fisher Sci.), according to the manufacturer
349 protocol. qRT-PCR reactions were performed in triplicates and fluorescence detection was
350 performed using QuantStudio™ 7 Real-Time PCR (Thermo Fisher Sci.). RNA expression was
351 normalized to the level of *gyrA*, a housekeeping gene control. The relative gene expression
352 levels of *wcaJ* were calculated from cycle threshold (CT) values using the $2^{-\Delta C}$ method, where
353 ΔC = CT(*wcaJ*) – CT(*gyrA*) [66].

354

355 **Colanic acid extraction**

356 Colanic acid (CA) was extracted and quantified by modification of the following method [67]:
357 50 µl of prepared extracts were then mixed with 4.5ml of H_2SO_4/H_2O (6:1 v/v) and incubated
358 at 100℃ for 20 min. The mixture was cooled to the RT and absorbances measured at 396 nm
359 and 427 nm. 100µl of 1M cysteine hydrochloride (cys) were then added and the absorbances
360 measured again at 396 nm and 427 nm. Final CA concentration was measured using the
361 following equation.

362 $$[OD_{396}\ \text{cys} - OD_{396}\ \text{pre-cys}] - [OD_{427}\ \text{cys} - OD_{427}\ \text{pre-cys}]$$

363 **Extraction and silver staining of LPS**

364 LPS was extracted by hot phenol-water method as described previously with some
365 modifications [43]. To remove protein and nucleic acids from cell extracts, proteinase K (50
366 µg/mL) (Roche, Mannheim, Germany), RNase (40 µg/mL) (Roche, Mannheim, Germany), and
367 DNase (20 µg/mL) (Roche, Mannheim, Germany) were added to the cell extract in the
368 presence of 1 µL/mL 20% $MgSO_4$ and incubated at 37°C overnight. An equal volume of hot
369 (65°C) 90% phenol was added to the mixtures followed by vigorous shaking at 65°C for 15
370 min. Suspensions were then cooled, transferred to 1.5 mL polypropylene tubes and
371 centrifuged at 8500×g for 15 min. Phenol phases were re-extracted by 300 µL distilled water.
372 Sodium acetate at 0.5 M final concentration and 10 volumes of 95% ethanol were added to
373 the extracts and samples were stored at -20°C overnight. After centrifugation, pellets were
374 washed twice with 95% ethanol and resuspended in 120µl Laemmli Sample Buffer (33 mM
375 Tris-HCl, pH 6.8, 1% SDS, 13.3% (w/v) glycerol, 0.005% bromophenol blue). Samples were
376 then heated at 100℃ for 20 min. 10 µL of each sample ($1x10^9$ cells) was separated on 15%
377 SDS polyacrylamide gel with a 5% stacking gel at 100 mA for 1.5h. Silver and Coomassie blue
378 staining of the gels was performed according to the standard protocols [68].

379

380 **Biofilm assay using Crystal Violet**

381 Biofilm was quantified by using the following method [69].

382

383 **Contact angle measurement**

384 Water or bacterial culture supernatant drops 10 µl in volume were placed on the outer side of
385 the bottom part of 100 × 15-mm plastic Petri dishes (0875712; Fisher Scientific). The Petri
386 dishes were washed with ethanol. For small angles of contact, where sideview images are not
387 accurate, i.e. <30º, the diameter of each drop was determined by taking a close-up, top-view

picture of the drop using a 24.2-megapixel camera (EOS R6 Mark II, Canon) with a 60-mm lens. Pictures were taken 5 min after drop deposition. The angle of contact, θ (°), between drop and surface was determined using a spherical-cap-shaped approximation for small angles ($\theta < 30°$) through $\theta=720 \cdot V\pi 2 \cdot a3$ (where V is a set volume of the drop (10 μl), and a is the radius (mm) of the circle formed by the drop base) as previously described [22].

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Main Figures

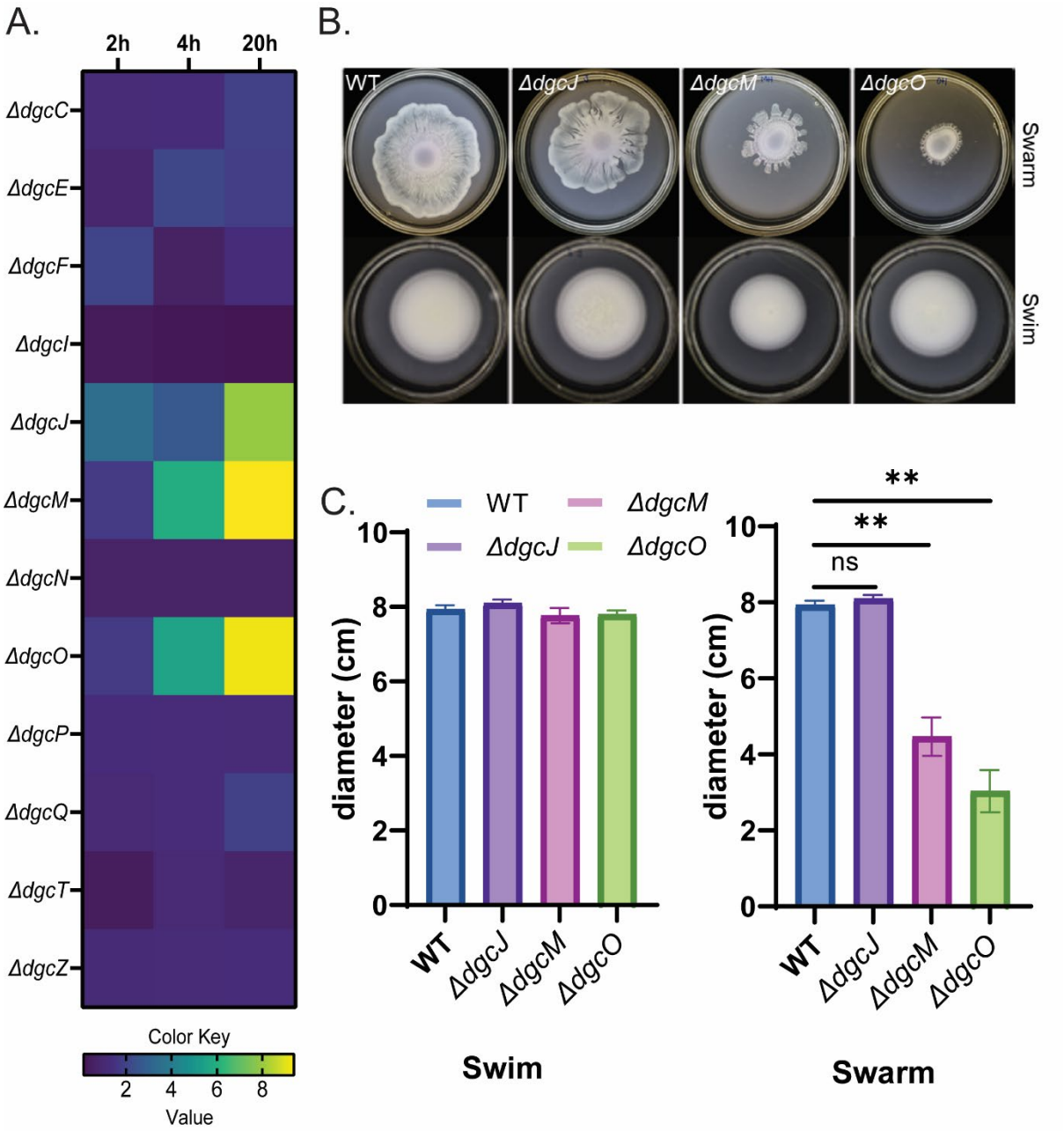

Figure 1. Identification of DGCs that contribute to swarming motility. (A) Comparison of fold changes in gene expression of WT *E. coli* DGCs during the time course of swarming. RNA-Seq data collected at 2, 4 and 20h were normalized to those from planktonic cultures (n=4). (B) Comparison of swimming (0.3% agar) and swarming (0.5% agar) motility of indicated DGC deletion mutants in the WT strain. Plates were incubated at 30℃ for 18h (n=3). (C) Plot of diameters across the zones of bacterial swimming or swarming shown in B. Calculated P values are indicated: *, <0.05, **, <0.01, or ***, <0.0001. NS, not statistically significant.

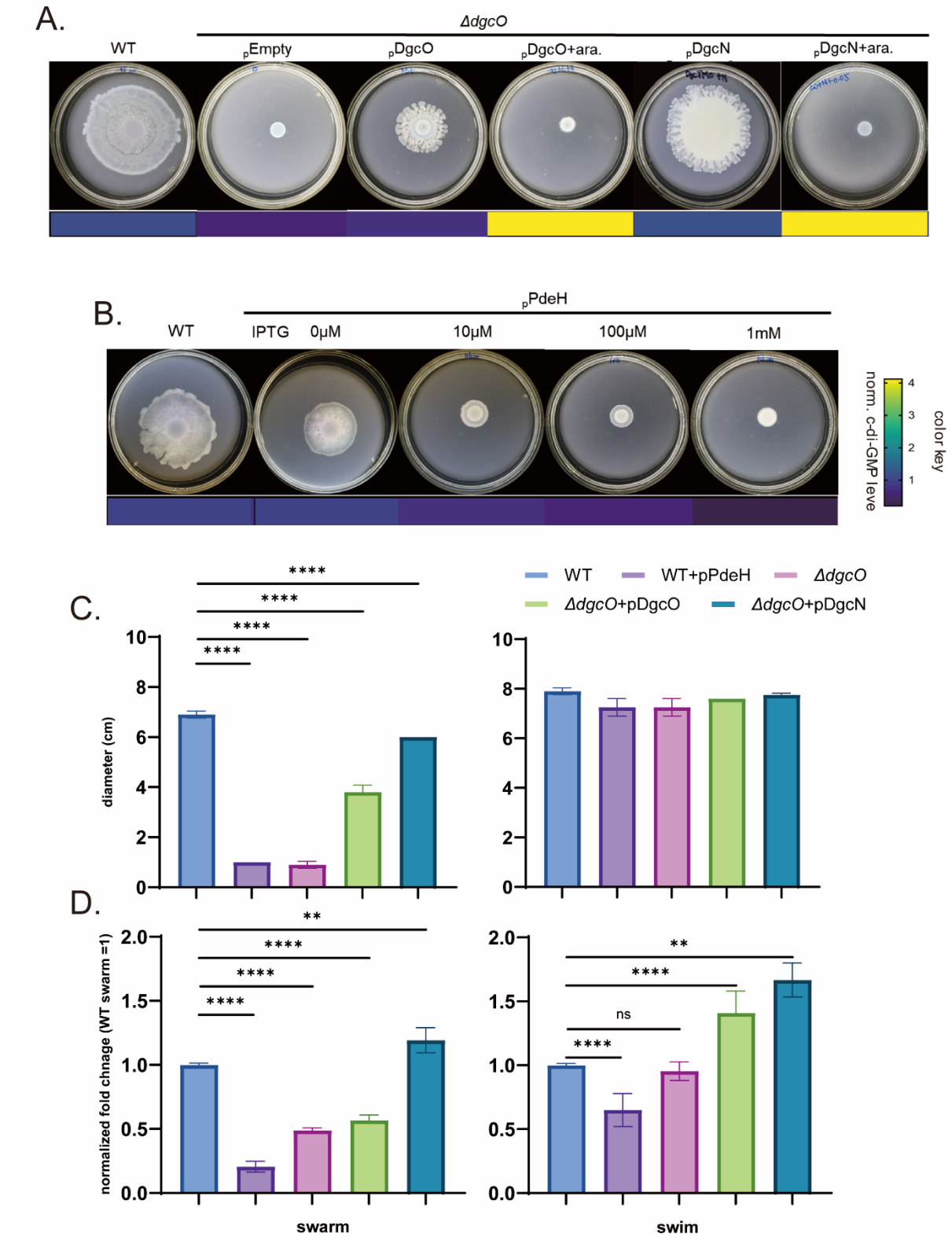

Figure 2. c-di-GMP is required for swarming. (A) Plasmids encoding *dgcO* (pDgcO), *dgcN* (pDgcN) or vector alone (pEmpty) under the control of pBAD promoter, were introduced to the *ΔdgcO* strain, and swarming compared to WT, with (+ara) or without addition of 0.05% arabinose (n=4). The color key beneath the plates corresponds to relative c-di-GMP levels measured using the riboswitch-based c-di-GMP sensor, shown in B as heatmap (see Table 1). (B) A plasmid encoding *pdeH* (pPdeH) driven from the T5-*lacO* promoter was introduced into WT and swarming monitored at indicated IPTG concentrations (n=2). C-di-GMP levels for

all strains in this figure are found in Table1, represented here by a heat map beneath the plates. (C) Diameters of swim and swarm zones of strains indicated by a color key (n=4); pDgcO and pDgcN data are without added inducer, and pPdeH with 10μM IPTG. (D) Cells from swim/swarm plates in C were collected and c-di-GMP levels measured using the c-di-GMP biosensor. The values were normalized to the c-di-GMP levels in WT cells. Calculated *P* values are indicated: *, <0.05, **, <0.01, or ***, <0.0001. NS, not statistically significant. Color key as in C.

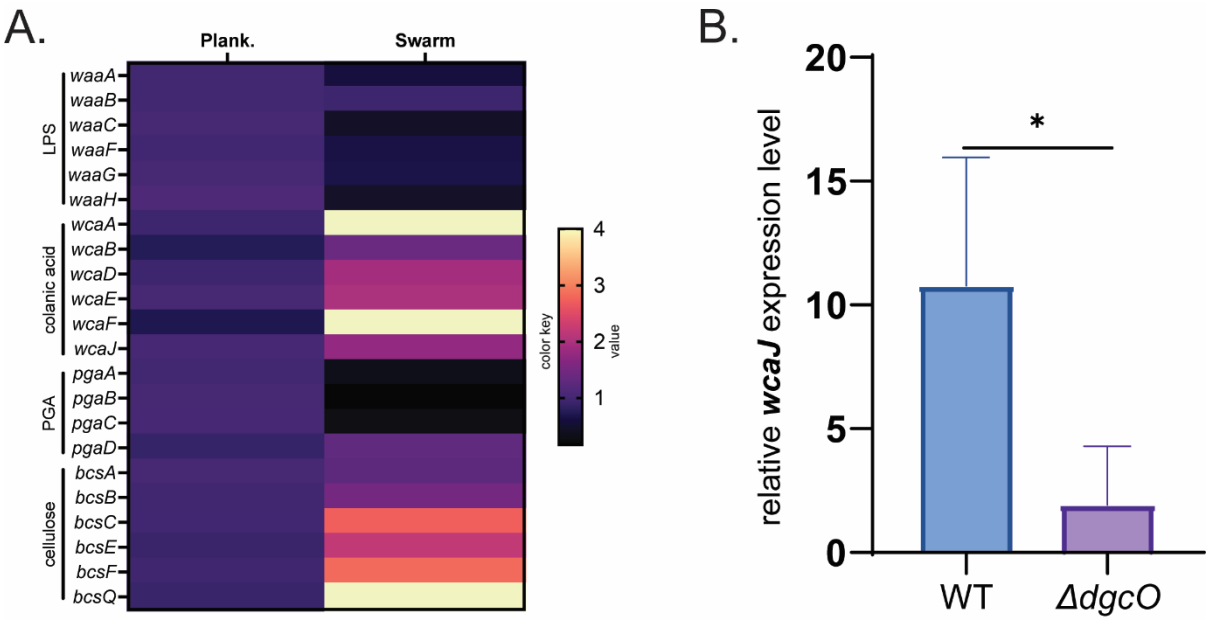

Figure 3. Exopolymer expression profiles during swarming. (A) Comparison of fold changes in gene expression of the four exopolymers of *E. coli* (LPS, CA, PGA, and cellulose). RNA-Seq data collected at 20h during the time course of swarming were normalized to those from planktonic cultures (n=4). (B) Comparison of *wcaJ* expression levels relative to the housekeeping gene *gyrA* in WT and *ΔdgcO,* using qRT-PCR from swarm cells collect at 20h (see Methods); (n=3).

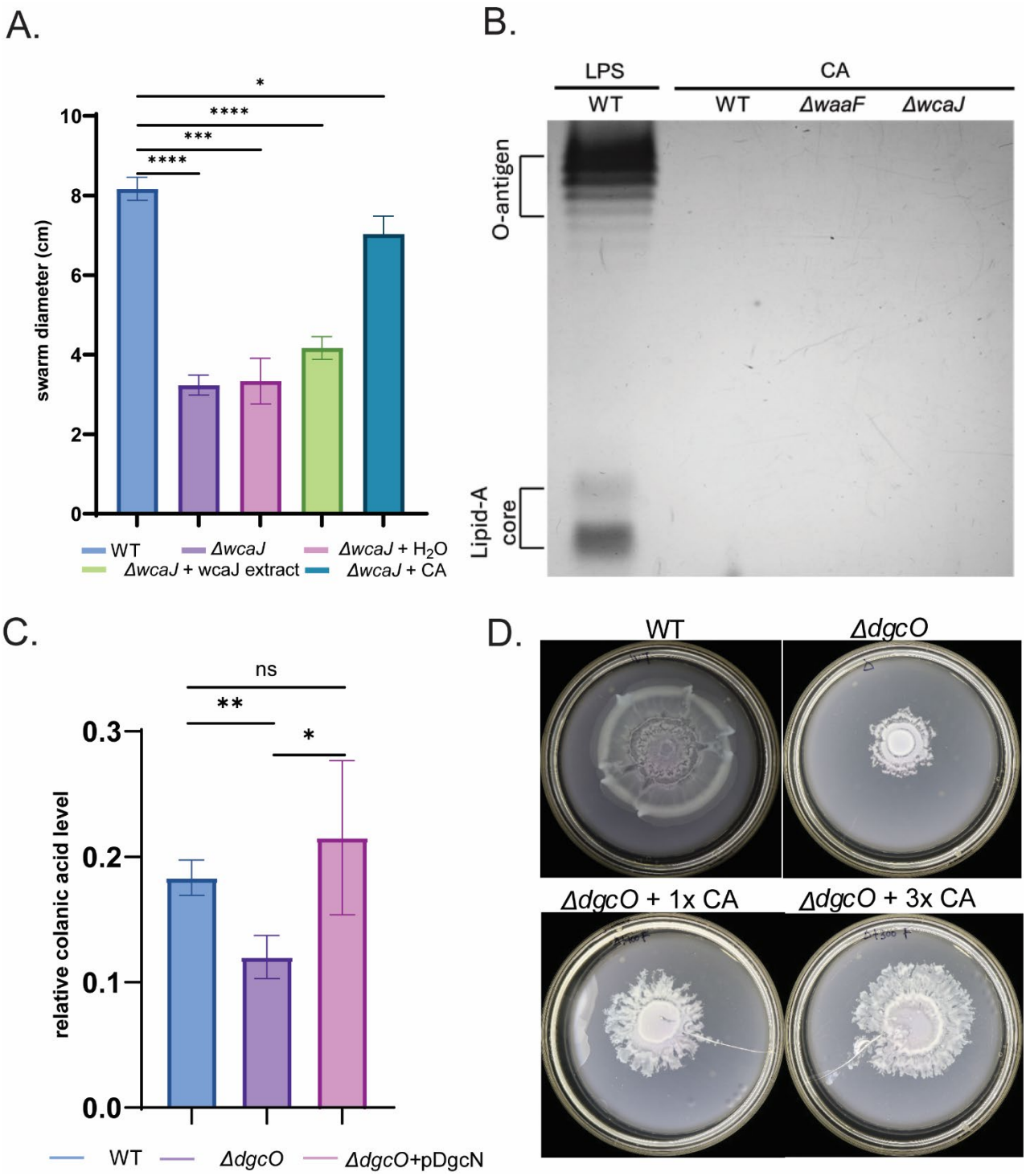

Figure 4. Colanic acid (CA) is required for swarming. (A) Swarming diameters at 20h were compared between WT, *ΔwcaJ* and *ΔwcaJ* supplemented with 100µl of either water, *ΔwcaJ* extract, or CA extract from *ΔwaaF* (n=3). (B) LPS extract from WT and CA extracts from *ΔwaaF* and *ΔwcaJ* strains were fractionated on SDS-PAGE gel, followed by silver staining (see Methods). (C) Comparison of relative CA levels between WT, *ΔdgcO* and *ΔdgcO* complemented with pDgcN (n=3). CA levels were measured as described under Methods. (D) Rescue of swarming in *ΔdgcO* by addition of indicated concentrations of CA extract from *ΔwaaF*.

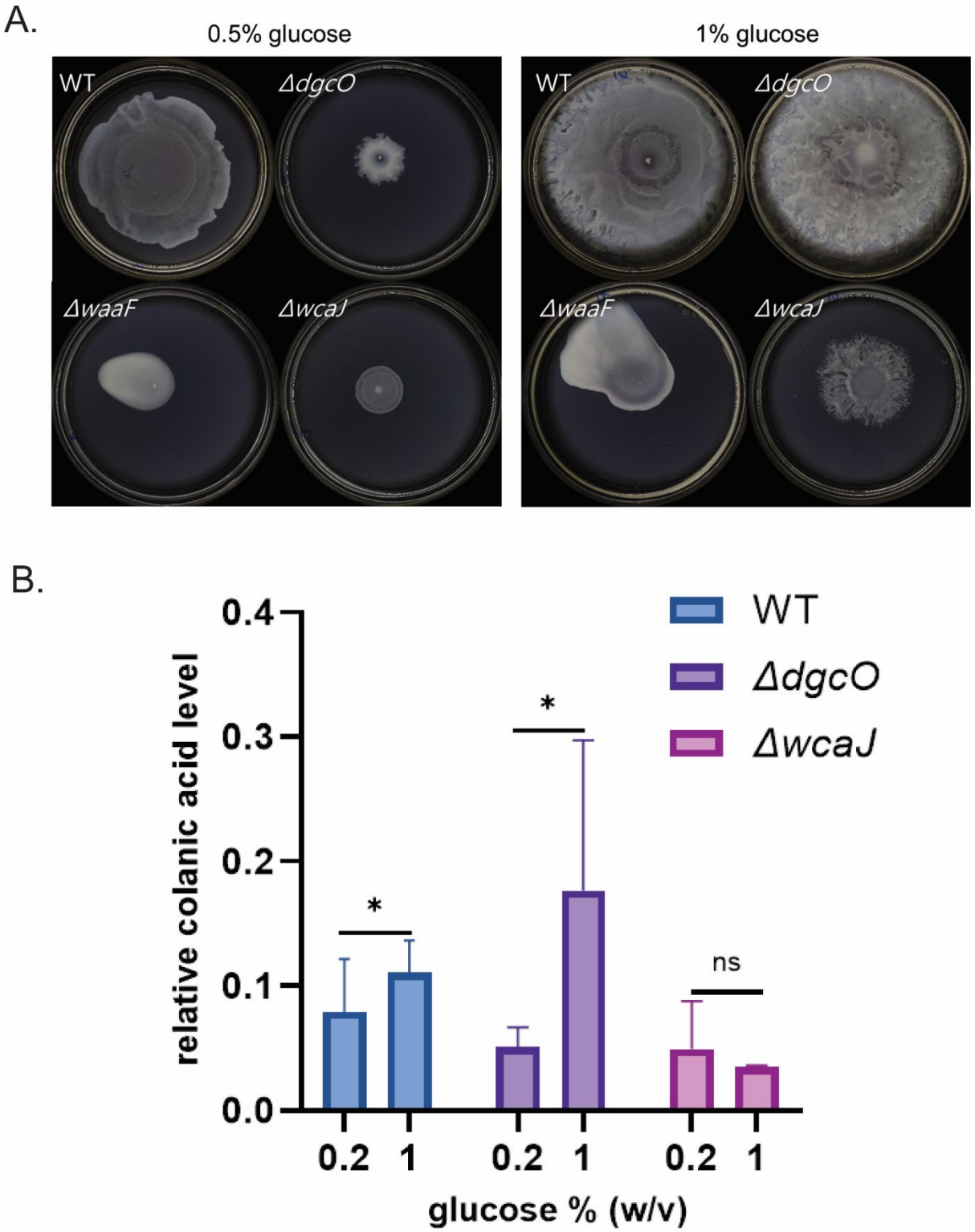

Figure 5. Addition of glucose rescues *ΔdgcO* swarm defect

(A) Swarming recorded at 20h on plates supplied with two different glucose concentrations (0.5% or 1%) and inoculated with indicated strains. (B) Comparison of relative CA levels between WT, *ΔdgcO* and *ΔwcaJ* (n=3). CA levels were measured as described under Methods

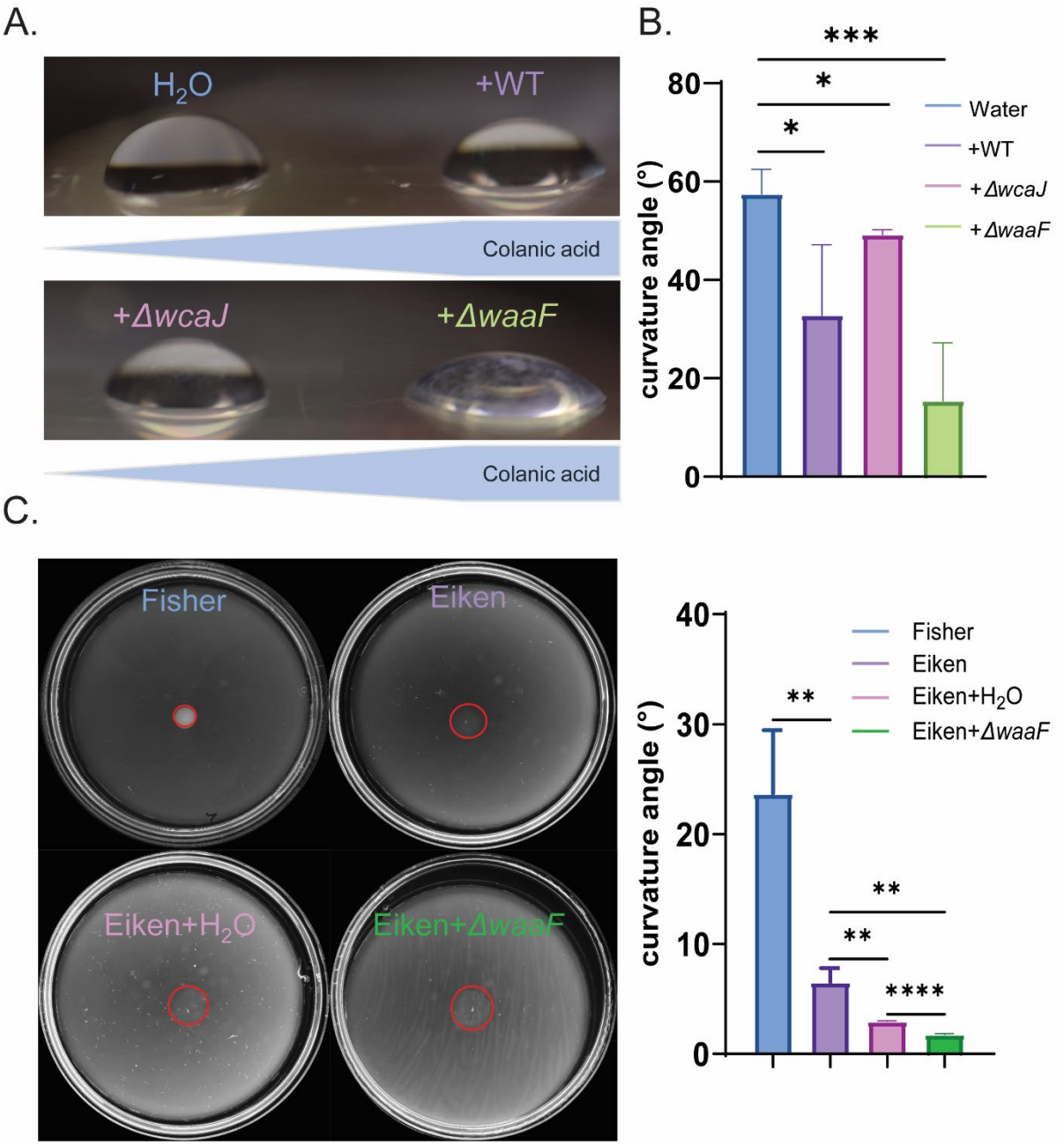

Figure 6. Colanic acid acts as a surfactant. (A) A side view of a 10-µl water drop or CA extracts in water from indicated strains, deposited on a plastic surface. Extracts with more CA are indicated in the blue ribbon below the images. The large contact angles could be measured directly from the image (see Methods). (B) A summary of measurements of contact angles from A. (C) Same as A-B, except water drops were placed directly on swarm media set with Fisher or Eiken agar (top row of plates), or water drops on Eiken agar compared with those of Δ*waaF* CA extract (bottom row of plates).

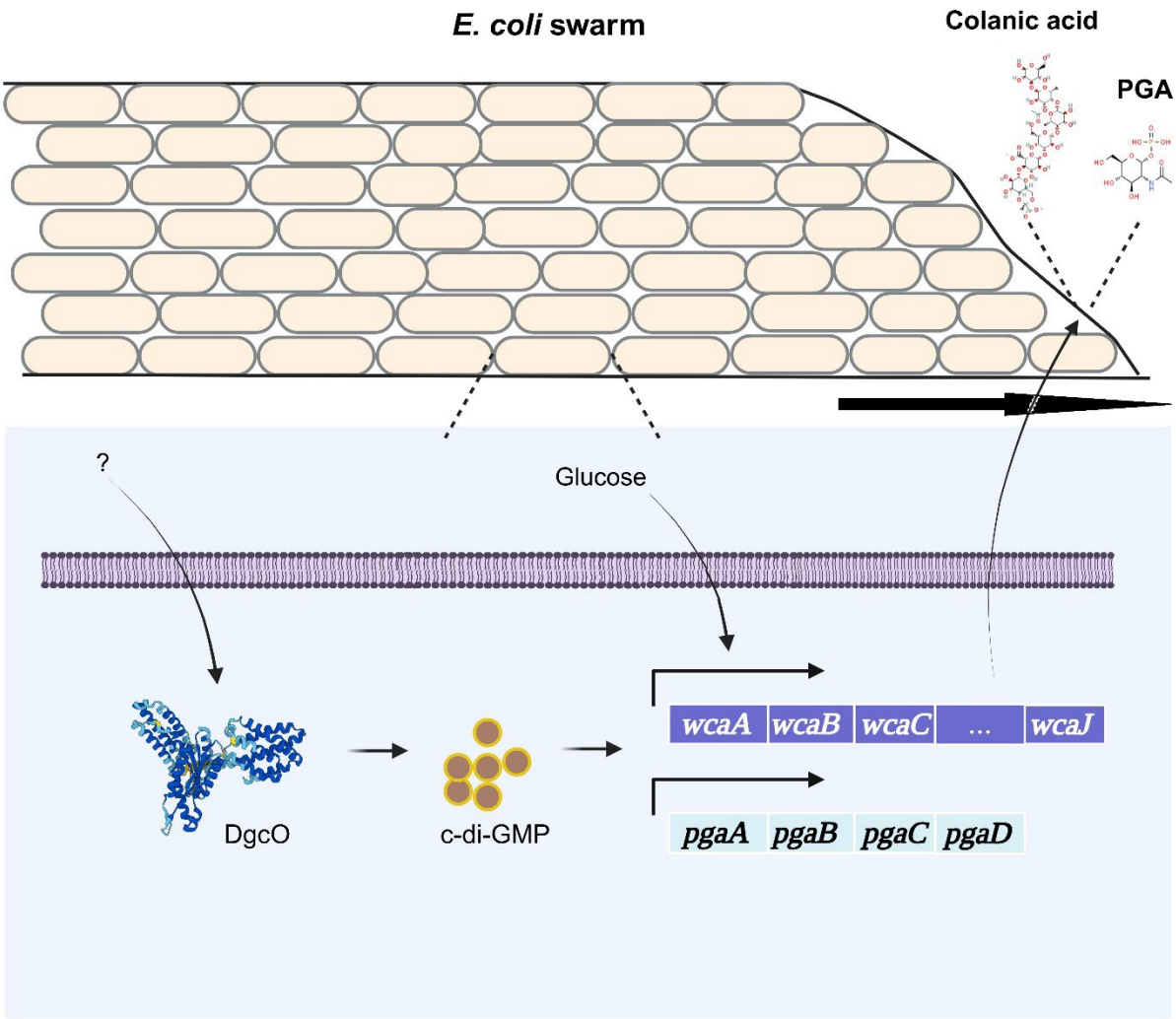

Figure 7. Summary of the role of DgcO in swarming. DgcO positively regulates swarming in *E. coli* by upregulating *wca* and *pga* operons engaged in the production of CA and PGA (this work and [37]. Glucose stimulates CA synthesis [41]. Depending on wet/dry surface conditions, these secreted exopolymers appear to function as wetting agents and surfactants to facilitate swarming.

Supplementary Figures and Tables

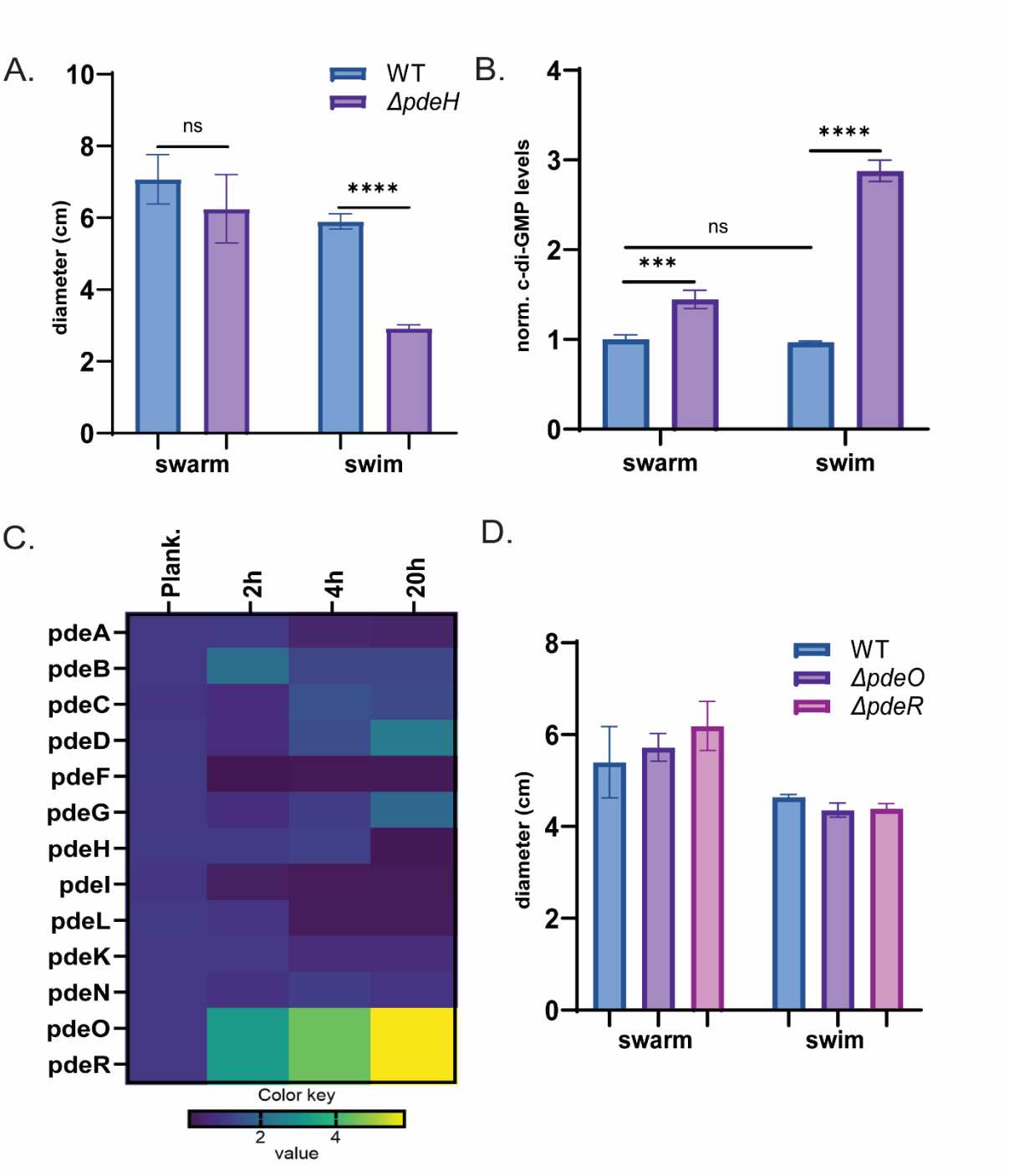

Figure S1. Differential c-di-GMP levels in swim and swarm cells. (A) Comparison of motility in swim (0.3% agar) or swarm (0.5% agar) plates of WT (MG1655) and Δ*pdeH* strains. Plates were incubated at 30℃ for 18h (n=3). (B) Measurement of c-di-GMP levels using a riboswitch-based c-di-GMP sensor (see Methods) in cells collected from swim and swarm plates shown in A, normalized to those of WT swim or swarm cells (see Table 1). Color coding of strains as in A. (C) Comparison of fold-changes in gene expression of all the *E. coli* PDEs during the time course of swarming. RNA-Seq data collected at 2, 4 and 20 h of swarming were normalized to those from planktonic cultures (n=4). (D) Swim and swarm motility

comparison of WT, Δ*pdeO* and Δ*pdeR* strains as in A.

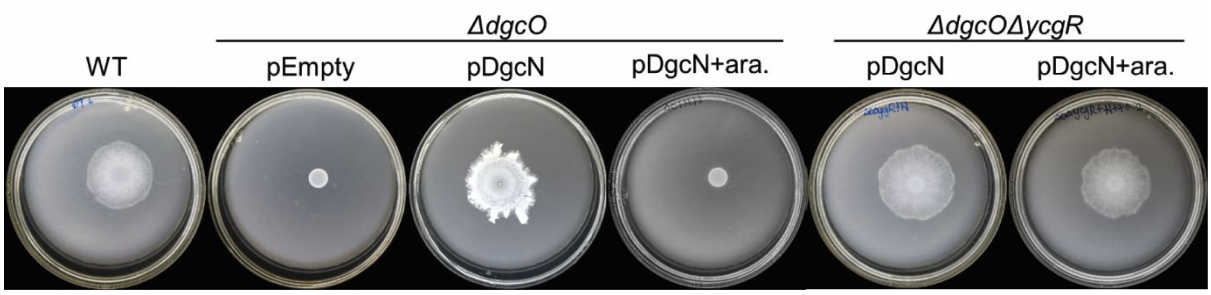

Figure S2. Swarming inhibition of *ΔdgcO* by pDgcN induction is through YcgR. Experimental setup as in Figure 2A except also monitored in a *ΔdgcOΔycgR* strain.

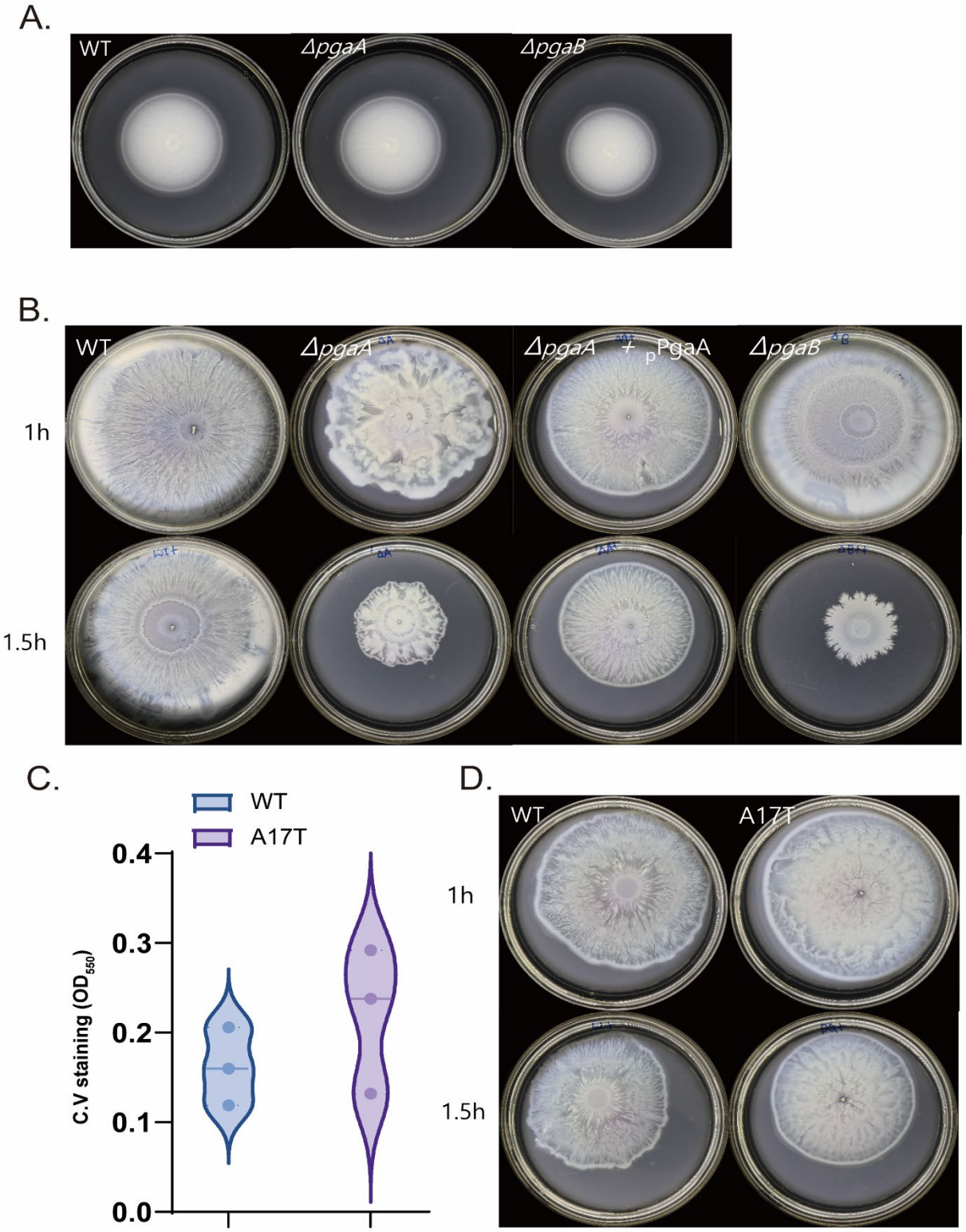

Figure S3. PGA, but not cellulose, assists swarming motility. (A-B) Comparison of swimming and swarming of indicated *pga* deletion mutants of MG1655. Plates were incubated at 30℃ for 18h (n=3) swarm or 37℃ for 12h (n=3) swim. Swarm plates were either dried under the hood for 1h (standard condition) or 1.5h. (C) Biofilm levels measured levels at OD_{550nm} by crystal violet staining of WT and *bcsQ*(A17T). (D) Swarming in indicated strains. Plates were dried for either 1 or 1.5h before cells were inoculated and incubated at 30℃ for 18h.

Movie S1. Phase contrast view of the edge of a WT swarm. Cells inoculated on swarm agar (see Methods) were viewed under phase contrast after 4h of incubation at 30ºC, a time when movement is generally first observed. The movie is in real time, at 40x magnification.

Movie S2. Phase contrast view of the edge of a Δ*dgcO* swarm. Experimental protocol was as described in Movie S1.

Movies S3 and S4. Movement at the edge WT *E. coli* swarm under different glucose concentrations. Experimental conditions as described for Movie S1, except plates were supplemented with either 0% glucose (Movie S3) or 0.5% glucose (Movie S4).

Table 1. c-di-GMP levels estimated from a riboswitch-based sensor

Strain	Relative c-di-GMP level to WT	Condition
WT(MG1655)*	1±0.008	Swim
Δ*pdeH*	2.88±0.11	Swim
ΔdgcO	0.82±0.03	Swim
WT+pPdeH (10μM IPTG)	0.75±0.04	Swim
ΔdgcO + pDgcO (0% ara)	1.28±0.37	Swim
ΔdgcO + pDgcN (0% ara)	1.5±0.09	Swim
ΔdgcO + pDgcN+ (0.05% ara.)	5±0.56	Swim
WT(MG1655)	1±0.093	Swarm
Δ*pdeH*	1.25±0.031	Swarm
ΔdgcM	.89±0.0305	Swarm
ΔdgcO	0.57±0.048	Swarm
WT+pPdeH (0μM IPTG)	0.82±0.111	Swarm
WT+pPdeH (10μM IPTG)	0.67±0.18	Swarm
WT+pPdeH (100μM IPTG)	0.62±0.08	Swarm
WT+pPdeH (1mM IPTG)	0.21±0.04	Swarm
ΔdgcO + pDgcO (0% ara)	0.70±0.003	Swarm
ΔdgcO + pDgcN (0% ara)	1.48±0.013	Swarm
ΔdgcO + pDgcO (0.05% ara)	2.56±0.234	Swarm
ΔdgcO + pDgcN+ (0.05% ara.)	4.17±0.400	Swarm

*Estimates of c-di-GMP levels from WT cells taken directly from a swim and swarm plates (see Methods) were set to 1 for comparison with all other strains and conditions tested. Based on reported data for planktonic *E. coli* [37], these values are roughly equivalent to 8pmol/mg protein. We are concerned here only with relative, not absolute, c-di-GMP values.

Table S1

Strains	**Genotype or Description**	**Reference**
MG1655	K12 wild-type (WT) strain: F^-, λ^-, rph-1	Laboratory Collection
JH702	MG1655 Δ*dgcM*	This work
JH703	MG1655 Δ*dgcO*	This work
JH1011	MG1655 Δ*dgcO* + pBAD30_empty	This work
JH1012	MG1655 Δ*dgcO* + pBAD30_DgcO	This work
JH1013	MG1655 Δ*dgcO* + pBAD30_YfIN	This work
JH706	MG1655 Δ*waaF*	This work
JH707	MG1655 Δ*wcaJ*	This work
JH711	MG1655 + ASKA_pdeH	This work
JH1422	MG1655 Δ*dgcM* + pBAD30_empty	This work
JH1423	MG1655 Δ*dgcM* + pBAD30_DgcM	This work
JH1408	MG1655 + cdiGsens	This work
JH1409	MG1655 *ΔdgcM*+ cdiGsens	This work
JH1410	MG1655 *ΔdgcO*+ cdiGsens	This work
JH1418	MG1655 *ΔpdeH*+ cdiGsens	This work
JH1105-1114	MG1655 *ΔdgcO* suppressors	This work
JH1419	MG1655 ASKA_PdeH+cdiG sens	This work
JH1201	MG1655 Δ*dgcO*Δ*fhuA*	This work
JH1202	MG1655 Δ*dgcO*Δ*stfP*	This work
JH1203	MG1655 Δ*dgcO*Δ*yhbX*	This work
JH1204	MG1655 Δ*dgcO*Δ*fhuA*+cdiG sens	This work
Plasmids	Expressed Proteins	
pKD4	Kanamycin resistance gene template	[62]
pKD46	λ Red Recombinase	[62]
pCP20	FLP recombinase	[62]
pBAD30	Cloning vector; $_P$BAD; Amp^R	[67]

pBAD30_YfiN	pBAD::YfiN	[67]
pBAD30_DgcO	pBAD:;DgcO	This study
pBAD30_DgcM	pBAD:;DgcM	This study
ASKA_PdeH	T5-*lacO* (pca24N)::PdeH	[68]
cdiG sens	pRP0122-P*be-amcyan_Bc4_turborfp*	[37]