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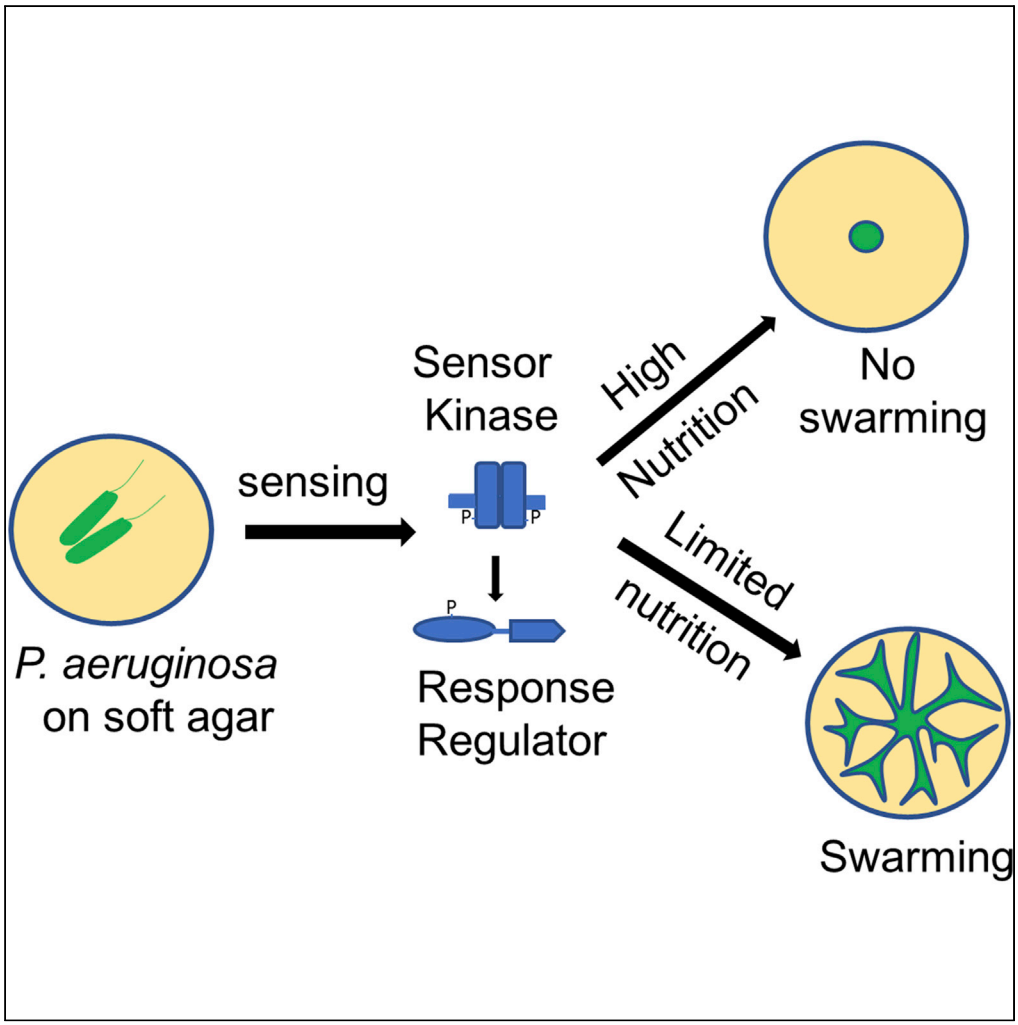

Ameen M. Kollaran, Shubham Joge, Harshitha S. Kotian, ..., Ayushi Mishra, Manoj Varma, Varsha Singh

varsha@iisc.ac.in

HIGHLIGHTS

The swarm pattern of *Pseudomonas aeruginosa* is plastic and growth media dependent

P. aeruginosa swarming motility is promoted by nutrient limitation

Forty-four *P. aeruginosa* genes encoding two-component system modulate swarming

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Context-Specific Requirement of Forty-Four Two-Component Loci in *Pseudomonas aeruginosa* Swarming

Ameen M. Kollaran,[1,5] Shubham Joge,[1,5] Harshitha S. Kotian,[2] Divakar Badal,[3] Deep Prakash,[1] Ayushi Mishra,[1] Manoj Varma,[2,4] and Varsha Singh[1,3,6,*]

SUMMARY

Swarming in *Pseudomonas aeruginosa* is a coordinated movement of bacteria over semisolid surfaces (0.5%–0.7% agar). On soft agar, *P. aeruginosa* exhibits a dendritic swarm pattern, with multiple levels of branching. However, the swarm patterns typically vary depending upon the experimental design. In the present study, we show that the pattern characteristics of *P. aeruginosa* swarm are highly environment dependent. We define several quantifiable, macroscale features of the swarm to study the plasticity of the swarm, observed across different nutrient formulations. Furthermore, through a targeted screen of 113 two-component system (TCS) loci of the *P. aeruginosa* strain PA14, we show that forty-four TCS genes regulate swarming in PA14 in a contextual fashion. However, only four TCS genes—*fleR*, *fleS*, *gacS*, and *PA14_59770*—were found essential for swarming. Notably, many swarming-defective TCS mutants were found highly efficient in biofilm formation, indicating opposing roles for many TCS loci.

INTRODUCTION

Swarming is a rapid bulk translocation behavior observed in many bacterial species, typically over semisolid agar surfaces (Harshey and Matsuyama, 1994; Henrichsen, 1972; Kearns, 2010). In many instances, bacterial swarm populations generally exhibit characteristic, macroscopic swarm patterns, which are easily recognizable (Kearns, 2010). The opportunistic human pathogen *Pseudomonas aeruginosa* displays a dendritic-type swarm pattern while swarming on soft agar surfaces. Flagella and quorum sensing (QS) are essential for *P. aeruginosa* swarming (Kohler et al., 2000; Overhage et al., 2008). Rhamnolipids, a class of glycolipid biosurfactants implicated in virulence and biofilm formation, are also critical for *P. aeruginosa* swarming, including tendril avoidance (Caiazza et al., 2005; Morris et al., 2011; Xavier et al., 2011). Several lines of evidence indicate that nutrient formulation, such as a change in either carbon or nitrogen sources, can have a drastic impact on rhamnolipid production (Bains et al., 2012; Kohler et al., 2000; Shrout et al., 2006). Most studies of *P. aeruginosa* swarming are primarily reported under minimal media conditions (M8, M9, and BM2) or in some instances, under complex media formulations such as nutrient broth, brain heart infusion (BHI), or fastidious anaerobe broth (FAB) (Baker et al., 2016; Kohler et al., 2000; Morales-Soto et al., 2015; Overhage et al., 2008; Rashid and Kornberg, 2000; Tremblay and Déziel, 2008). However, despite gross conservation in the dendritic swarm pattern on these different media formulations, the *P. aeruginosa* swarms often appeared distinct. Hence, these widespread, yet little described, observations strongly urge one to further examine whether the nutritional components of the growth medium can have an impact on *P. aeruginosa* swarm ability, particularly pattern formation.

Previously, two independent transcriptome studies showed marked dysregulation of very distinct sets of genes, virulence factors in one versus translation and energy metabolism in the other, in *P. aeruginosa* swarming motility (Overhage et al., 2008; Tremblay and Déziel, 2010). Such a notable divergence between these two studies might have resulted from the use of two different media (BM2 and M9), which differ in nutritional composition (Tremblay and Déziel, 2010). There also exists at least one instance where a *P. aeruginosa* mutant displays contrasting swarming phenotypes. A pili mutant, *pilA,* of *P. aeruginosa* is described as a non-swarmer on M8 agar (Kohler et al., 2000), reported as a swarmer on nutrient broth agar (Rashid and Kornberg, 2000), and reported as a hyper-swarmer on FAB agar (Shrout et al., 2006). These observations also suggested the possible impact of nutrient components on *P. aeruginosa* swarming and also the conditional requirement of several genetic regulators. An interesting question is how *P. aeruginosa* cells sense such changes in the nutrition that affect swarm phenotype. Environmental signals

[1]Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka 560012, India

[2]Centre for Nano Science and Engineering, Indian Institute of Science, Bangalore, Karnataka 560012, India

[3]Biosystems Science and Engineering, Indian Institute of Science, Bangalore, Karnataka 560012, India

[4]Robert Bosch Centre for Cyber Physical Systems, Indian Institute of Science, Bangalore, Karnataka 560012, India

[5]These authors contributed equally

[6]Lead Contact

*Correspondence: varsha@iisc.ac.in

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	M8	M9	BM2	PGM
Magnesium sulfate ($MgSO_4$)	1.0 mM	1.0 mM	2.0 mM	1.0 mM
Sodium chloride (NaCl)	8.6 mM	8.6 mM	–	50 mM
Calcium chloride ($CaCl_2$)	–	1.0 mM	–	1.0 mM
Ammonium chloride (NH_4Cl)	–	20 mM	–	–
Potassium phosphate (KPO_4)	–	–	62 mM	10 mM
Potassium dihydrogen phosphate (KH_2PO_4)	22 mM	22 mM	–	–
Disodium hydrogen phosphate (NA_2HPO_4)	12 mM	12 mM	–	–
Ferrous sulfate $FeSO_4.7H_2O$	–	–	10 μM	–
D-glucose	0.2%	0.2%	0.4%	–
Casamino acids	0.5%	0.5%	0.1%	–
Peptone	–	–	–	0.32%
Cholesterol	–	–	–	5 μg/ml

Table 1. Media Composition
The composition of different nutrient formulations used in this study.

such as nutrition influence the activation of the three QS circuits—Rhl, Las, and Pqs—in *P. aeruginosa* (Dekimpe and Déziel, 2009; Duan and Surette, 2007; Wagner et al., 2003; Welsh and Blackwell, 2016). A few bypass signaling circuits, including a two-component system (TCS), have been implicated in such contexts (Welsh and Blackwell, 2016). For instance, the PhoB-PhoR TCS can directly activate the Rhl QS circuit under phosphate-limited condition (Jensen et al., 2006). However, a comprehensive analysis of the possible impact of various nutrients or the importance of bacterial nutrient sensors in *P. aeruginosa* swarming is yet to be carried out.

In the present study, we analyzed the swarming behavior of *P. aeruginosa* strain PA14 across six different nutrient agars and defined swarm features that can be quantified easily. We show that swarm patterns vary considerably across media with reproducible, medium-specific features. We also show that 44 genes encoding TCSs including several poorly characterized or unstudied sensor kinases (SKs) and response regulators (RRs) are required for *P. aeruginosa* swarming. Among these, four TCS genes are essential for swarming on all media, whereas the remaining have context-specific functions. We also find that several positive regulators of swarming have an opposite effect on biofilm formation.

RESULTS

Phenotypic Plasticity *in P. aeruginosa* Swarming Is Nutrition Dependent

To understand whether nutrition had an impact on swarming, we analyzed *P. aeruginosa* swarming on six different media—Luria Bertani (LB), BHI, M8, M9, peptone growth media (PGM), and BM2—the nutrient formulations often described for *P. aeruginosa* growth or swarming studies (Kohler et al., 2000; Morris et al., 2011; Overhage et al., 2008, 2007; Yeung et al., 2009) (Table 1). Peptone growth media (PGM), also called *slow-killing medium*, is used for *P. aeruginosa* growth in *Caenorhabditis elegans* infection studies (Singh and Aballay, 2006; Sun et al., 2011; Tan et al., 1999). As shown in Figures 1A–1F, we found that four media supported dendritic swarm pattern for *P. aeruginosa* PA14. However, BHI and LB media did not support dendrite formation, a characteristic of *P. aeruginosa* swarming (Figures 1A and 1B). LB and BHI agar also poorly supported isometric swarm expansion pattern exhibited by other species such as *Escherichia coli*, *Salmonella typhimurium*, or *Bacillus subtilis* (Harshey and Matsuyama, 1994; Kearns and Losick, 2003; Patrick and Kearns, 2009). Thus three minimal media (M8, M9, and BM2) and one undefined medium (PGM) supported swarming with distinct dendrites. All four media also supported multiple (1–3) levels of branching (Figures 1C–1F) but had medium-specific or plastic patterns.

Furthermore, we analyzed the planktonic growth kinetics of PA14 in all the six media mentioned above. Both LB and BHI broth supported better growth of PA14 (Figure 1G) compared with M8, M9, and PGM

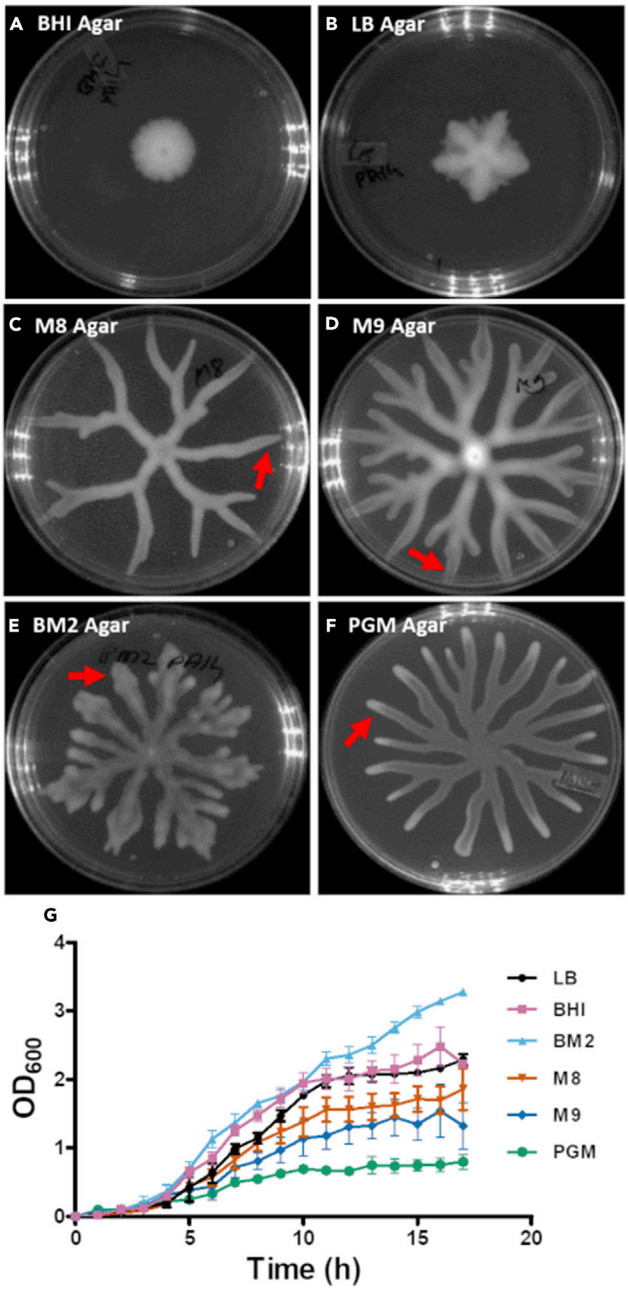

Figure 1. *P. aeruginosa* PA14 Swarm Pattern on Different Swarming Agar

(A–F) *P aeruginosa* swarm obtained at 37°C for 24 h on (A) brain heart infusion (BHI), (B) Luria Bertani (LB), (C) M8, (D) M9, (E) BM2, and (F) peptone growth media (PGM) with 0.6% agar; 6–20 plates were used for each medium.

(G) PA14 wild-type planktonic growth kinetics on various media is shown. Dendrites are indicated with arrow in (C–F).

broth. The BM2 broth was also able to support good planktonic growth like LB and BHI broth. M8 and M9 broth supported moderate growth, whereas the PGM broth supported poor growth for PA14. Hence, except for the BM2 medium, our data suggest an inverse relationship between planktonic growth and propensity for swarming in *P. aeruginosa* PA14. Taken together, analysis of swarm agar and broth phase growth of PA14 in three undefined media and three minimal media suggests that poor media, probably lacking specific nutrients, promote swarming. These data also provide evidence for nutrition-dependent plasticity in *P. aeruginosa* swarm pattern.

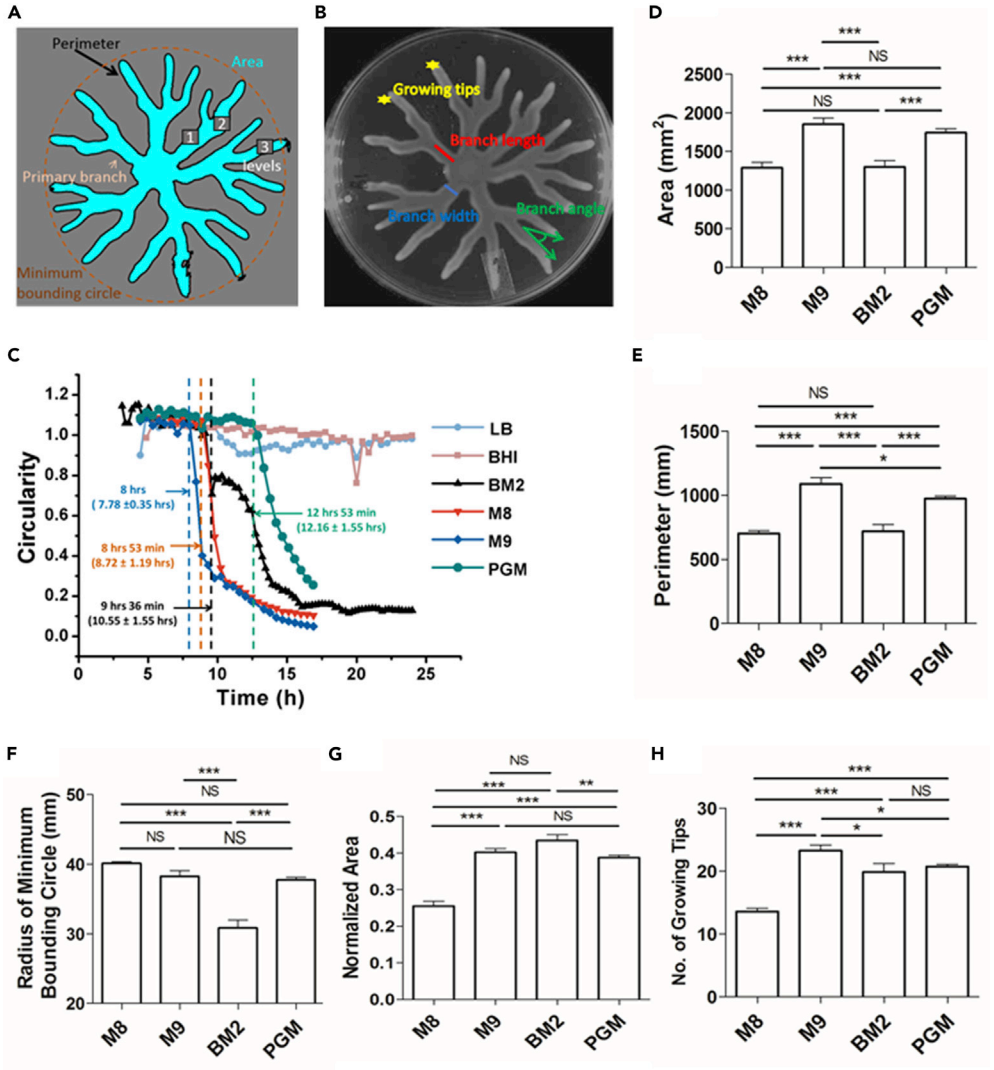

Figure 2. Macroscale Features of *P. aeruginosa* Swarm

Quantifiable features of the swarm (A) Perimeter, area, primary branch, branching levels and minimum bounding circle, and (B) Growing tips, branch length, branch width and branch angle are indicated. (see Methods for definition). (C–H) (C) Circularity plot for swarm expansion on LB, BHI, M8, M9, BM2, and PGM swarm agar. End of swarm lag is indicated. Histogram for (D) swarm area, (E) perimeter, (F) radius of minimum bounding circle, (G) normalized area, and (H) number of growing tips. Pairwise comparison between every two media was performed by Tukey test. $p > 0.05$, ns, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Videos S1, S2, S3, S4, S5, and S6 and Figure S1.

Macroscale Features Define the Plasticity of *P. aeruginosa* Swarm

To characterize the plasticity in PA14 swarm patterns across different media, we set out to define features of the swarm that could be quantified. We found that the conventional approach of comparing a single feature, such as the swarm diameter or bacterial cell number (Overhage et al., 2007; Xavier et al., 2011; Yeung et al., 2009), was not suitable to differentiate dendritic patterns observed on four different media in our study (Figures 1C–1F). By analyzing several swarm images for each of the media, we defined 11 measurable features of *P. aeruginosa* swarm illustrated in Figures 2A–2C. These included branch angle, branch width, number of growing tips, area of the swarm, swarm perimeter, normalized area, etc. (Figures 2A and 2B, see Methods). Swarm lag, the time taken to initiate branching from the time of spotting, is the shortest on M9 followed by on M8, BM2, and PGM agar (Videos S1, S2, S3, S4, S5, and S6). This is represented in Figure 2C as circularity versus time. Drop in circularity below the value of 1.0 marks the end of the swarm lag and initiation of branching. We find that the branching

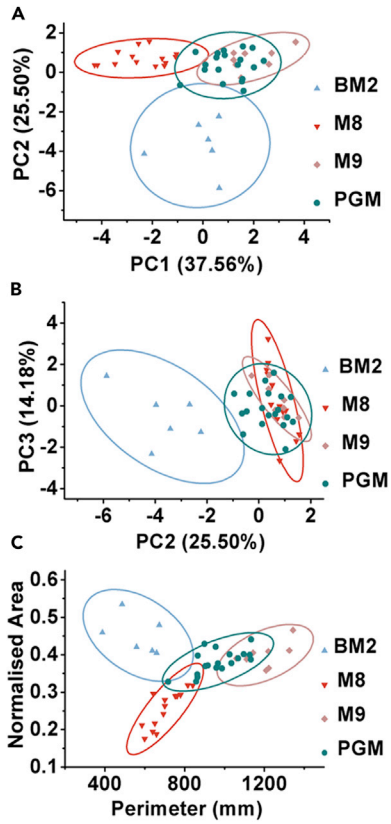

Figure 3. Principal-Component Analysis of Swarm Features

(A and B) (A) PC1 (branch angle, area, perimeter, normalized area, and growing tips) versus PC2 (branch width and radius of minimum bounding circle), and (B) PC2 versus PC3 (branch length, number of primary branches and number of levels) plot for dendritic swarm pattern on BM2, M8, M9, and PGM agar.

(C) Perimeter versus normalized area plot for swarm patterns on BM2, M8, M9, and PGM agar.

Centroids for each medium-specific swarm are indicated in (A–C). See also Figures S2 and S3.

starts first in M9, followed by M8, and then on BM2 agar. The swarm lag is longest on PGM agar (Figure 2C). Our observations suggest that medium influences the regulatory program that dictates the set time to initiate branching.

We utilized one-way ANOVA to isolate features that account for variance across swarm patterns observed on four media that promote dendritic swarming. All 10 macroscale features—area (***), perimeter (***), radius of minimum bounding circle or RMBC (***), normalized area (***), branch length (**), branch angle (***), branch width (***), number of levels (*), number of primary branches (*), and number of growing tips (***)—could explain the variance across swarms on these media. Furthermore, we utilized Tukey's post hoc test to identify features that vary significantly between any two media (Figures 2D–2H, Figure S1). For example, perimeter and area coverage (mean $\pm$ SEM) values for swarm were significantly different (Figure 2D) for most pairwise comparisons, as well as the number of growing tips and normalized area. However, to understand the contribution of each feature to the swarm plasticity across different media, we performed a principal-component analysis (PCA). For the PCA, we used all the 10 macroscale features other than circularity of several swarms on each of the four media (Methods). Principal component 1 (branch angle, area, perimeter, normalized area, and growing tips) contributed 37% to the variance, whereas component 2 (branch width and radius of minimum bounding circle) contributed to 25% (Figure 3A) of the variance across media. Principal component 3 (branch length, number of primary branches, and number of levels) contributed only 14% to the variance (Figure 3B). Normalized area versus perimeter could also distinguish PA14 swarm patterns on M8, M9, BM2, and PGM agar into distinct centroids (Figure 3C). We have used these two features in the rest of this study. Taken together, we could define multiple quantifiable features of *P. aeruginosa* PA14 swarm that can be used to analyze perturbations to swarm patterns.

Several Two-Component Genes of *P. aeruginosa* Are Conditional Modulators of Swarming

Media-dependent plasticity in PA14 swarming patterns strongly suggested that nutritional cues, in the growth media, might be critical for inducing swarming in *P. aeruginosa*. We then set out to ask how does *P. aeruginosa* sense such changes to execute swarming? In prokaryotes, the TCSs are predominantly involved in sensing environmental signals such as nutrition, change in pH, redox state, osmolarity, and light (Laub and Goulian, 2007; Stock et al., 2000; Zschiedrich et al., 2016). The *P. aeruginosa* PA14 genome encodes 160 TCS genes (Lee et al., 2006; Liberati et al., 2006) and is thought to confer exceptional adaptability of this bacterium to various environmental niches and a wide range of hosts, including humans, *Drosophila*, *C. elegans*, and plants (Barreteau et al., 2009; Clatworthy et al., 2009; D'argenio et al., 2001; Francis et al., 2017; Rodrigue et al., 2000; Tan et al., 1999).

In a previous genetic screen for swarming in *P. aeruginosa* PA14, 12 of the candidate genes identified were TCS class regulators (Yeung et al., 2009). This study was, however, under BM2 agar condition alone. We hypothesized that several TCSs are required to sense differential nutritional signals, which promote swarming. As the media used in this study vary in both macro- and micronutrients (Table 1), we expected to find distinct TCS genes to be required for swarming on different media. To test this hypothesis, we performed a targeted genetic screen for swarming, using transposon insertion mutations affecting 113 TCS loci of PA14 (Liberati et al., 2006). The screen was performed on all six media in duplicates. To assess the effect of TCS genes on swarming, we extracted three macroscale features—area, perimeter, and normalized area—from 681 swarms using MATLAB (see Figures S2 and S3). As shown in perimeter versus normalized area plot for four media in Figure 4A–4D, many TCS genes were required for swarming (Table S1). Many of the swarm regulators were orphan, whereas there were four pairs (Table S2; images in Figure S4).

Irrespective of difference in nutrient formulations, we found that four TCS genes were essential for swarming on all media, whereas nine TCS genes were required for swarming on at least two media. However, the largest number of TCS genes (31 of 44) displayed medium specific role in swarming. Notably, 13 SK- and 7 RR-encoding genes were essential for swarming exclusively on BM2 agar; only 3 RR- and 2 SK-encoding genes were found essential for swarming on M8 agar alone. Swarming on PGM agar required six TCS genes, whereas a single TCS gene encoding an RR was found essential exclusively in swarming on M9 agar. Media-dependent requirement of TCS is displayed in a Venn diagram (Figure 4E). These observations suggest that swarming on the BM2 medium is dependent on signaling from many SKs, whereas swarming on other media is less reliant on them. It is also interesting to point out that in 23 cases either the RR or the SK, but not both, was enriched in the screen, indicating a possible cross talk in *P. aeruginosa* TCS signaling for swarming (Table S2). However, we did find four cognate pairs that displayed phenotypic correlation for swarming.

Majority of TCS mutants displayed a strong, but context-dependent, swarming phenotype across media. A few striking examples are presented in Figure 4F. PA3947/rocR had a BM2-agar-specific function (Figure 4F). Similarly, PA3271 and PA1611 had specific function in swarming on PGM agar and M8 agar, respectively. PA14_59790/*pvrR*, an RR found exclusively in *P. aeruginosa* PA14 and PA7 genomes, showed a role in swarming on M9 and weakly on PGM agar (Figure 4F). As a control, we checked the context-dependent requirement for PA3587/*metR*, a previously described transcriptional regulator of swarming on BM2 agar (Yeung et al., 2009). We found that *metR* was indeed swarming deficient on BM2 agar but swarming proficient on M8, M9, and PGM agar (Figure 4F). Interestingly, *phoB* required for swarming in low-phosphate (2 mM) BM2 medium (Bains et al., 2012) was swarming proficient on all the four media we tested (Figure 4F). This was expected as all media we used contain phosphate concentration of 24 mM or above.

Flagella and QS are essential for swarming in PA14 strain (Kohler et al., 2000; this study), although they have muted phenotype in PA01 strain (Gellatly et al., 2018). Indeed, quorum-defective mutant, *rhlR*, was a non-swarmer on all media we tested. However, *rhlR* had a wild-type swimming phenotype (Figure 4G). In contrast, essential swarming regulators of the TCS class were swimming defective (Figure 4G). *fleS* and *fleR* mutant showed moderate to severe swimming defect (Figure 4G), as also shown earlier (Ritchings et al., 1995). An analysis of transposon insertions in 26 flagellar genes (Liberati et al., 2006) also showed non-swarming phenotype (data not shown). Taken together, these experiments suggested that swimming ability is indeed essential for swarming under all conditions.

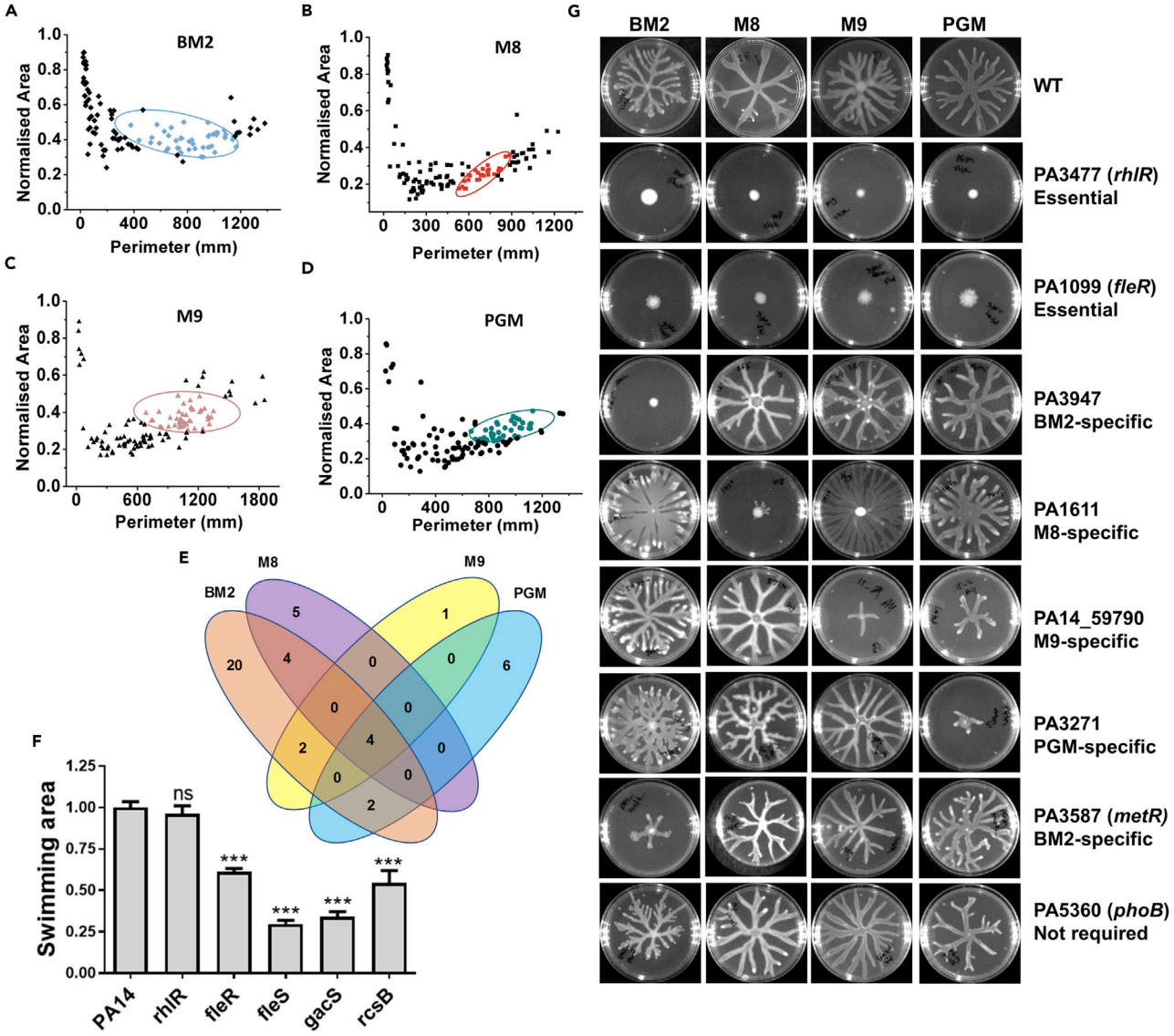

Figure 4. Two-Component Systems of *P. aeruginosa* Regulate Swarming

(A–D) Perimeter versus normalized area plot for PA14 and 113 TCS mutants on (A) BM2 agar, (B) M8 agar, (C) M9 agar, and (D) PGM agar. Centroid for wild-type PA14 on respective media is shown. All mutants outside the centroids represent weak swarmers or non-swarmers.

(E) Venn diagram to show media-specific non-swarmer TCS mutants (also see Table S1).

(F) Representative images of swarm of six TCS mutants, *rhlR* and *metR* strains on BM2, M8, M9, and PGM media.

(G) Swimming phenotype of essential swarm regulators on LB-0.3% swim agar.

Mean values were compared by unpaired *t* test ($p > 0.5$, ns or not significant; ***$p < 0.001$). See also Figures S2–S4, and Table S1.

In all, our observations showed that 44 TCS genes are required for swarming, but in a conditional manner. The results indicated that many nutritional or environmental cues promote swarming by activating specific two-component signaling circuits.

TCS Genes Differentially Regulate Swarming and Biofilm Formation

Biofilm and swarming constitute the sessile and motile population, respectively, but both rely on QS. In *P. aeruginosa* certain cellular components such as flagella are required for both swarming and biofilm formation (Kohler et al., 2000; O'Toole and Kolter, 1998). In contrast, some regulatory components important for biofilm formation negatively regulate swarming motility (Ueda and Wood, 2009; Bhuwan et al., 2012;

Kuchma et al., 2007). For instance, higher cellular level of cyclic di- guanosine monophosphate (GMP) molecule is considered a major switch for biofilm formation in many bacteria, including *P. aeruginosa* (Baker et al., 2016; Romling et al., 2013; Valentini and Filloux, 2016), while suppressing swarming. Indeed, some of the genes discovered as swarm regulator in our study—gacA/gacS, *retS*, *sagS*, *bfiS*, *wspR,* and *hptB*—are known to be regulators of biofilm formation (reviewed in Francis et al., 2017). Yeung et al. had examined 35 swarming-defective strains and found that 19 formed better biofilm. Of these, five were TCS components—*cbrA*, *gacS*, *ntrC*, PA4398, and *algR* (Yeung et al., 2009). This raised the question whether swarm regulators discovered in this study regulate biofilm formation in PA14 and in what manner.

To understand the impact of TCS genes on biofilm formation, we assayed biofilm formation by PA14 wild-type and TCS mutants as described (O'Toole, 2011) at 24 h. We used M63 medium recommended for quantification of biofilm in addition to M8, M9, BM2, and PGM broth (Table S3). Figure 5A shows swarm phenotype of all 44 swarm mutants (see perimeter values in Table S1) and *rhlR* mutant on four different media in colored disk format. Figure 5B shows biofilm phenotype based on crystal violet stain of TCS mutant on five media, again in colored disk format. We found that biofilm formation was not influenced by a change in media (compare disk color in each column in Figure 5B, values in Table S3). Only three mutants were unable to form the biofilm on all media (dark blue disks, Figure 4B). These included *fleS* and *fleR* mutants defective in flagella biogenesis and *wspR*. sagS and bfiS are known to regulate old biofilm in PA01 strain (Petrova and Sauer, 2011), but we found no phenotype for them in young biofilm in PA14 strain. However, four TCS mutants (*cheA, creC, cpxA, and tctE*) showed hyper-biofilm phenotype (deep yellow disks in Figure 4B) and thus appear to be negative regulators of biofilm formation, in a media-independent manner. There were few media-specific regulators of biofilm formation as well. For instance, *ntrB* was essential for biofilm formation in M63 and PGM broth, whereas *kinB* was found essential only in M8 broth. The relationship between biofilm formation and swarm formation phenotypes is displayed in a double-faced Janus droplet map for four media in Figure 5C. The left half of the droplet represents swarm phenotype, whereas the right half represents swarming phenotype under the same condition (medium). We found that in 38 instances non-swarmer TCS mutants or a weak swarmer produced significantly better biofilm (2- to 5-fold increase in crystal violet stain) than the wild-type PA14 (16 on BM2, 8 on PGM, 9 on M9, 5 on M8, and 8 on PGM). There were 12 instances of coordinate regulation of swarming and biofilm formation, *fleR*, *fleS*, *wspR*, PA4781, and *kinB,* for both swarming and biofilm formation. In three instances, *erdR* and *tctE* were negative regulators of both biofilm and swarm (yellow Janus droplet in Figure 5C). All other events appear to indicate that TCS genes regulate one process (swarm or biofilm) but not the other. Taken together, the systematic analysis of swarm and biofilm formation on four different media (176 one-on-one comparisons) indicated that several, but not all, TCS circuits regulate switch between swarming and biofilm formation.

In summary, this study provides evidence that many TCS genes are critical for swarming in *P. aeruginosa* in a contextual manner (Figure 6). We find that PA14 swarming under one condition, such as BM2 agar, requires input from several TCS systems, whereas swarming on M8, M9, and PGM media (condition II) rely on fewer TCS circuits. Nutritionally rich media, LB and BHI, do not support dendritic swarming in *P. aeruginosa* PA14. Thus extrinsic nutritional cues in conjunction with bacterial SK/RR systems are critical in the modulation of *P. aeruginosa* swarming.

DISCUSSION

In this study, we show that *P. aeruginosa* exhibits a remarkable, context-dependent plasticity in its swarming behavior. This arises due to nutrient limitation in growth media and is sensed by the TCS class of SKs and their partners called *response regulators*. We provide a number of macroscale features of *P. aeruginosa* swarm to differentiate media-specific swarm patterns into distinct populations.

We were able to establish that swarm lag, the time to initiate branching or dendrite formation, is a quantifiable feature of *P. aeruginosa* swarming that can be represented as a change in circularity in time. Indeed, swarm lag was significantly different between M8, M9, BM2, and PGM agar. The circularity remained close to 1 for the entire duration in non-dendritic growth on LB and BHI, again making it a valuable feature. Circularity, as well as other macroscale features described in this study, can be utilized to quantify the effect of the environmental factors or genetic regulators on the swarm pattern.

One important question raised by this study is what are the nutritional cues that promote swarming? Boyle et al. (Boyle et al., 2015) have suggested that iron limitation is a requirement for swarming. Among the four

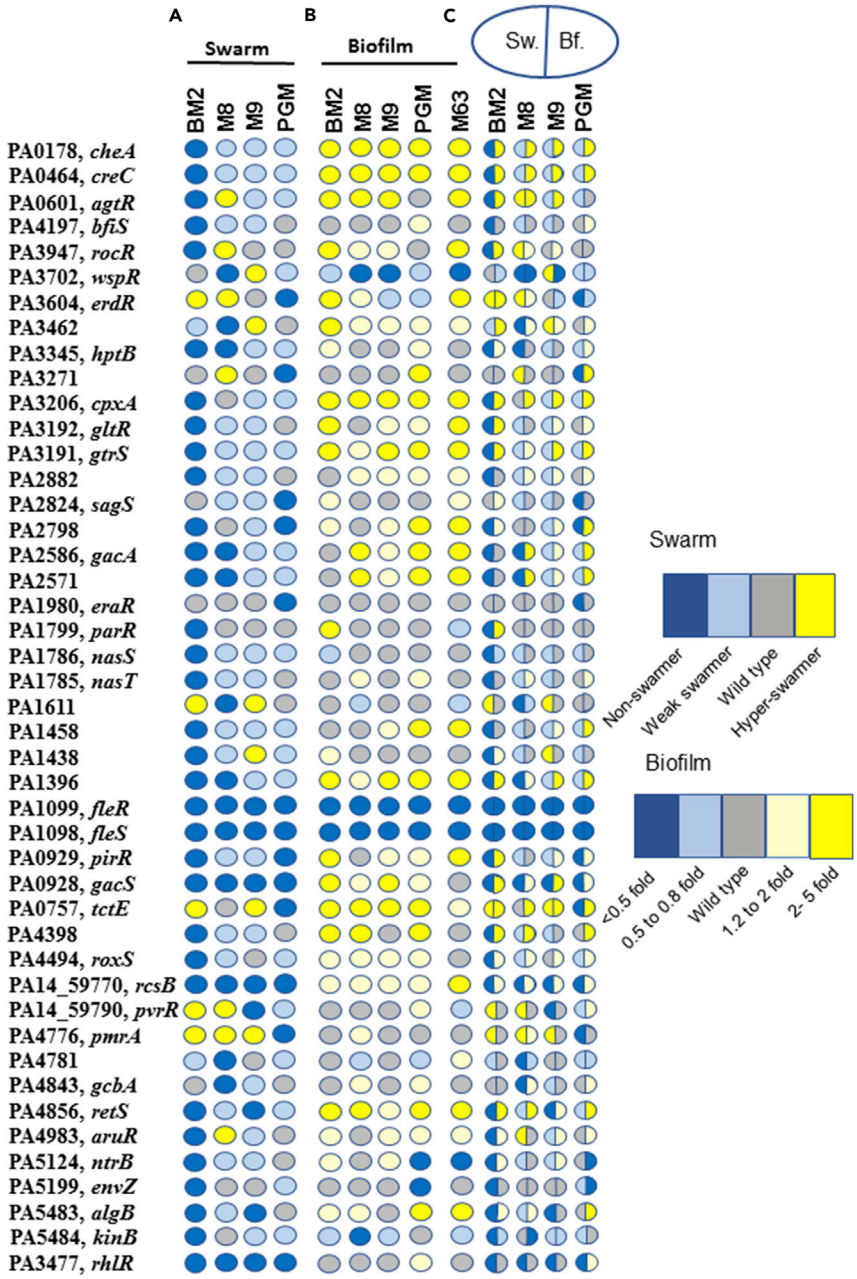

Figure 5. Media-Dependent Role of TCS Genes in Swarming and Biofilm Formation

(A) Disk heatmap of swarm phenotype of 44 TCS mutants and *rhlR*. Heatmap is based on perimeter values in Table S1. Swarm phenotype: non-swarmer, perimeter less than 20% of PA14 swarm perimeter; weak swarmer, >20% but less than mean − SD of PA14 perimeter; hyper-swarmer, > mean + SD of PA14 swarm perimeter.

(B) Biofilm formation by 44 TCS mutants and *rhlR* in BM2, M8, M9, PGM, and M63 broths, measured by crystal violet stain (values in Table S3).

(C) Janus droplet representation of media-dependent biofilm and swarm phenotype. Left face shows swarming phenotype, whereas the right face reflects biofilm phenotype. Crystal violet staining for biofilm is represented as fraction of biofilm formation by PA14.

See also Figure S4, Tables S1–S3.

media we used, only BM2 had the iron supplement. However, it does support PA14 swarming with a medium-specific pattern. The nitrogen content of the BM2 medium, however, is lower than that of M8 and M9 media (Table 1), suggesting that nitrogen limitation could also be a driver for initiating swarming under

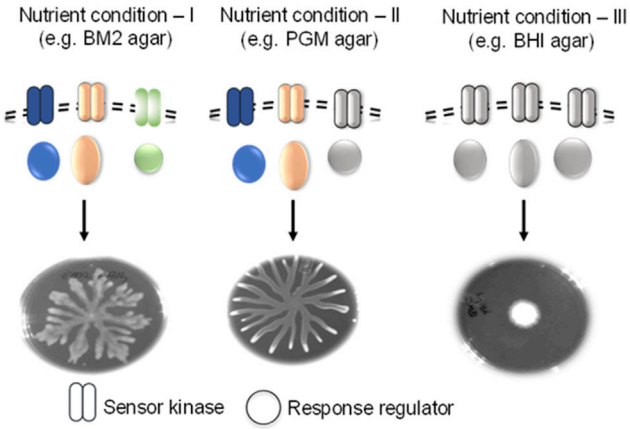

Figure 6. Media-Dependent Plasticity in *P. aeruginosa* Swarming

Growth media vary in macro- and micronutrient contents. Some growth media (condition I) depend on several TCS modules to allow swarming of *P. aeruginosa*. Another set of media (condition II) promote swarming but rely on a smaller but specific set of TCS genes for swarming. Certain other growth media (condition III) do not allow dendritic swarming of *P. aeruginosa*.

BM2 condition. Indeed, nitrogen-related TCSs—*ntrB* and *nasS*/*nasT*—were essential for swarming on BM2 media alone. These mutants also displayed weak swarming on other three media, suggesting that nitrogen limitation may be a contributing factor to swarming in those media as well. Phosphate limitation is a known driver for swarming (Bains M, Fernández L, 2012), but it was not relevant for the four media we tested due to the presence of phosphate in the media and dispensability of phosphate-specific TCS *phoB* and *phoR* (Table 1, Figure 4F) for swarming in this study. Involvement of low Mg^{2+} and cationic-peptide-inducible *PmrA* on swarming on PGM agar suggests that Mg^{2+} and cationic peptides may become relevant on certain media. Addition or removal of specific macro- and micronutrients (Table 1) to or from these four media will serve to decipher additional nutritional cues that drive TCS genes to modulate swarming. Do similar drivers exist in a *P. aeruginosa* infection setting in humans? A careful analysis of nutrients in body fluids of the host in different pathologies such as cystic fibrosis and diabetic foot ulcer can provide better insight into *P. aeruginosa* pathogenesis.

Our study raises a second question: How does nutrition impact swarming? It could be via modulation of flagellar output, modulation of rhamnolipid production, or novel pathways required for hitherto unidentified effector molecules, necessary for swarming. In planktonic growth, rhamnolipid production is induced at the end of the lag phase, which corresponds with onset of nutrient limitation (Caiazza et al., 2005). In addition, Xavier et al. have shown that rhamnolipid production is regulated by nitrogen limitation in minimal media (Xavier et al., 2011). We find that rhamnosyl transferase chain A (RhlA) transcription in much higher in PGM-broth-grown PA14 than in LB-broth-grown PA14. Transcription of *rhlA* is further induced on PGM-0.6% swarm agar than PGM broth (data not shown). This indicated that rhamnolipid production is dependent on media as well as surface contact. Careful analyses of rhamnolipid production in response to removal or limitation of specific nutrient one at a time would be instructive in understanding the regulation of swarming. Some of the TCS components may regulate swarming via the modulation of cyclic di-GMP levels. We found that PA4398 is a positive regulator of swarming on BM2 medium and a negative regulator of biofilm formation (Figures 5A and 5B). This is in agreement with an earlier report wherein the mutation in PA4398 led to a 50% increase in intracellular cyclic-di-GMP, which is linked to enhanced biofilm formation (Strehmel et al., 2015). Indeed, six RRs (*RocR*, *WspR*, *ErdR*, *PvrR*, PA4781, and PA4843), we identified as swarm regulator genes, do possess diguanylate cyclase (GGDEF) or phosphodiesterase (EAL and HD-GYP) domains for regulation of cyclic-di-GMP turnover in bacteria. There is also some evidence for the role of nitrogen limitation in swarming. A TCS pair (*NasS*/*nasT*) involved in nitrate assimilation is essential for swarming on BM2. In addition, SK *NtrB* and two ntrC domains containing RR are also essential for swarming (Table S2). A systematic study of each TCS component in a medium-specific context will help decipher the possible mechanism of modulation of swarming. Over time, it would be possible to build up the regulatory program for swarming in *P. aeruginosa*.

A growing body of evidence suggests that environmental signals, particularly nutritional cues, can differentially influence QS in *P. aeruginosa* (Duan and Surette, 2007; Jensen et al., 2006; Wagner et al., 2003; Welsh and Blackwell, 2016). The TCS class signaling was one of the few bypass activation circuits implicated in such contexts (Jensen et al., 2006; Welsh and Blackwell, 2016). We were surprised to find 32 TCS modulators of swarming on BM2 agar when compared with 12 genes uncovered in a previous screen (Yeung et al., 2009). We believe that additional TCS regulators of swarming were found due to the utilization of a large (90-mm) dish format for each strain in our study when compared with the 96-well multiplicator format deployed for the primary screen in the previous report (Yeung et al., 2009). We find that avoidance zone between two swarms occurs at about 5 mm distance, and 96-prong multiplicator does not allow enough expansion of swarm to detect all non-swarmers in our laboratory (data not shown). A recent study from our laboratory shows that *P. aeruginosa* can detect both bacteria and non-biological obstacles on PGM swarm agar (Kotian et al., 2018) reiterating that swarming in *P. aeruginosa* is sensitive to environmental cues including proximity to isogenic bacteria.

P. aeruginosa genomes exhibit expansion of the TCS-encoding genes to 160, one of the largest TCS repertoires among eubacteria. The GacS network, along with HptB and SagS branches, control biofilm, virulence, and motility. We found that six components of this extended network (*gacS*, *gacA*, *retS*, PA1611, *hptB*, *sagS*, and *bfiS*) were regulators of swarming in *P. aeruginosa*. Only gacS was an essential regulator of swarming. On the other hand, some of the conditional swarm modulators uncovered in this study are linked to nutrient assimilation (e.g., *ntrB*, *nasS*, and *nasT*). Several TCS mutants previously described as motility-, swarming-, or biofilm-related loci including *PA3702*/*wspR* (Chen et al., 2014), *PA3345*/*hptB* (Bhuwan et al., 2012; Hsu et al., 2008) *PA14_59770* (*rcsB*) (Giraud et al., 2009), *PA14_59790* (*pvrR*) (Giraud et al., 2009; Zheng et al., 2016), and *PA4398* (Strehmel et al., 2015) enriched as swarm regulator, at least under one condition, in our study. To the best of our knowledge, the others are not known to have any swarming-related function. A very recent study analyzed TCS genes for cytotoxicity in a cell line model (Gellatly et al., 2018) and found 27 TCS genes. Only six of these were enriched as swarm regulators in our study. This indicates that TCS genes control different biological processes. Based on our study, we would like to propose that some of the *P. aeruginosa* TCS genes may have evolved, and would have been retained in the genome, to modulate swarming motility.

One of the surprises from this study came in the form of antagonistic regulation of swarm and biofilm formation by TCS genes and strengthens previous reports (Yeung et al., 2009). There were 38 cases of an inverse relationship between biofilm and swarm. This indicates that several TCS signaling circuits might promote swarming while suppressing biofilm formation. This leads to an interesting hypothesis that *P. aeruginosa* can possibly only exist in one state (either swarming or biofilm) at one time. In future, we would like to study the molecular signatures of the two states. Swarming bacteria are believed to be antibiotic resistance (Butler et al., 2010; Overhage et al., 2008). One of the directions to pursue will be to ask if swarming population of *P. aeruginosa* is more susceptible to antibiotics than *P. aeruginosa* in a biofilm. If so, could we change the state of the bacteria and make them more susceptible to antibiotics. This could be done by pharmacological intervention or simply by perturbation of host body fluids? A comprehensive analysis of transcriptional events that initiate biofilm versus initiate swarm will add to better understanding of the differences between these two quorum-dependent processes.

Limitations of the Study

Our study of the swarm pattern is limited to six different media conditions, although additional media have been described for *P. aeruginosa* growth. Although there are about 160 predicted two-component-related loci in *P. aeruginosa* PA14 genome, our study covers only 113 available in the transposon insertion library. Because of the broad-based nature of our study, we were not able to provide mechanistic details behind each of our observations. We strongly believe that our findings would be of interest to *P. aeruginosa* community as well as broader swarming research community.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.02.028.

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AUTHOR CONTRIBUTIONS

A.M.K, S.J., D.P., H.S.K., D.P., A.M., M.V., and V.S. conceptualized the study. S.J., A.M.K., D.P., and A.M. performed all the experiments. S.J., A.M.K., D.P., and A.M. analyzed the data. H.K. and D.B. performed MATLAB-based processing of images and videos. H.K. and D.B. derived swarm features from swarm images and performed PCA analysis. S.J., A.M.K., H.K., D.P., M.V., and V.S. wrote the manuscript.

DECLARATION OF INTERESTS

Authors declare no conflict of interest.

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ISCI, Volume *13*

Supplemental Information

Context-Specific Requirement of Forty-Four Two-Component Loci in *Pseudomonas aeruginosa* Swarming

Ameen M. Kollaran, Shubham Joge, Harshitha S. Kotian, Divakar Badal, Deep Prakash, Ayushi Mishra, Manoj Varma, and Varsha Singh

Figure S1

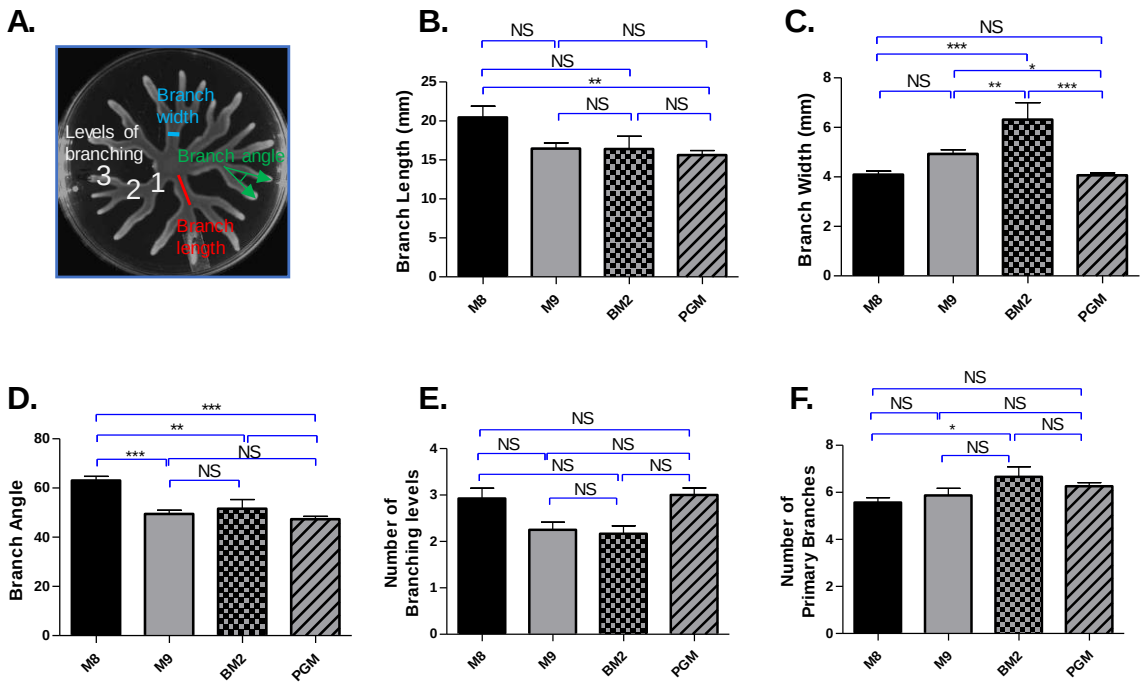

Figure S1: Macroscale features of *P. aeruginosa* swarm, Related to Figure 1 and Figure 2. (A) swarm features are indicated. Histograms for (B) branch or dendrite length, (C) branch width, (D) branch angle, (E) branching levels, and (F) number of primary branches. Pairwise comparison between every two media was performed by Tukey test. (P>0.05, ns; P<0.05, *; p<0.01, **, P<0.001 ***).

Figure S2

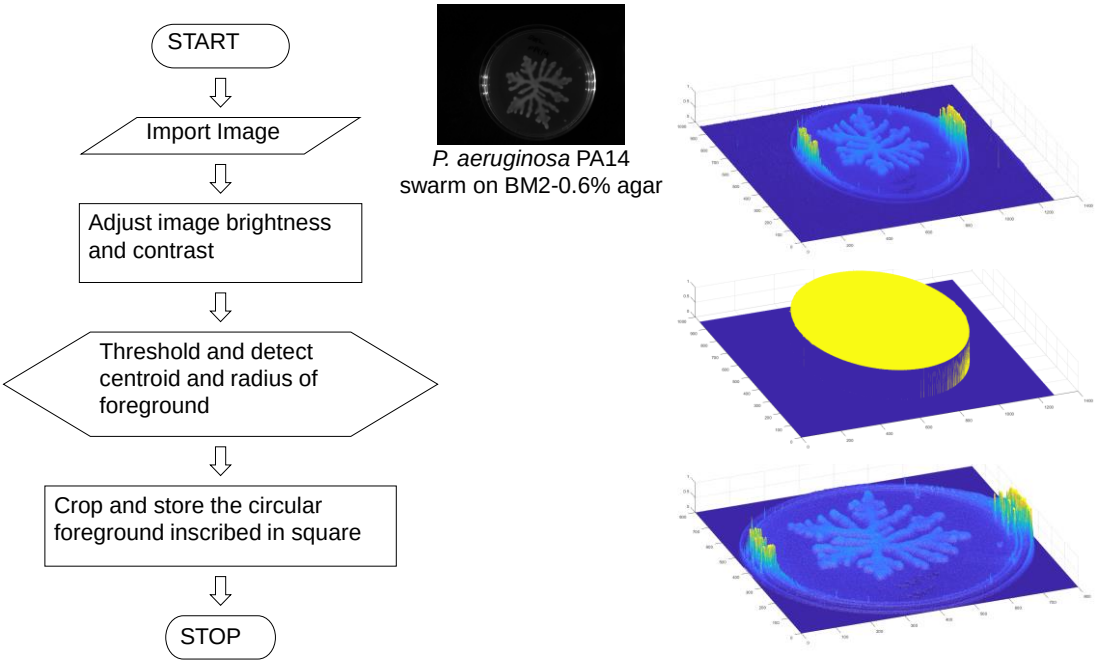

Figure S2. MATLAB workflow for swarm image processing, Related to Figure 3 and Figure 4.

Figure S3.

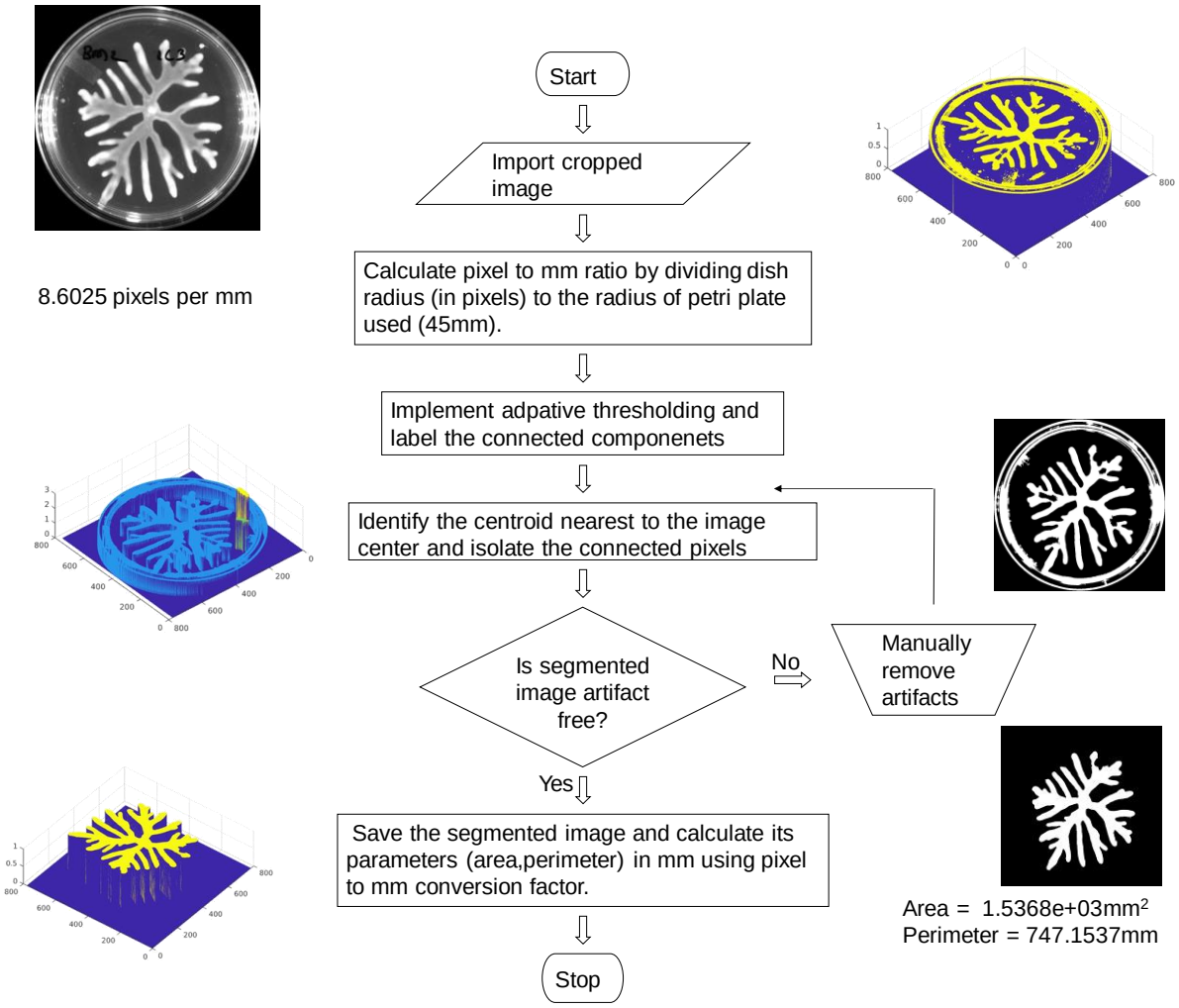

Figure S3. MATLAB workflow for feature extraction from processed swarm image, Related to Figure 3 and Figure 4.

Figure S4. Swarming of 44 TCS mutant strains on six different growth media, Related to Figure 4 and Figure 5.

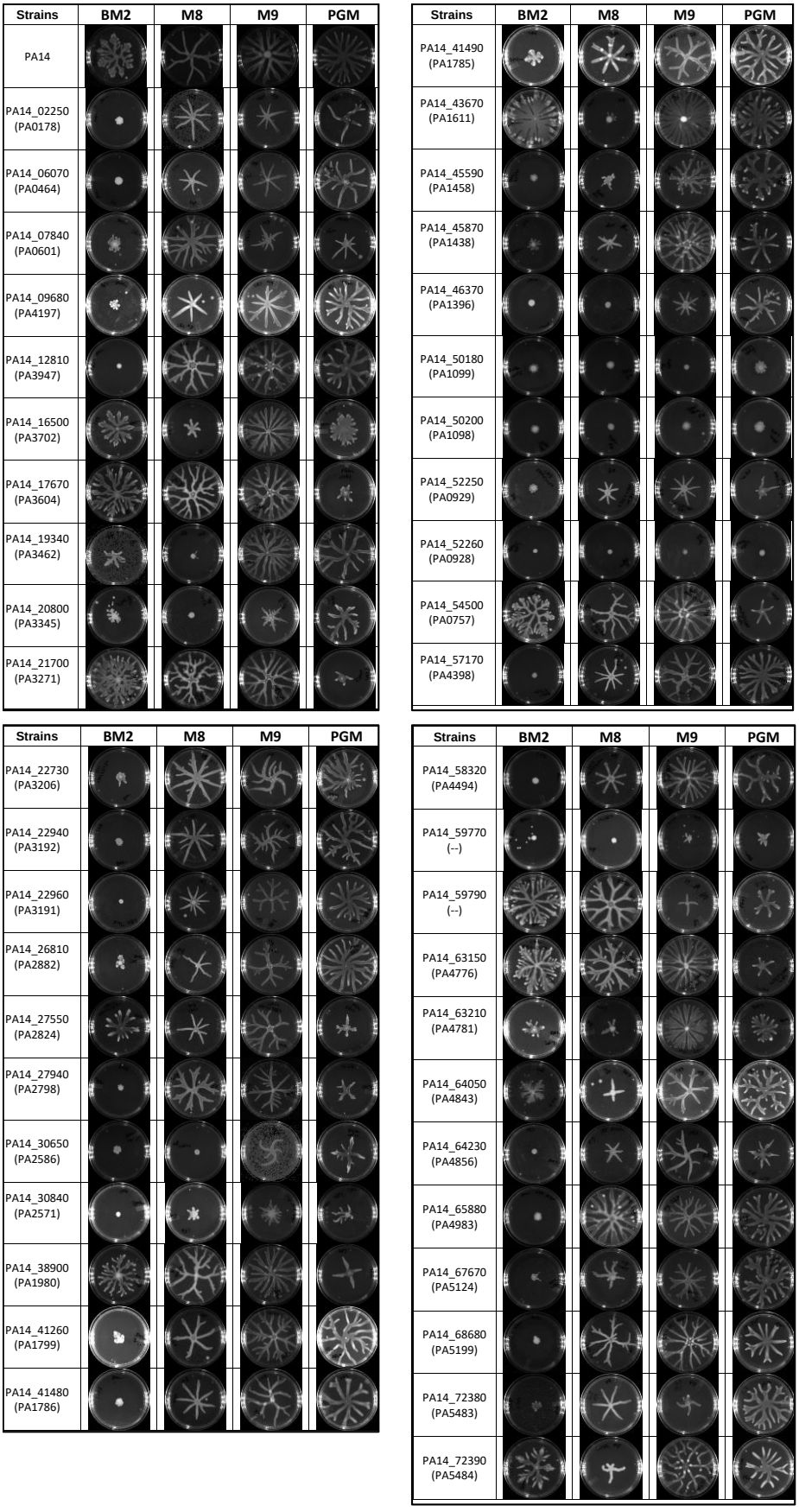

Table S1. Perimeter values[#] for swarms of TCS mutants.

TCS Sl No.	Locus ID	MEDIA				TCS Sl No.	Locus ID	MEDIA			
		PGM	M9	M8	BM2			PGM	M9	M8	BM2
	PA14	100±11	100±11	100±9	100±24	57	PA14_43350	72	132	128	116
	rhlR	3	3	4	4	58	PA14_43670	104	161	11	174
1	PA14_00430	42	118	110	113	59	PA14_45590	71	67	15	8
2	PA14_02250	30	32	67	6	60	PA14_45870	52	140	24	16
3	PA14_02260	69	86	66	68	61	PA14_45880	78	195	121	159
4	PA14_05320	119	95	42	116	62	PA14_46370	51	30	4	5
5	PA14_05330	61	193	97	30	63	PA14_46980	97	113	27	36
6	PA14_06060	103	37	45	168	64	PA14_47390	108	54	29	58
7	PA14_06070	56	32	32	6	65	PA14_48160	142	111	83	167
8	PA14_07820	93	76	111	88	66	PA14_49170	53	46	50	97
9	PA14_07840	23	25	136	18	67	PA14_49180	25	185	32	37
10	PA14_09680	84	56	40	9	68	PA14_49420	45	111	110	44
11	PA14_09690	90	137	31	67	69	PA14_49440	121	155	70	157
12	PA14_10770	48	72	38	99	70	PA14_50180	9	3	8	13
13	PA14_11120	127	122	106	196	71	PA14_50200	7	7	6	10
14	PA14_11630	83	107	68	148	72	PA14_52240	88	49	99	126
15	PA14_11680	81	44	22	44	73	PA14_52250	15	33	27	7
16	PA14_12780	106	62	31	60	74	PA14_52260	4	3	3	3
17	PA14_12810	99	118	122	4	75	PA14_54500	18	120	85	177
18	PA14_12820	71	128	103	21	76	PA14_54510	108	49	61	15
19	PA14_13740	62	67	53	180	77	PA14_55780	110	110	133	39
20	PA14_16350	61	117	34	112	78	PA14_55810	88	159	159	111
21	PA14_16500	31	133	20	83	79	PA14_56950	88	90	140	40
22	PA14_17670	12	109	150	176	80	PA14_57140	94	126	157	152
23	PA14_19340	80	138	7	29	81	PA14_57170	93	77	50	5
24	PA14_20780	92	78	135	125	82	PA14_58300	106	106	61	155
25	PA14_20800	43	27	5	17	83	PA14_58320	59	97	50	5
26	PA14_20820	83	21	30	41	84	PA14_59770	10	7	4	4
27	PA14_21700	10	108	137	147	85	PA14_59780	77	71	120	72
28	PA14_22730	75	60	102	11	86	PA14_59790	25	12	140	213
29	PA14_22940	86	60	80	6	87	PA14_59800	78	71	26	36
30	PA14_22960	66	42	49	3	88	PA14_60250	50	102	101	105
31	PA14_24340	84	108	83	181	89	PA14_60260	64	131	140	184
32	PA14_24350	79	63	24	130	90	PA14_62530	128	107	89	201
33	PA14_24710	145	69	41	48	91	PA14_62540	118	59	60	142
34	PA14_24720	98	136	124	148	92	PA14_63150	17	142	142	153
35	PA14_26810	118	50	36	11	93	PA14_63160	94	37	133	34
36	PA14_27550	16	70	41	58	94	PA14_63210	33	114	18	24
37	PA14_27800	65	72	83	140	95	PA14_64050	81	50	17	51
38	PA14_27810	92	114	91	129	96	PA14_64230	28	18	74	4
39	PA14_27940	17	75	89	5	97	PA14_65860	119	108	109	178
40	PA14_29360	44	114	138	149	98	PA14_65880	82	69	183	6
41	PA14_29740	107	96	174	157	99	PA14_67670	89	52	34	9
42	PA14_30650	29	26	4	6	100	PA14_67680	113	86	172	133
43	PA14_30700	75	88	67	102	101	PA14_68230	105	79	39	119
44	PA14_30830	67	78	89	142	102	PA14_68250	83	128	99	101
45	PA14_30840	21	34	13	4	103	PA14_68680	55	114	87	6
46	PA14_31950	64	106	157	39	104	PA14_69470	77	121	110	179
47	PA14_31960	50	56	124	43	105	PA14_69480	56	28	150	81
48	PA14_32580	57	92	38	197	106	PA14_70750	58	108	136	118
49	PA14_36420	109	112	72	166	107	PA14_70760	103	119	145	205
50	PA14_37690	35	105	55	23	108	PA14_70790	110	21	71	112
51	PA14_38900	20	104	116	116	109	PA14_72380	93	16	46	12
52	PA14_40570	44	129	153	117	110	PA14_72390	56	131	21	91
53	PA14_41260	89	115	67	10	111	PA14_72720	40	131	154	46
54	PA14_41270	65	160	59	27	112	PA14_72740	112	87	38	55
55	PA14_41480	74	66	59	6	113	PA14_73020	33	94	5	65
56	PA14_41490	97	53	55	19						

Table S1. Perimeter values[#] for swarms of TCS mutants, Related to Figure 4 and Figure 5.

[#]Deep blue (non-swarmer), perimeter < 20% of PA14 WT; light blue (weak swarmer), perimeter >20% to < mean - SD of PA14 WT; grey (wild type), mean ± SD of PA14 WT; yellow (hyper-swarmer) > mean + SD of PA14 WT.

Table S2. Description of TCS class of bacterial swarm regulators of *P. aeruginosa* PA14

Sl. No	PA14 ID	PAO1 ID	Gene name[&]	Domain features[*]
	TCS pairs			
1	PA14_22940	PA3192	*gltR*	REC-wHTH
2	PA14_22960	PA3191	*gtrS*	HAMP-HisK
3	PA14_41480	PA1786	*nasS*	(no hit found)
4	PA14_41490	PA1785	*nasT*	REC-ANTAR
5	PA14_50180	PA1099	*fleR*	REC-AAA-Fis
6	PA14_50200	PA1098	*fleS*	PAS-HisK
7	PA14_72380	PA5483	*algB*	REC-AAA-Fis
8	PA14_72390	PA5484	*kinB*	HAMP-HisK
	TCS pairs[#]			
9	PA14_02250	PA0178	*cheA*	HPt-HisK-cheW
10	PA14_06070	PA0464	*creC*	HAMP-HisK
11	PA14_07840	PA0601	*agtR*	REC-HTH
12	PA14_09680	PA4197	*bfiS*	PAS-PAC-PAS-HisK
13	PA14_12810	PA3947	*rocR*	REC-EAL
14	PA14_16500	PA3702	*wspR*	REC-GGDEF
15	PA14_22730	PA3206	*cpxA*	HAMP-HisK
16	PA14_26810	PA2882		CBS-HisK
17	PA14_30840	PA2571		HisK
18	PA14_38900	PA1980	*eraR*	REC-HTH
19	PA14_41260	PA1799	*parR*	REC-wHTH
20	PA14_45590	PA1458		Hpt-HisK-CheW
21	PA14_45870	PA1438		HAMP-HisK
22	PA14_52250	PA0929	*pirR*	REC-wHTH
23	PA14_54500	PA0757	*tctE*	HAMP-HisK
24	PA14_57170	PA4398		HAMP-PAS-HisK
25	PA14_58320	PA4494	*RoxS*	HisK
26	PA14_63150	PA4776	*pmrA*	REC-wHTH
27	PA14_65880	PA4983	*aruR*	REC-wHTH
28	PA14_67670	PA5124	*ntrB*	HisK
29	PA14_68680	PA5199	*envZ/amgS*	HAMP-HisK
30	PA14_59770	N/A	*rcsB*	REC-HTH
31	PA14_59790	N/A	*pvrR*	REC-EAL
	Hybrids			
32	PA14_19340	PA3462		HisK-REC
33	PA14_21700	PA3271		Na:Solute_symport-HisK-REC
34	PA14_27550	PA2824	*sagS*	HisK-REC
35	PA14_43670	PA1611		HAMP-HisK-REC
36	PA14_46370	PA1396		HisK-REC
37	PA14_52260	PA0928	*GacS*	HAMP-HisK-REC-HPt
38	PA14_64230	PA4856	*RetS*	HisK-REC-REC
	Orphans			
39	PA14_17670	PA3604	*erdR*	REC-HTH
40	PA14_27940	PA2798		REC
41	PA14_63210	PA4781		REC-HD-GYP
42	PA14_64050	PA4843	*gcbA*	REC-REC-GGDEF
43	PA14_30650	PA2586	*gacA*	REC-HTH
	HPt Protein			
44	PA14_20800	PA3345	*hptB*	HPt

Table S2. Description of TCS class of bacterial swarm regulators of *P. aeruginosa* PA14, Related to Figure 5. * Domain descriptions are based on prosite (https://prosite.expasy.org/scanprosite/) protein domain searches. # TCS genes with known interacting partner. However, the partner did not enrich in this screen. & Those in bold letters are newly proposed *bsw* names, based on this study. Sensor kinases are shown in grey. All others are response regulators or histidine phosphor transfer protein.

Table S3. Crystal Violet based quantification[#] of biofilm formation in *P. aeruginosa* PA14.

Strain	PA01 ID		PGM	M9	M8	BM2	M63
PA14			1.0 ± 0.0, n=45	1.0 ± 0.0, n=45	1.0 ± 0.0, n=45	1.0 ± 0.0, n=45	1.0 ± 0.1, n=41
pelA	PA3064		0.3 ± 0.0, n=40	0.4 ± 0.0, n=41	0.2 ± 0.0, n=42	0.4 ± 0.0, n=39	0.4 ± 0.0, n=36
PA14_02250	PA0178	cheA	3.0 ± 0.4, n=9	1.7 ± 0.1, n=9	2.2 ± 0.2, n=9	2.2 ± 0.2, n=9	2.1 ± 0.2, n=9
PA14_06070	PA0464	creC	4.6 ± 0.8, n=9	2.5 ± 0.3, n=9	3.0 ± 0.6, n=9	4.6 ± 0.6, n=9	3.0 ± 0.3, n=9
PA14_07840	PA0601		1.0 ± 0.2, n=9	2.7 ± 0.3, n=12	2.9 ± 0.4, n=9	3.9 ± 0.5, n=6	2.2 ± 0.6, n=13
PA14_12810	PA3947		0.7 ± 0.1, n=6	1.2 ± 0.2, n=6	1.2 ± 0.2, n=3	2.8 ± 0.9, n=3	0.9 ± 0.1, n=6
PA14_17670	PA3604		0.6 ± 0.1, n=10	0.7 ± 0.1, n=8	1.8 ± 0.3, n=12	2.9 ± 0.4, n=15	2.2 ± 0.3, n=11
PA14_19340	PA3462		1.8 ± 0.2, n=15	2.0 ± 0.2, n=15	1.9 ± 0.2, n=15	2.4 ± 0.3, n=15	2.0 ± 0.2, n=15
PA14_21700	PA3271		2.0 ± 0.4, n=9	2.1 ± 0.3, n=9	1.5 ± 0.3, n=9	5.5 ± 0.8, n=9	2.4 ± 0.4, n=9
PA14_22730	PA3206		2.9 ± 0.2, n=15	2.3 ± 0.2, n=15	2.3 ± 0.2, n=15	3.2 ± 0.2, n=15	3.4 ± 0.5, n=14
PA14_22940	PA3192	gtlR	1.4 ± 0.1, n=9	2.0 ± 0.2, n=9	1.2 ± 0.2, n=9	3.5 ± 0.8, n=9	2.2 ± 0.2, n=9
PA14_22960	PA3191		2.5 ± 0.2, n=18	2.3 ± 0.2, n=18	2.0 ± 0.2, n=18	4.2 ± 0.8, n=12	2.1 ± 0.2, n=16
PA14_26810	PA2882		1.7 ± 0.3, n=9	1.3 ± 0.1, n=9	1.7 ± 0.2, n=9	1.0 ± 0.1, n=9	1.8 ± 0.1, n=9
PA14_27550	PA2824		1.1 ± 0.1, n=15	1.1 ± 0.2, n=15	1.1 ± 0.1, n=15	1.4 ± 0.1, n=14	1.8 ± 0.4, n=15
PA14_27940	PA2798		2.8 ± 0.5, n=12	1.9 ± 0.1, n=12	1.1 ± 0.1, n=12	1.8 ± 0.2, n=12	2.9 ± 0.4, n=12
PA14_30650	PA2586	gacA	2.1 ± 0.3, n=9	1.9 ± 0.1, n=9	2.3 ± 0.2, n=12	1.4 ± 0.2, n=9	2.5 ± 0.2, n=9
PA14_30840	PA2571		1.4 ± 0.2, n=17	1.2 ± 0.2, n=18	1.8 ± 0.4, n=12	3.4 ± 0.3, n=18	1.6 ± 0.1, n=12
PA14_41260	PA1799		0.9 ± 0.1, n=9	0.9 ± 0.1, n=9	1.1 ± 0.2, n=9	0.2 ± 0.0, n=6	0.7 ± 0.2, n=9
PA14_41480	PA1786	nasS	0.8 ± 0.1, n=9	1.0 ± 0.2, n=9	0.8 ± 0.1, n=9	0.8 ± 0.1, n=7	0.8 ± 0.2, n=8
PA14_45590	PA1458		2.7 ± 0.4, n=9	1.8 ± 0.2, n=9	1.4 ± 0.4, n=9	1.0 ± 0.2, n=8	3.7 ± 0.7, n=8
PA14_46370	PA1396		1.9 ± 0.2, n=12	2.2 ± 0.2, n=12	1.7 ± 0.3, n=11	2.5 ± 0.5, n=12	2.1 ± 0.2, n=12
PA14_50180	PA1099	fleR	0.3 ± 0.0, n=9	0.2 ± 0.0, n=9	0.3 ± 0.1, n=9	0.5 ± 0.1, n=9	0.2 ± 0.0, n=3
PA14_50200	PA1098	fleS	0.2 ± 0.0, n=9	0.2 ± 0.0, n=9	0.4 ± 0.1, n=8	0.3 ± 0.0, n=9	0.2 ± 0.0, n=3
PA14_52250	PA0929		1.4 ± 0.2, n=9	1.6 ± 0.2, n=9	1.3 ± 0.2, n=9	2.5 ± 0.3, n=6	3.1 ± 0.6, n=9
PA14_52260	PA0928	GacS	1.6 ± 0.3, n=9	3.4 ± 0.2, n=9	1.9 ± 0.3, n=9	2.8 ± 0.6, n=5	1.0 ± 0.2, n=9
PA14_54500	PA0757		0.8 ± 0.1, n=9	1.3 ± 0.1, n=9	1.4 ± 0.0, n=6	2.8 ± 0.6, n=5	2.3 ± 0.3, n=9
PA14_57170	PA4398		1.6 ± 0.3, n=12	1.2 ± 0.3, n=12	2.0 ± 0.3, n=6	2.9 ± 0.3, n=6	1.6 ± 0.4, n=12
PA14_58320	PA4494		1.5 ± 0.2, n=15	1.7 ± 0.2, n=15	1.3 ± 0.1, n=15	1.8 ± 0.2, n=12	1.7 ± 0.3, n=15
PA14_59770	NOT PRESENT	rcsB	1.9 ± 0.3, n=18	2.4 ± 0.4, n=18	2.5 ± 0.5, n=15	3.6 ± 0.5, n=12	2.5 ± 0.3, n=12
PA14_63210	PA4781		0.6 ± 0.1, n=9	0.8 ± 0.1, n=9	0.6 ± 0.1, n=9	1.0 ± 0.0, n=9	1.7 ± 0.2, n=9
PA14_64230	PA4856		3.4 ± 0.4, n=9	1.7 ± 0.1, n=9	2.5 ± 0.4, n=9	2.4 ± 0.1, n=9	3.3 ± 0.0, n=3
PA14_65880	PA4983		1.6 ± 0.3, n=12	1.8 ± 0.1, n=12	1.2 ± 0.2, n=12	1.4 ± 0.1, n=12	1.9 ± 0.2, n=12
PA14_67670	PA5124	ntrB	0.5 ± 0.0, n=15	1.5 ± 0.3, n=18	0.9 ± 0.2, n=18	1.4 ± 0.1, n=12	0.5 ± 0.1, n=15
PA14_72380	PA5483	algB	2.7 ± 1.2, n=9	1.3 ± 0.3, n=9	1.5 ± 0.3, n=8	1.3 ± 0.2, n=6	3.4 ± 1.1, n=9
PA14_72390	PA5484		1.0 ± 0.2, n=9	1.1 ± 0.1, n=9	1.1 ± 0.2, n=6	1.4 ± 0.3, n=6	0.5 ± 0.1, n=9

Table S3. Crystal Violet based quantification[#] of biofilm formation in *P. aeruginosa* PA14, Related to Figure 5.

[#] Grey cells represent values not significantly different from PA14 control (p> 0.05); deep blue (defective), CV stain < 50% of PA14 control; light blue (weak CV stain), 50% to mean - SD of PA14 control; light yellow, CV stain up to 2-fold of PA14 control; deep yellow, CV stain 2 to 6-fold of PA14 control. Mean values of CV stain in each strain was compared with mean value of CV stain in PA14 control by unpaired *t* test. N represents number of replicates.

TRANSPARENT METHODS

Bacterial strains and growth conditions

Pseudomonas aeruginosa PA14 was used as the wild-type strain. All the mutant strains used in this work are from the transposon insertion mutant library of PA14 (Liberati et al., 2006). Unless otherwise mentioned, all strains were grown in Luria Bertani (LB) broth under standard laboratory conditions. For selection of transposon mutants, gentamycin (50 µg mL^{-1}) antibiotic was used. No antibiotics were used in the swarm dishes. Additional growth media used in this study are BHI, BM2, M9, M8 (a modified M9 media without NH_4Cl and $CaCl_2$), PGM and M63 medium (100 µM KH_2PO_4, 15.14 mM $(NH_4)_2SO_4$, 0.36 µM $FeSO_4.H_2O$, 1 mM $MgSO_4$ and 4% arginine). Media composition for the rest is provided in Table 1.

Swarming assay and screening

Swarming motility assays were performed as previously described (Overhage et al., 2008; Yeung et al., 2009), with additional modifications. Appropriate medium (LB, BHI, M8, M9, BM2 or PGM) was solidified with 0.6% Bacto™ agar (BD) and inoculated after 16 hours. All plates were inoculated at the centre with 2 µL of overnight bacterial culture in LB broth (OD_{600} = 2.8 - 3.0) and incubated at 37^0C for 24 hours. All the no-swarmer phenotypes were confirmed at least in three independent experiments.

Swimming Assay

For swimming tests, PGM containing 0.3% Bacto™ agar (BD) was used. A 5 µl of 2 ml overnight culture was inoculated into 5 ml LB broth (secondary culture) and incubated at 37^0C for 5 hours (or OD_{600} = 1). Using sterile toothpicks, the secondary culture was introduced into the centre of swim agar plate by puncturing into the agar but without touching the base of the plates. Plates were incubated at 37^0C for 24 hours right side up. Swimming proficiency were analysed by measuring the swim area covered.

Biofilm quantification

Biofilm formations were assayed as previously described (O'Toole, 2011). Briefly, *P. aeruginosa* strains were grown overnight in 2 mL of LB broth at 37^0C. Overnight culture was then diluted with biofilm media (M63) or swarming media (M9, M8, BM2 or PGM) at a ratio of 1:100. 100 µL of the dilution were added to each well of a 96-well microtiter plate, in triplicate. Microtiter plate was then incubated at 37^0C for 24 hours. After incubation, the plates were rinsed with tap water to remove the planktonic cells and air dried. 0.1% solution of crystal violet in water was added to each well of microtiter dish for staining, followed by 15 minutes incubation at room temperature. After rinsing the excess stain by vigorous shaking and tapping of the plate on paper towels, plates were dried for a few hours. Crystal violent stain in each well was solubilized in 125 µL 30% acetic acid followed by 15 minutes incubation at room temperature. Biofilm formation was quantified by measuring absorbance at 550 nm. PA14 WT was taken as reference; a mutant strain for EPS, *pelA,* was taken as control. The results are represented in table S3 as mean value (standard error of mean) and sample size (n). All strains were stained 3 - 4 independent times, with three replicates on every occasion.

Principal component analysis for swarm features

Principal component analysis (PCA) was performed on different parameters of the colony pattern of wild type PA14 in different nutrient media. Based on the gross appearance of the patterns, we divided these patterns into two classes - branching and non-branching patterns. The wild type PA14 produces non-branching patterns on LB and BHI swarm agar but produces branching patterns on BM2, M8, M9 and PGM swarm agar. We defined the ten following parameters to describe a branching pattern of PA14 swarm:

Branch length (BL): Length of all dendrites in the pattern

Branch Angle (BA): Angle between two dendrites in the pattern

Branch width (BW): Dendrite width of all branches in the pattern

Area (A): Area covered by the swarm

Perimeter (P): Perimeter of the pattern formed by the swarm

Radius of Minimum bounding circle radius (RMBC): Radius of the circle that can encircle the pattern

Number of levels (NL): Number of levels of branching a dendrite undergoes

Normalised Area (NA): Ratio of Area of the pattern and Area of the minimum bounding circle

Number of primary branches (NPB): Number of branches originating from the point of inoculation

Growing tips (GT): Total number of dendrite tips present in the swarm at 24 hours.

The non-dendritic patterns (on LB and BHI agar) are characterized by Area (A), Perimeter (P), Radius of minimum bounding circle (RMBC), and the Normalized Area (NA). We chose two independent parameters to differentiate patterns of dendritic swarm. The parameters chosen i.e. Perimeter and Normalised area are also defined for patterns on LB and BHI.

Statistical analysis: One-way ANOVA was used to analyse variance of swarming traits across swarms on different media. For comparison of means of traits, post hock Tukey test was performed in Figure 2. In all other cases, unpaired student's t test was performed to compare mean ± SEM as indicated.

Video S1. Video of *P. aeruginosa* PA14 swarming along with trace for area, perimeter, and circularity on BHI-0.6% agar, Related to figure 2.

Video S2. Video of *P. aeruginosa* PA14 swarming along with trace for area, perimeter, and circularity on LB-0.6% agar, Related to figure 2.

Video S3. Video of *P. aeruginosa* PA14 swarming along with trace for area, perimeter, and circularity on M8-0.6% agar, Related to figure 2.

Video S4. Video of *P. aeruginosa* PA14 swarming along with trace for area, perimeter, and circularity on M9-0.6% agar, Related to figure 2.

Video S5. Video of *P. aeruginosa* PA14 swarming along with trace for area, perimeter, and circularity on BM2-0.6% agar, Related to figure 2.

Video S6. Video of *P. aeruginosa* PA14 swarming along with trace for area, perimeter, and circularity on PGM-0.6% agar, Related to figure 2.