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EDITOR'S CHOICE

# *Pseudomonas aeruginosa* biofilm formation on endotracheal tubes requires multiple two-component systems

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**Abstract**

**Introduction.** Indwelling medical devices such as endotracheal tubes (ETTs), urinary catheters, vascular access devices, tracheostomies and feeding tubes are often associated with hospital-acquired infections. Bacterial biofilm formed on the ETTs in intubated patients is a significant risk factor associated with ventilator-associated pneumonia. *Pseudomonas aeruginosa* is one of the four frequently encountered bacteria responsible for causing pneumonia, and the biofilm formation on ETTs. However, understanding of biofilm formation on ETT and interventions to prevent biofilm remains lagging. The ability to sense and adapt to external cues contributes to their success. Thus, the biofilm formation is likely to be influenced by the two-component systems (TCSs) that are composed of a membrane-associated sensor kinase and an intracellular response regulator.

**Aim.** This study aims to establish an *in vitro* method to analyse the *P. aeruginosa* biofilm formation on ETTs, and identify the TCSs that contribute to this process.

**Methodology.** In total, 112 *P. aeruginosa* PA14 TCS mutants were tested for their ability to form biofilm on ETTs, their effect on quorum sensing (QS) and motility.

**Results.** Out of 112 TCS mutants studied, 56 had altered biofilm biomass on ETTs. Although the biofilm formation on ETTs is QS-dependent, none of the 56 loci controlled quorum signal. Of these, 18 novel TCSs specific to ETT biofilm were identified, namely, AauS, AgtS, ColR, CopS, CprR, NasT, KdpD, ParS, PmrB, PprA, PvrS, RcsC, PA14_11120, PA14_32580, PA14_45880, PA14_49420, PA14_52240, PA14_70790. The set of 56 included the GacS network, TCS proteins involved in fimbriae synthesis, TCS proteins involved in antimicrobial peptide resistance, and surface-sensing. Additionally, several of the TCS-encoding genes involved in biofilm formation on ETTs were found to be linked to flagellum-dependent swimming motility.

**Conclusions.** Our study established an *in vitro* method for studying *P. aeruginosa* biofilm formation on the ETT surfaces. We also identified novel ETT-specific TCSs that could serve as targets to prevent biofilm formation on indwelling devices frequently used in clinical settings.

## INTRODUCTION

Ventilator-associated pneumonia (VAP) is a hospital-acquired pulmonary infection in the patients' intubated with endotracheal tubes (ETTs) with an estimated 13% mortality [1, 2]. The ETT is the site of biofilm formation by bacteria that can dislodge into the lungs to infect the tissue [3–9]. *Acinetobacter baumannii, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* are the most common bacterial species in the biofilm found in infected patients [10, 11]. *P. aeruginosa* alone is responsible for 35.8% mortality seen in VAP patients [12]. With the increasing multi-drug resistance in *P. aeruginosa*, the mortality rate is on the rise [12]. The increasing fatality underscores the need to understand the regulation of biofilm formation to provide targets for preventive and therapeutic interventions.

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**Keywords:** C4-HSL; polyvinyl chloride; RhlR; type IV pilus; twitching.

**Abbreviations:** CV, Crystal Violet; EPS, Exopolysaccharide; ETT, Endotracheal Tube; HSL, Homo-serine Lactone; PVC, Polyvinyl Chloride; QS, Quorum Sensing; TCS, Two-component System; VAP, Ventilator-associated Pneumonia.

One supplementary figure and three supplementary tables are available with the online version of this article.

Biofilm is a surface-attached aggregate of micro-organisms encased in self-produced exopolymeric substances (EPSs), which constitutes 90% of the volume of the biofilm [13]. In *P. aeruginosa* biofilm, EPS is composed of polysaccharides such as Pel, Psl and alginate, extracellular DNA and water [14, 15]. Past studies on biofilm have used hyper-biofilm producer mutant Δ*retS* and the non-producer Δ*pel*Δ*psl*Δ*algD* as positive and negative controls, respectively [16, 17]. Although *P. aeruginosa* isolates from cystic fibrosis patients produce alginate constitutively [18], loss of alginate production does not affect biofilm growth [19]. However, Pel, Psl and alginate do increase the survival of bacteria during infection [20–22].

Several studies on biofilm formed on a glass surface, polystyrene, flow cell chamber, etc., have led to our current understanding that a network of regulators and effectors are involved in the formation of *P. aeruginosa* biofilm. The network includes quorum sensing, cyclic di-GMP signalling and motility appendages [23–38]. The exact role of the physical environment in initiating or controlling biofilm formation remains a topic of contemporary interest, especially for indwelling devices.

The ability to sense and adapt to diverse environments makes *P. aeruginosa* a versatile and ubiquitous micro-organism. The two-component systems (TCSs), typically composed of a membrane-associated sensor kinase and an intracellular response regulator, endow bacteria with the ability to sense and adapt to external cues including metabolites, trace elements, antimicrobial peptides, pH and surfaces [39]. We have recently reported that 44 TCS encoding genes in *P. aeruginosa* PA14 regulate swarming behaviour in a media-dependent fashion, underscoring their role in sensing and responding to changing environmental cues [40]. One of the well-studied TCS is the GacA-GacS system in *P. aeruginosa* [41, 42], which can regulate the secretion of Psl polysaccharide via RsmA [43]. The activity of the GacA-GacS system also regulated by LadS and RetS histidine kinases [16, 28, 44]. AlgR is a response regulator that is linked not only with alginate production but also with the synthesis of type-IV pili involved in surface sensing [45, 46]. AlgR also regulates cyclic di-GMP levels [47]. Another response regulator WspR is part of a chemosensory system of *P. aeruginosa* involved in surface sensing and biofilm formation in a flow-cell system by modulating cyclic di-GMP levels [29]. Many TCSs involved in the sensing of magnesium, zinc and cationic peptides provides resistance to antimicrobial peptides [48–51], although their role in biofilm formation is yet to be investigated.

Many studies on the biofilm of *P. aeruginosa* have focused on the biofilm formed on polystyrene or glass surface, flow cell or as colony biofilm on agar [16, 40, 52]. However, a systematic study of genetic regulators of biofilm formation on a clinically relevant surface such as endotracheal tubes or urinary catheter has not been performed. To fill this lacuna, we established an *in vitro* ETT biofilm assay for *P. aeruginosa* PA14 in this study and assessed the requirement of TCSs in biofilm formation. We find that *P. aeruginosa* PA14 forms an easily visible and quantifiable biofilm on pieces of ETT *in vitro*. Our study shows that biofilm formation on ETTs is regulated by 56 TCSs. These TCSs affect flagella-dependent swimming motility and type-IV-pilus-dependent twitching motility. These TCSs can be organized into roles related to fimbriae synthesis, PA14-specific chemosensory network, GacS network and antimicrobial peptide resistance.

## METHODS

### Strains used

*E. coli* XL-1 Blue strain harbouring pECP61.5 reporter, having *rhlR* under *tac* promoter along with *rhlA-lacZ* fusion, was used for the detection of *P. aeruginosa* butyryl-HSL in X-gal substrate conversion assay [53]. All the insertion mutants including *pelA (PA14_24480/PA3064), flgM (PA14_20730/PA3351), pilW (PA14_60290/PA4552), rhlI (PA14_19130/PA3476*) and *rhlR (PA14_19120/PA3477*) and TCS mutants used in this study are from the *P. aeruginosa* PA14 transposon insertion library [please see Table S1 (available in the online version of this article) for the gene names] from Fred Ausubel at Massachusetts General Hospital [54]. We would like to caution that these studies are not done with clean deletion mutants. Some transposon insertion mutants used in this study can have polar effects. These are denoted with * in Table S1.

### Biofilm growth on endotracheal tubes

Commercially available PORTEX brand ETTs with an inner diameter of 8 mm were used to set up an *in vitro* assay for biofilm growth. The ETTs were cut into pieces 7–10 mm in size using a sterilized razor blade. M63 growth medium [ammonium sulfate 15.13 mM, monopotassium phosphate 22.04 mM, dipotassium phosphate 40.18 mM, magnesium sulfate heptahydrate 1 mM, and L-Arginine 0.4% (w/v)] described previously [55], were used for biofilm assays. A 25 ml M63 medium in a 90 mm polystyrene Petri plate was inoculated with 0.25 ml of PA14 culture at $OD_{600}$ of 1. Four pieces of ETT were placed in the petri-plate at 1 cm from one another. These plates were incubated at 37 °C for 12, 24, 36, 48 and 72 h under static condition, as indicated in Fig. 1.

### Quantification of biofilm formed on endotracheal tubes

Biofilm biomass was estimated using a positively charged dye, crystal violet (CV) [56]. After a period of biofilm growth, the ETTs were taken out of *P. aeruginosa* PA14 culture dishes and submerged into deionized water using sterile forceps. The ETTs were gently transferred into a 35 mm petri plate containing 5 ml of 0.1% CV solution for 15 min, incubated at room temperature. The excess CV was washed off by dipping stained ETTs into the water, followed by 6 h drying at room temperature. The ETTs with CV stain was imaged. The CV bound to ETTs was dissolved with a 30% glacial acetic acid solution, and the absorbance was measured at 550 nm. Each experiment had at least four

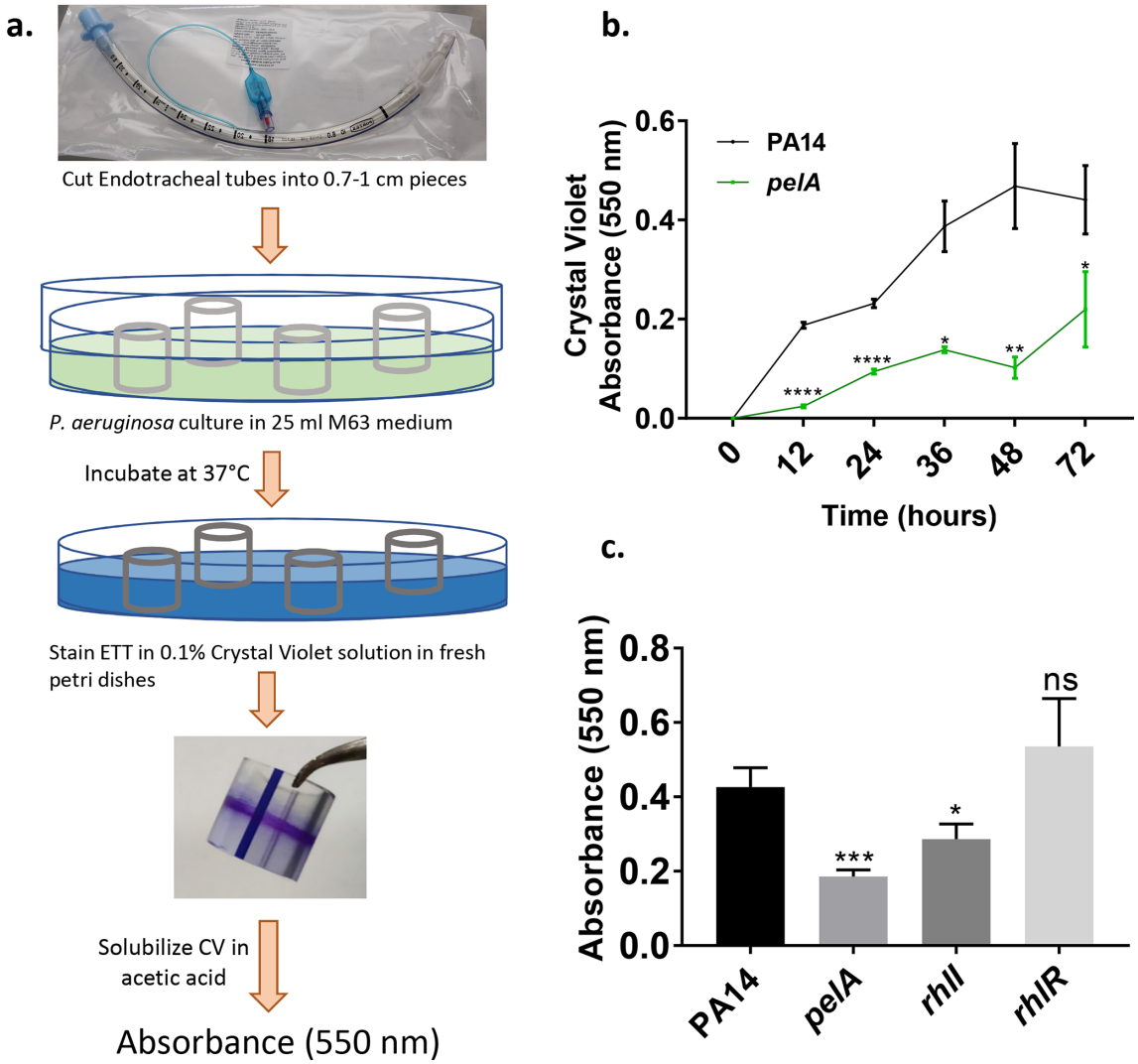


**Fig. 1.** *P. aeruginosa* forms biofilm on the ETT *in vitro*. (a) Schematic for biofilm formation on pieces of sterile ETT. (b) ETT-associated biofilm formed by *P. aeruginosa* PA14 and PA14*pelA* strain at 12, 24, 36, 48 and 72 h at 37 °C in static culture in M63 medium. (c) Biofilm biomass of PA14, PA14*pelA*, PA14*rhlI* and PA14*rhlR* strains on ETTs after 24 h. Data in (b) and (c) are represented as mean±SEM of crystal violet absorbance at 550 nm. Statistical comparisons between PA14 and mutants by Student's *t*-test (*P*-values *≤0.05; **≤0.01, ***≤0.001).

pieces of ETT per strain (mutants and wild-type) within each experiment. The significance of the CV data tested using Student's *t*-test (see Table S1). The experiment was repeated at least seven times for each of the mutants described in Table S1.

### C4 homoserine-lactone (HSL) reporter bioassay

The C4-HSL production by *P. aeruginosa* strains was screened using a plate-based bioassay that was modified from a previously published protocol [53, 57, 58]. Briefly, *E. coli* XL-1 harbouring *rhlA-lacZ* reporter was used for the detection of butyryl-HSL in the X-gal substrate conversion assay [53]. Using a 1.2 cm edge of a sterile glass slide *E. coli* reporter cells were streaked on a 35 mm plate containing LB with 1% agar supplemented with 40 µg/ml X-gal and 1 mM IPTG. Perpendicular to *E. coli* reporter strain, and at 5 mm, *P. aeruginosa* cells were streaked using a 2.5 cm long edge of a sterile glass slide. The double-printed plates were incubated in the dark at 37 °C for 21 h, followed by imaging, as shown in Figs 2a and S1. In this modified assay, the production of butyryl-HSL was defined by the appearance of blue colour across the *E. coli* reporter. A *P. aeruginosa rhlI* mutant was used as a control, and as expected did not produce any C4-HSL in the bioassay.

### Swimming and twitching motility assays

Swimming and twitching assays were performed as described previously [37]. For swimming tests, LB containing 0.3% Bacto

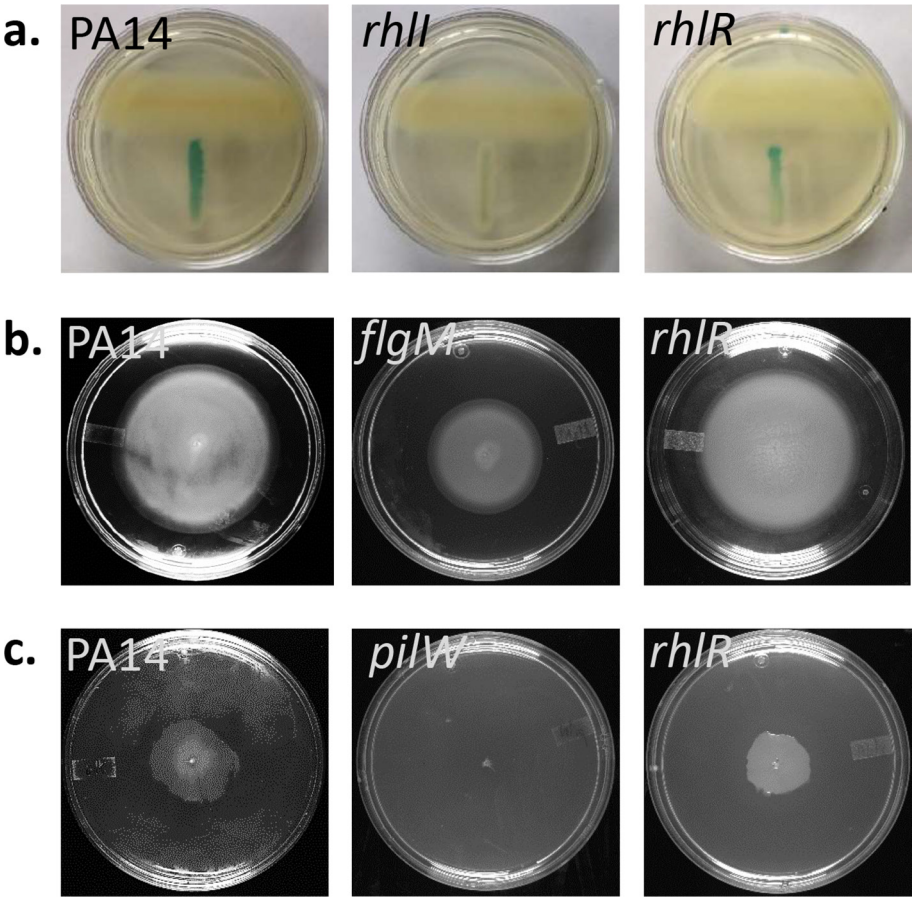


**Fig. 2.** Motility and autoinducer production by the wild-type *P. aeruginosa* PA14 and its isogenic mutants. (a) Plate-based swimming motility assay for PA14, *flgM* and *rhlR* strains on LB containing 0.3% agar. (b) Plate-based swimming motility assay for PA14, *pilW* and *rhlR* strains on LB containing 1.5% agar. (c) Substrate conversion bioassay for butyryl homoserine lactone production by PA14, *rhlI* and *rhlR* strains. See the methodology for details.

agar (BD) was used. A 5 µl of an overnight culture of *P. aeruginosa* was inoculated into 5 ml LB broth (secondary culture) and incubated at 37 °C for 5 h or till $OD_{600}$ reached 1. The secondary culture was introduced into the centre of the swim agar plate by puncturing into the agar but without touching the base of the plates using sterile toothpicks. The plates were incubated at 37 °C for 24 h right side up. Swimming proficiency was analysed by measuring the diameter of the swim area, followed by the calculation of the area covered.

For twitching motility assay, the secondary culture of bacteria was introduced under the LB agar containing 1% agar but above the surface of the Petri dish and incubated at 37 °C. After 48 h of incubation, bacteria on the surface of the agar were washed off, and the area covered by twitching bacteria was measured. Swimming (or twitching) area for each strain was represented as percent of PA14 swimming (or twitching) area. The data is presented as the mean±SEM of each mutant and compared against *P. aeruginosa* PA14 using Student's *t*-test (see Tables S3 and S4).

## Data representation

Mean value for crystal violet absorbance for biofilm growth (Table S1, Fig. 3), for swimming motility (Table S3, Fig. 4) and for twitching motility (Table S4, Fig. 5) was imported against locus ID of genes in references *P. aeruginosa* strain PA14, from http://www.pseudomonas.com/ [59], in R studio as previously described at https://www.r-project.org. The imported data was then transformed into R studio data frame, each containing biofilm growth and phenotypes (hyper, hypo and normal) against each locus. To generate a circular barplot, the angle of rotation for each locus was estimated by 360 by the number of PA14 strains used. The 'tidyverse' (https://ggplot2.tidyverse.org/) packages were used to transform bar graphs from cartesian to polar coordinate. To represent the overlapping phenotype of TCS mutants amongst swimming, twitching and biofilm, Venn diagrams were generated using 'eluerr' (https://github.com/jolars/eulerr) as shown in Fig. 6.

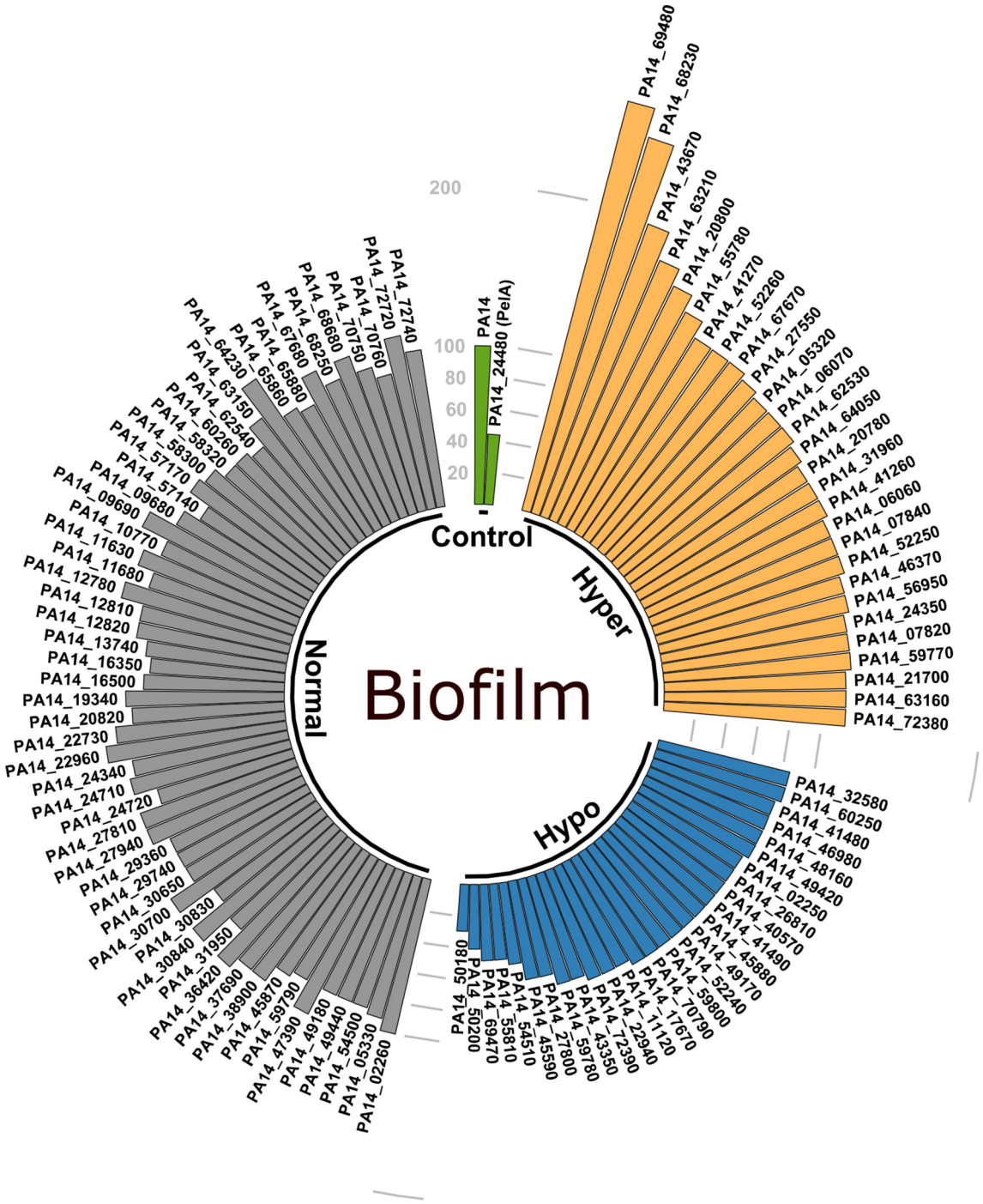


**Fig. 3.** Biofilm formation phenotype of TCS mutants. Circular barplot was plotted using 'R' showing the biofilm formation of the *P. aeruginosa* TCS mutants. The PA14*pelA* (PA14_20730) mutant is against the control PA14 shown in green. The PA14*fleR* (PA14_50180) mutant is highlighted in red. Hypo (blue) and hyper (yellow) represent mutants with biofilm biomass less or more than PA14, respectively. TCS mutants with wild-type biofilm formation are represented in grey. Also, see Table S1 for crystal violet absorbance value (mean±SEM) and statistics.

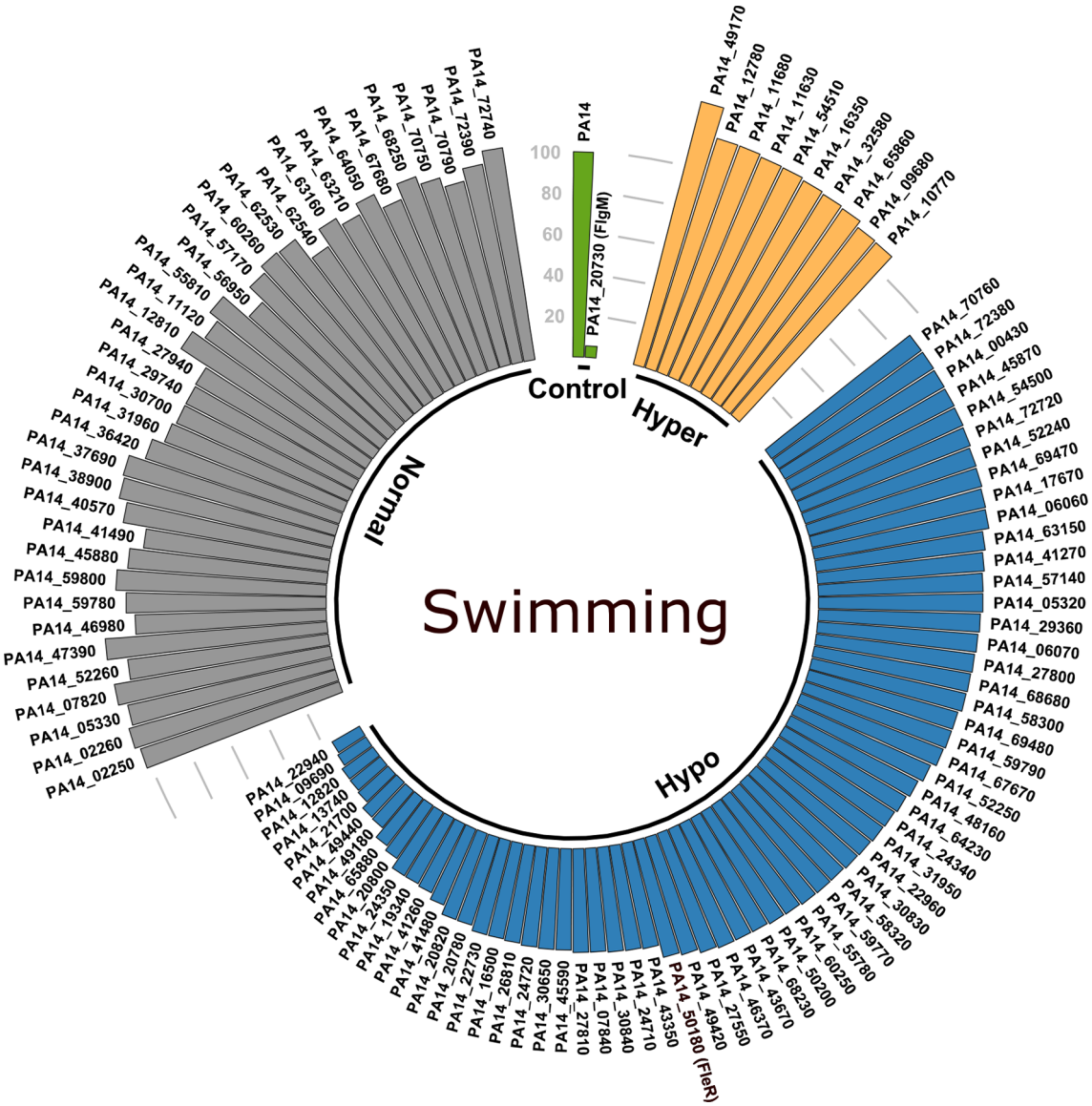


**Fig. 4.** Swimming motility phenotype of *P. aeruginosa* PA14 TCS mutants. Circular barplot of swimming motility of TCS mutants of *P. aeruginosa* plotted using 'R'. The parent PA14 and the negative control PA14*flgM* (*PA14_20730*) mutant are shown in green next to the control. Hypo (blue) and hyper (yellow) represents mutants with reduced or enhanced motility, respectively, compared to PA14. TCS mutants with wild-type motility are represented in grey. See Table S3 for swimming area values (mean±SEM) and the associated statistics.

### Statistical analyses

The data for biofilm on ETTs, swimming and twitching are presented as the mean±SEM of each mutant and compared against *P. aeruginosa* PA14 using unpaired Student's *t*-test [35, 60].

## RESULTS

### *P. aeruginosa* PA14 efficiently forms biofilm on ETTs *in vitro*

ETTs made of polyvinyl chloride were used as a substrate for the formation of *P. aeruginosa* PA14 biofilm. We used PA14 and *pelA* mutant of PA14*pelA*::Tn to study the kinetics of biofilm formation. PelA is essential for pellicle production, one of the three major components of EPS in *P. aeruginosa* biofilm [61]. At the indicated time points, the ETT pieces were stained with crystal violet and imaged or quantified (schematic in Fig. 1a) (see methodology for details). A biofilm formed at the air–liquid interface on the luminal surface of ETTs, as shown in Fig. 1a. An increase in bacterial biomass attached to ETTs was observed with time for both strains (Fig. 1b). However, the increase in biomass was significantly lower *in* PA14*pelA* than the wild-type PA14 at all the time points post-inoculation, suggesting

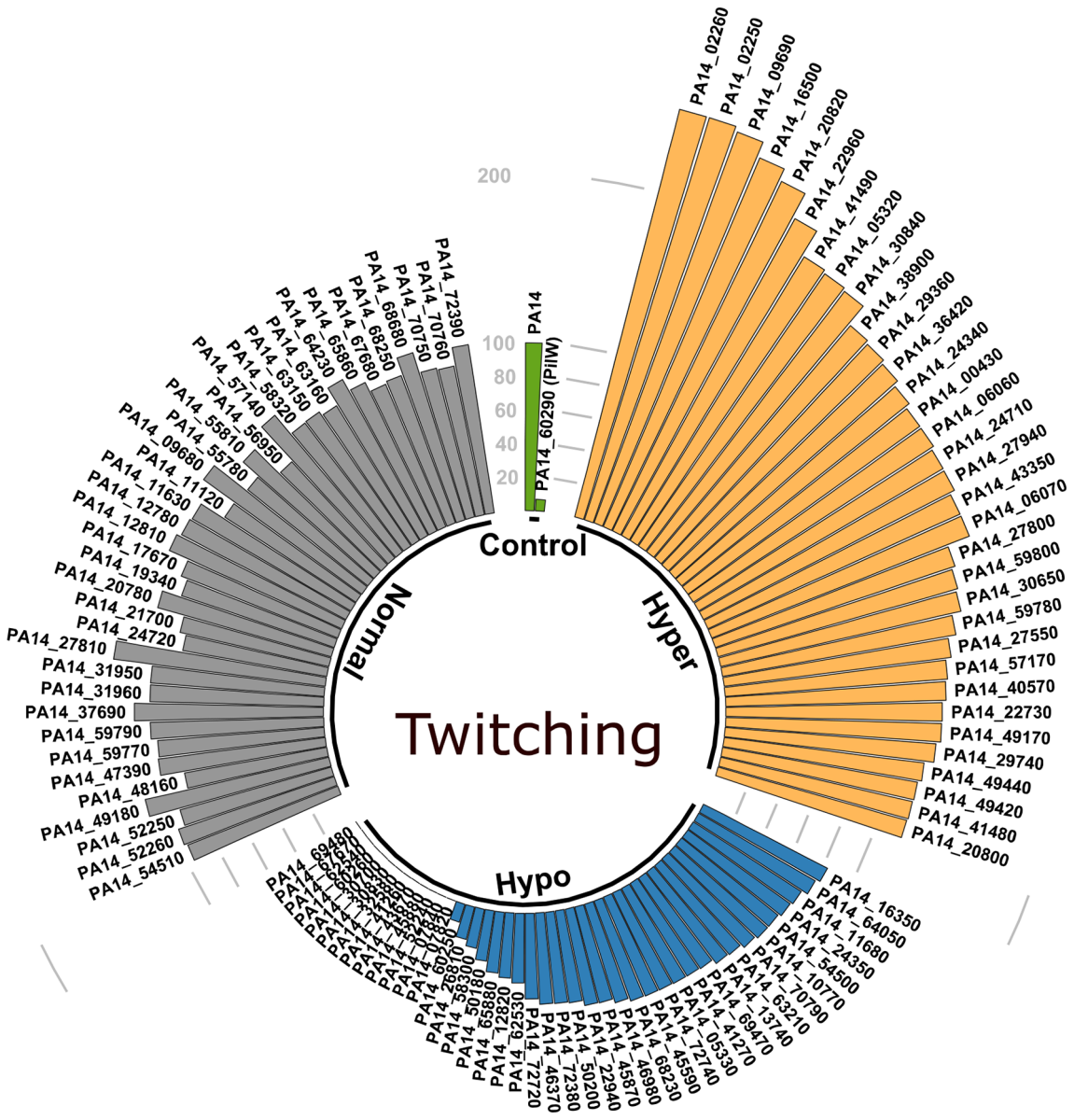


**Fig. 5.** Twitching motility of *P. aeruginosa* PA14 TCS mutants. Circular barplot twitching motility of TCS mutants of *P. aeruginosa* plotted using 'R'. PA14*pilW (PA14_60290)* mutant is shown in green next to the control PA14. Hypo (blue) and hyper (yellow) represents reduced and enhanced motility respectively, compared to PA14. TCS mutants with wild-type motility are represented in grey. See Table S4 for twitching area values (mean±SEM) and the associated statistics.

that the *pel* operon is necessary for biofilm formation on the ETT surface and can be used as a control in future studies.

The *rhlI-rhlR* QS system that is based on C4-HSL is essential for biofilm formation in *P. aeruginosa* [62]. The PA14 *rhlI* defective in C4-HSL synthesis, had reduced biofilm biomass on ETTs (Fig. 1c); however, the *rhlR* mutant did not. Thus, *P. aeruginosa* PA14 forms a quantifiable biofilm on ETTs within 24 h, which is dependent on *pel* operon as well as C4-HSL.

## TCSs are required for the *P. aeruginosa* biofilm formation on ETTs

Several TCSs of *P. aeruginosa* have been linked to biofilm formation. However, their role in ETT-associated biofilm has not been elucidated. To understand if specific TCSs enable *P. aeruginosa* to sense the surface environment, biofilm formation of 112 transposon insertion mutants in TCS (43 sensor kinases, 48 response regulators, 15 sensor/regulators hybrid and HptB) were analysed. In clinical settings, ETTs are removed from intubated patients every 24 h; therefore, we examined biofilm for 24 h.

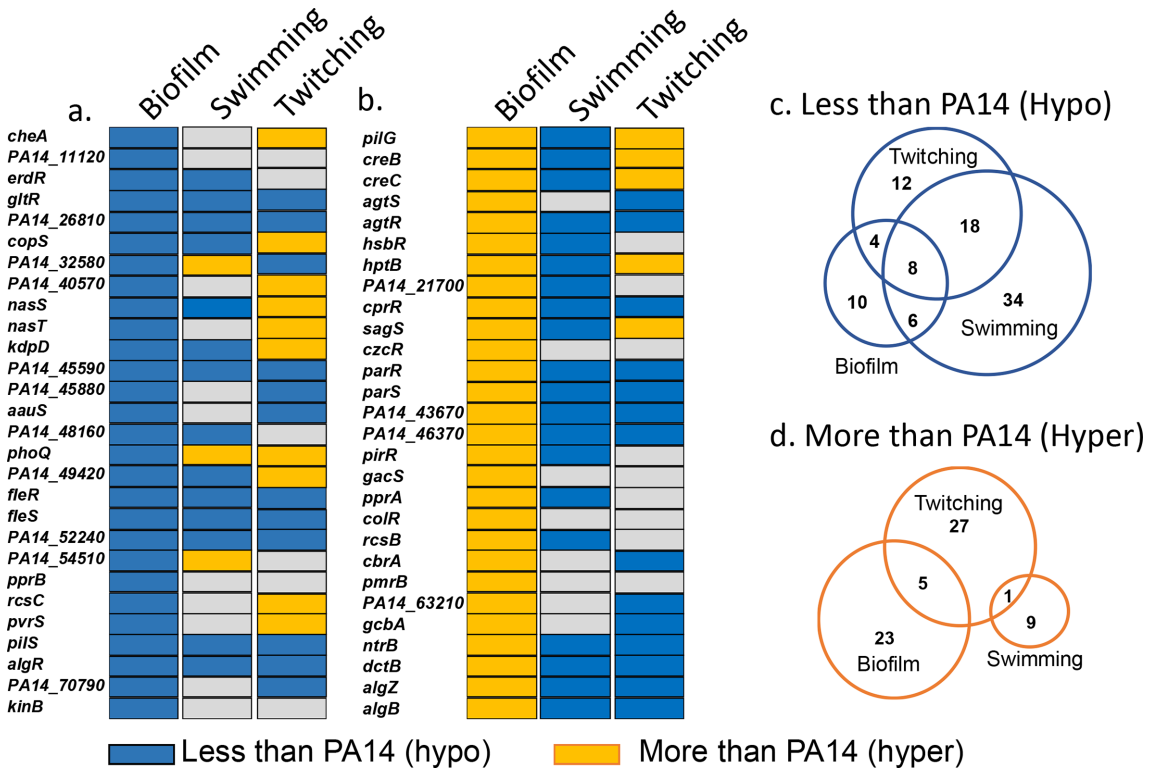


**Fig. 6.** Common regulators of biofilm formation and motility in *P. aeruginosa*. Comparison of biofilm and motility phenotypes in *P. aeruginosa* PA14 TCS mutants. (a) The TCS mutants with reduced (hypo, colored blue) biofilm biomass compared against swimming and twitching motilities. (b) The TCS mutants with increased (hyper, yellow) biofilm biomass compared against swimming and twitching motilities. Venn diagrams showing the number of TCS genes which have (c) reduced biofilm biomass and reduced swimming as well as twitching and (d) increased biofilm biomass and increased swimming as well as twitching.

Twenty-eight TCS mutants had reduced biofilm biomass on the ETT surface. As many as 28 TCSs mutants had enhanced biofilm growth on ETTs than the wild-type PA14 (Fig. 3, also see Table S1). Eighteen TCS genes that were not previously associated with enhanced biofilm formation were identified. These genes were *agtS, PA14_11120, cprR, copS, colR, PA14_32580, parS, nasT, kdpD, PA14_45880, aauS, PA14_49420, PA14_52240, pprA, rcsC, pvrS, pmrB, PA14_70790*.

## Effect of TCS mutations on flagella-dependent swimming motility

The initiation of biofilm formation has been shown to require flagella [37]. For example, FleR-FleS regulates flagellum biogenesis [63], and this TCS was found to be essential for biofilm formation on the ETT surface (Fig. 3), consistent with another study of biofilm on polystyrene surface [40]. We postulated that these 112 TCS mutants with reduced biofilm might have defective flagella. To confirm this postulate, the 112 TCS mutants were tested in a quantitative bacterial swimming assay comparing against the parent PA14. The PA14*fleR* and PA14*flgM* mutants were used as positive and negative controls, respectively (Fig. 2a–c). As expected, the *flgM* mutant was defective in swimming motility [64, 65]. A small number of mutants (10 out of 112) had a better swimming phenotype than the parent PA14. A majority of TCS mutants were defective in swimming motility (Fig. 4, Table S3) while 26 TCS mutants had swimming motility even less than the *fleR* response regulator mutant.

## Effect of TCS mutations on type-IV-pilus-dependent twitching motility

Type-IV pilus was shown to be necessary for biofilm formation on polyvinylchloride (PVC) plastic [37]. We hypothesized that TCS mutants with lower biofilm biomass may have a defective type-IV pilus. To test this, we performed a quantitative twitching motility assay for all the 112 TCS mutants and compared with their isogenic parent *P. aeruginosa* PA14. As controls, the parent PA14*rhlR* and PA14*pilW* were used as positive and negative controls, respectively [66]. As expected, the parent PA14 and PA14*rhlR* were capable of twitching but not the PA14*pilW* mutant (Fig. 2d–f). We examined all 112 TCS mutants for twitching motility (Table S4). Many TCS mutants displayed enhanced twitching motility (Fig. 5) while 11 TCS mutants (*PA14_30700, PA14_30830, PA14_32580, parR, PA14_43670, PA14_45880, pirS, pilR, cbrB, ntrB* and *algZ/fimR*) had lost their twitching motility.

### Effect of TCS mutations on C4-HSL-dependent quorum sensing

The *P. aeruginosa* biofilm formation is a well-studied example of social behaviour relying on quorum [67]. Since *the* loss of *rhlI- rhlR* and C4-HSL autoinducer production resulted in reduced biomass on the ETT surface (Fig. 1c), we tested the effect of TCS mutations on C4-HSL production. We examined the autoinducer production by *P. aeruginosa* PA14, PA14*rhlI* and PA14*rhlR* using a C4-HSL reporter strain of *E. coli* [53]. As expected, the autoinducer production was observed in PA14 but was abolished in the PA14*rhlI*, and was reduced in PA14*rhlR* (Fig. 2g–i). None of the 112 TCS mutants had any impact on C4-HSL production (Fig. S1).

Comparison of motility and biofilm-formation phenotype of PA14 TCS mutants indicated that eight, TCS-*PA14_52240* (*PA0930*), *PA14_45590* (*PA1458*), *fleR, fleS, gltR, pilS, algR, PA14_26810* (*PA2882*), were necessary for biofilm formation on ETTs, for swimming and for twitching motility (Fig. 6a–c) suggesting shared regulation of biofilm formation and motility appendages. There were six additional TCS mutants [*erdR, copS, nasS, kdpD, PA14_48160* (*PA1243*), *PA14_49420* (*PA1158*)] with reduced swimming and reduced biofilm phenotype suggesting that these TCS may affect biofilm formation by their effect on flagella (Fig. 6a–c, Tables S1 and S3). As many as 14 TCS mutants [*agtS, agtR, cprR, parR, parS, PA14_43670*(*PA1611*), *PA14_46370*(*PA1396*), *cbrA, PA14_63210*(*PA4781*), *gcbA, ntrB, dctB, algZ* and *algB*], with increased biofilm biomass had reduced twitching motility or no twitching motility at all (Fig. 6b) suggesting a possible antagonistic relationship between type-IV pili and biofilm formation. Taken together our results suggested that functions of a large fraction of TCSs of *P. aeruginosa* PA14 include swimming motility, surface sensing, chemosensation, fimbriae synthesis, and antimicrobial peptide resistance, and biofilm formation on ETTs (Fig. 7). Of these, 18 TCS have not been linked to biofilm formation before (Fig. 7) suggesting that these may be ETT-specific regulators of biofilm formation.

## DISCUSSION

Two-component systems allow bacteria to sense their environment facilitating rapid adaptability and survival. In this study, we show that *P. aeruginosa* PA14 TCSs regulates its ability to form biofilm on ETTs. Using a set of 112 PA14 TCS transposon insertion mutants, we show that 35 sensor kinases, 20 response regulators and one histidine phosphotransfer protein regulate biofilm formation on ETTs (Table S1). Although the C4-HSL quorum signal is required for biofilm formation on ETTs, none of the 112 TCSs regulate C4-HSL autoinducer production (Fig. S1) . Further, we show that some of the TCS regulators of biofilm regulate flagellum-dependent swimming motility and type-IV-pilus-dependent twitching motility (Tables S3 and S4). Our data suggest that TCS loci may control biofilm formation via multiple mechanisms.

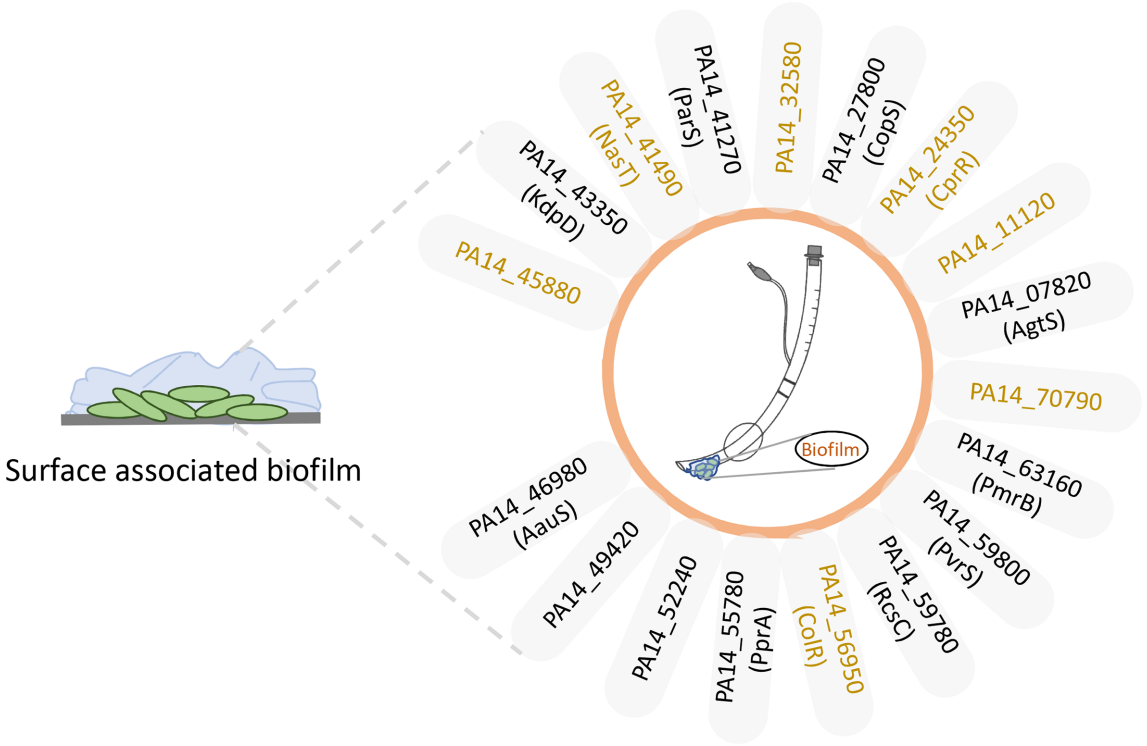


**Fig. 7.** Diagrammatic representation of *P. aeruginosa* PA14 TCSs specifically involved in biofilm formation on the ETT. Of the 18 TCSs specific to ETT-associated biofilm, 11 sensor kinases (black), and seven response regulators in (yellow) are shown.

A growing body of evidence suggests that some environmental signals can influence quorum sensing in *P. aeruginosa* [68–71] although the role of environmental signals in biofilm formation is less explored. Although TCSs are primary responders to environmental signals, none of the 112 TCS mutants we tested had altered C4-HSL autoinducer production, suggesting that this quorum signal is not regulated by these TCSs. Our results are supported by a recent study that demonstrated the RhlR effect on *P. aeruginosa* biofilm formation independent of C4-HSL [62]. Thus, the two findings argue for the presence of alternate pathways for governing biofilm formation.

We noted certain similarities between TCS genes involved in the biofilm formation on ETTs (this study) and those reported for biofilm formation on other surfaces such as polystyrene and glass [40, 72, 73]. This included the regulator of flagellum biogenesis FleR-FleS, phosphate response regulator PhoQ, and AlgR-KinB (Fig. 3, Table S1). Recent reports suggest that KinB regulates *pelA* transcription and is necessary for colony biofilm on congo red agar medium [74]. These five TCSs (FleR, FleS, PhoQ, AlgR and KinB) may be the core requirement of biofilm formation on solid surfaces.

Many TCSs involved in resistance to antimicrobial peptide and antibiotics had an impact on ETT-associated biofilm formation (Table S1). For example, increased biomass was seen with the *colR*, *parS-parA* and *pmrB* systems linked to zinc signalling, cationic peptide signalling and $Mg^{2+}$ sensing, respectively [48, 49]. A decreased biofilm biomass was observed with *phoQ, cprR* and *copR* systems associated with resistance to cationic antimicrobial peptides, polymyxin B and imipenem, respectively [48, 49, 75, 76]. Of these *phoQ* has been previously linked to *P. aeruginosa* biofilm formation [77].

Surface adhesins encoded by *cupA*, *cupB* and *cupC* genes are regulated by the Roc network and are required for the initial attachment stage of biofilm [78]. The TCSs in Roc network, RocS2-RocA2, and RocS1-RocR, did not have any impact on ETT biofilm formation (Table S1). The PvrS, RcsC-RcsB system controlling the *cupD* cluster of genes, specific to *P. aeruginosa* PA14 but lacking in PA01 [79, 80], affected the biofilm on ETTs. This finding suggests that fimbriae are important for the ETT biofilm formation.

The GacS network in *P. aeruginosa* regulates biofilm formation, motility and virulence via a set of 11 TCSs [16, 25, 28, 35, 51, 65, 81–84]. Of these, mutations in *gacS*, *PA14_43670* (*PA1611*), *sagS* and *hptB* had an impact on the biofilm on ETTs suggesting that part of the GacS network may be involved. We also found that ETT biofilm formation was not affected by TCSs related to phosphate and iron sensing, PfeS, CarS, PhoB-PhoR [85–87], and those involved in the control of carbon and nitrogen metabolism such as NarX, CbrB, NtrC and MfiS-MfiR [88–90].

*P. aeruginosa* responds to growth on surfaces by activating chemosensory systems namely, Wsp system and Chp/FimS/AlgR network, both relying on secondary messenger signaling [47, 51, 91]. We found that the WspR response regulator was dispensable, but the FimS-AlgR, PilH and PilG TCSs, affected biofilm formation on ETTs (Fig. 3, Table S1). This indicated that *P. aeruginosa* PA14 sensing of ETT surface likely relied on FimS-AlgR chemosensory system during biofilm formation. It is important to note that many TCS mutants with no twitching phenotype had increased biofilm biomass on ETTs (Fig. 6b). This indicated a possible antagonistic relationship between type-IV pili of PA14 and its ability to form biofilm on ETTs. Extensive experimentation will be needed at morphological, genetic and gene expression levels to test this possibility.

Our study allowed us to discover many TCSs involved in biofilm formation (Fig. 3, Table S1). Although a systematic analysis of the requirement of all TCS genes in biofilm formation has not been attempted in a single study, several studies have looked at the role of many TCS components in biofilm formation. The majority of these studies were done in microtitre plates (polystyrene material), and some on PVC surface or glass surface [11, 16, 25, 40, 73, 92–100]. These studies have reported many TCS mutants with increased biofilm biomass. Of these negative regulators previously reported, we found that mutants in 22 genes, *pilG, creB, creC, agtR, hsbR, hptB, PA3271, sagS, czcR, parR, PA14_43670* (*PA1611*), *PA14_46370* (*PA1396*), *PA14_52250* (*PA0929*), *gacS, rcsB, cbrA, PA14_63210* (*PA4781*), *gcbA, ntrB, dctB, algZ* and *algB* had increased biofilm biomass on ETT surface [16, 40, 73, 79, 92, 94, 98, 99, 101–104]. In addition, we found six new genes, *agtS, cprR, parS, pprA, colR* and *pmrB* with increased biofilm biomass. Of the 28 TCSs needed for the formation of biofilm on ETTs, FleR, FleS, PhoQ, AlgR and KinB have been reported to be involved in biofilm formation on other surfaces previously [34, 46, 72, 93]. The loss of *wspR* (*PA3702*) enhanced the biofilm formation in polystyrene plates [40, 73], but not on ETTs. This finding suggests that WspR chemosensory system may have a function in sensing harder surfaces such as polystyrene. This also suggested that other chemosensory TCSs such as FimS-AlgR might aid in sensing of PVC-based ETT surface. Importantly we found 12 new TCSs that positively influence biofilm formation, PA14_11120, CopS, PA14_32580, NasT, KdpD, PA14_45880, AauS, PA14_49420, PA14_52240, RcsC, PvrS and PA14_70790. This set of TCSs provides potential targets for inhibition and/or disruption of biofilm on ETTs in a clinical setting.

Our study design that included systematic analysis of mutants in large sample sizes (11 samples or more for each mutant, see Table S1) allowed us to uncover a larger set of TCSs involved in biofilm formation than reported before. Some of the TCSs discovered in this study are likely to be specific to the polyvinylchloride (PVC)-based surface of ETTs and other indwelling devices [105, 106], and thus of clinical importance. Reports suggest that commonly used polymers and glass vary widely in their physical properties such as surface charge, hydrophobicity, roughness, topography, stiffness and chemical propertires [107–110]. The difference in the biofilm phenotype of *gltR* and *retS* mutants [16, 40] on PVC, polystyrene and glass indicates that surface's material properties could be one of the governing

factors for bacterium's ability to adhere and form a biofilm. One such property is the modulus of elasticity, which is a measure of the stiffness of a material [111]. The major component of ETTs is PVC. The elastic moduli of PVC, polystyrene and borosilicate glass are 14.5, 3.3 and 64 GPa, respectively [107–109]. This suggests that PVC is likely the most elastic material (363.6% of elongation before yield) followed by polystyrene (1.5–3% of elongation before yield) and glass is least elastic [108, 112]. The hyper- and hypo-biofilm formation on polystyrene and ETT, respectively, by PA14*gltR* indicates that the GltR response regulator may respond to surfaces with low stiffness. This finding suggests that we need to investigate the molecular aspect of biofilm formation on low-stiffness surfaces used in indwelling devices in clinics. Discerning the mechanism of surface sensing by TCSs uncovered in this study will increase our understanding of biofilm formation and sensing. In the future, targeted investigation into individual TCSs can be performed to gain molecular and mechanistic insights into biofilm formation in clinical settings.

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Author contributions
D.B., A.K. and V.S. conceptualized the study. D.B., A.V.J., M.A.K. performed the experiments and analysed the data. D.B., A.V.J., M.A.K., A.K. and V.S. wrote the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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