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ChpC controls twitching motility-mediated expansion of *Pseudomonas aeruginosa* biofilms in response to serum albumin, mucin and oligopeptides

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Abstract

Twitching motility-mediated biofilm expansion occurs via coordinated, multi-cellular collective behaviour to allow bacteria to actively expand across surfaces. Type-IV pili (T4P) are cell-associated virulence factors which mediate twitching motility via rounds of extension, surface attachment and retraction. The Chp chemosensory system is thought to respond to environmental signals to regulate the biogenesis, assembly and twitching motility function of T4P. In other well characterised chemosensory systems, methyl-accepting chemotaxis proteins (MCPs) feed environmental signals through a CheW adapter protein to the histidine kinase CheA to modulate motility. The *Pseudomonas aeruginosa* Chp system has an MCP PilJ and two CheW adapter proteins, PilI and ChpC, that likely interact with the histidine kinase ChpA to feed environmental signals into the system. In the current study we show that ChpC is involved in the response to host-derived signals serum albumin, mucin and oligopeptides. We demonstrate that these signals stimulate an increase in twitching motility, as well as in levels of 3′–5′-cyclic adenosine monophosphate (cAMP) and surface-assembled T4P. Interestingly, our data shows that changes in cAMP and surface piliation levels are independent of ChpC but that the twitching motility response to these environmental signals requires ChpC. Furthermore, we show that protease activity is required for the twitching motility response of *P. aeruginosa* to environmental signals. Based upon our data we propose a model whereby ChpC feeds these environmental signals into the Chp system, potentially via PilJ or another MCP, to control twitching motility. PilJ and PilI then modulate T4P surface levels to allow the cell to continue to undergo twitching motility. Our study is the first to link environmental signals to the Chp chemosensory system and refines our understanding of how this system controls twitching motility-mediated biofilm expansion in *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative WHO categorised ‘priority pathogen’ due to its high levels of antibiotic resistance and health-care associated infections. In the lungs of cystic fibrosis (CF) patients this pathogen forms biofilms resulting in chronic lung infections that are the major cause of morbidity and mortality in these individuals [1]. *P. aeruginosa* is also commonly associated with chronic catheter-associated urinary tract infections (CAUTIs) [2]. The success of *P. aeruginosa* as an opportunistic pathogen is largely attributed to its ability to form biofilms [3] and to produce many cell-associated and secreted virulence factors [4]. Type IV pili (T4P) are a major cell-associated virulence factor which are located at the pole of the cell and are involved in biofilm formation and twitching motility-mediated active biofilm expansion via rounds of extension, surface attachment and retraction [5, 6]. Twitching motility is likely to facilitate active biofilm expansion by *P. aeruginosa* along the length of indwelling catheters [7–10].

The biogenesis, assembly and twitching motility function of T4P is regulated by a number of complex regulatory systems including a putative chemosensory system, the Chp system [5]. This system is encoded by the *pilGHIJK-chpABC* gene cluster [11–14], which is homologous to the *Escherichia coli*

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Abbreviations: BM, base media; BSA, bovine serum albumin; cAMP, 3'-5'-cyclic adenosine monophosphate; c-di-GMP, 3',5'-cyclic diguanylic acid; CF, cystic fibrosis; ELISAs, enzyme-linked immunosorbent assays; GlcNAc, N-acetylglucosamine; MCP, methyl-accepting chemotaxis protein; SBP, solute-binding protein; T4P, Type-IV pili.

Four supplementary figures are available with the online version of this article.

Che chemosensory system involved in regulating flagella-mediated swimming chemotaxis in response to environmental signals [15]. The *E. coli* chemosensory system detects environmental cues either by direct binding of the ligand by one of four methyl-accepting chemotaxis proteins (MCP) or binding of the ligand by a periplasmic solute-binding protein (SBP) that then interacts with an MCP. The signal is transduced via an adapter protein, CheW, to the histidine kinase CheA, which modulates flagella function via phosphorylation of the CheY response regulator.

In addition to the Chp chemosensory system, *P. aeruginosa* has three additional chemosensory systems, Che I and Che II that modulate swimming motility and the Wsp system that controls c-di-GMP production and in turn stimulates biofilm formation and represses swimming motility [16]. The *P. aeruginosa* genome also encodes 26 MCPs [17, 18] and 98 SBPs [19], many of which have been linked to specific ligands. Experimental and computational analyses have linked 23 of these MCPs to the Che I chemosensory system pathway [20–29], McpB to the CheII chemosensory system pathway [23, 30] and WspA to the Wsp system [23, 31].

The core signalling components of the Chp system that are required for twitching motility in *P. aeruginosa* include a putative CheA histidine kinase homolog, ChpA [14], the MCP PilJ [12], the CheW-like adapter protein, PilI and the CheY homolog PilG [12, 14]. The Chp system gene cluster also encodes another CheW homolog, ChpC [14]. This is not unprecedented as possession of multiple CheW homologs associated with one chemosensory system have been found in many bacteria [32]. While PilI is essential for twitching motility [14], ChpC is not [12]. Interestingly, ChpC and PilI have both been predicted to interact only with PilJ [23] though they share only 20% amino acid sequence identity. The periplasmic domain of PilJ has been shown to sense conformational changes in PilA, the T4P monomer unit, when the pilus is undergoing active extension, surface attachment and retraction and transducing this signal via PilI to ChpA [33]. Furthermore, PilJ has been shown to also interact with FimS [34], which is part of the two-component sensor FimS/AlgR that is involved in expression of *fimU-pilVWXY1Y2E* required for T4P biogenesis and assembly [5, 35].

In addition to its role in controlling twitching motility, the Chp system also positively regulates intracellular levels of the second messenger, 3′–5′-cyclic adenosine monophosphate (cAMP) via the major adenylate cyclase, CyaB, which synthesises cAMP [36]. The global regulator Vfr binds cAMP and activates expression of a number of genes required for T4P biogenesis [37, 38]. The ability of PilJ to sense retracted T4P monomer units has been shown to be required for modulation of intracellular cAMP levels [33].

FimL has also been linked to the Chp system-cAMP-T4P regulatory pathway in *P. aeruginosa*. Our previous work demonstrated that this protein is necessary for twitching motility, and while *fimL* mutants have wild-type levels of intracellular T4P, surface associated T4P levels are decreased [39]. In addition to the role of FimL in twitching motility, it has also been shown to be involved in autolysis [39]. More recent work by us and others has revealed that compensatory mutations in the cAMP phosphodiesterase *cpdA* (which breaks down cAMP) results in increased cAMP levels, restoring twitching motility in *fimL* mutants [40, 41]. Since this compensatory mutation in *cpdA* restores the twitching motility phenotype, FimL is likely to directly target CyaB activity, and not be involved in T4P biogenesis or assembly.

We have previously investigated the twitching motility response of *P. aeruginosa* to serum albumin (in the form of BSA), mucin and oligopeptides (in the form of tryptone) and shown that FimX is involved in the response to mucin and oligopeptides [42]. *P. aeruginosa* would encounter each of these host-signals in an infection setting: mucin, which is known to be elevated in a CF lung environment [43, 44], oligopeptides in urine [45–47] when *P. aeruginosa* infects a catheter-implanted urinary tract, and serum albumin in the vicinity of epithelial cells. The stimulation of twitching motility in response to these signals is likely to accelerate colonisation by *P. aeruginosa* of epithelial cells, CF lungs or implanted devices. The stimulatory response to these signals is not completely abolished in a *fimX* mutant [42] suggesting that there are additional regulators involved. In the current study we demonstrate that ChpC of the Chp chemosensory system is also involved in the twitching motility response to serum albumin, mucin and oligopeptides.

METHODS

Bacterial strains and media

The strains and plasmids used in this study and their relevant characteristics are listed in Table 1. *P. aeruginosa* was cultured on lysogeny broth (LB) solidified with agar at 1.5% (w/v) or 1% (w/v) (for interstitial biofilm expansion assays) and grown overnight (or for 24 h for interstitial biofilm expansion assays) at 37 °C. *P. aeruginosa* or *E. coli* cultures were grown in either cation-adjusted Mueller Hinton broth (CAMHB) or LB and incubated overnight at 37 °C, with shaking at 250 r.p.m. Agar plates for interstitial biofilm expansion assays consisted of base medium (0.5% (w/v) yeast extract (Oxoid), 0.05% (w/v) NaCl (Sigma)) solidified with 1% (w/v) bacteriological agar (Oxoid) with or without supplements BSA (0.1% (w/v); Research Organics, USA; filter sterilized and added to autoclaved base medium), mucin from porcine stomach (0.05% (w/v); Sigma), tryptone (3% (w/v); Oxoid), GlcNAc (50 mM), casamino acids (Fisher Biosciences, 3% (w/v); filter sterilized and added to autoclaved base medium) and individual amino acids (20 mM (v/w); filter sterilized and added to autoclaved base medium). Protease inhibitor cocktail (Sigma) was added to autoclaved base medium agar or broth (4% (v/v)). Antibiotics were used at the following concentrations as required: ampicillin 50 μg ml^{-1} and tetracycline 5 μg ml^{-1} for *E. coli* and carbenicillin 250 μg ml^{-1} and tetracycline 200 μg ml^{-1} for *P. aeruginosa*. *P. aeruginosa* was also grown on Vogel-Bonner medium (VBM) (10× solution contains

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant description	Reference or source
***P. aeruginosa* strains**		
PA14	Wild-type clinical *P. aeruginosa* strain	[57]
PAK	Wild-type *P. aeruginosa* strain	D.Bradley, Memorial University of Newfoundlands, St Johns, Canada
PA14Δ*chpC*	In frame deletion of *chpC* in wild-type strain PA14	This study
PAKΔ*chpC*	In frame deletion of chpC in wild-type strain PAK	This study
PAKpilA:TcR	*pilA* inactivated by allelic displacement with a tetracycline resistance cassette (TetR)	[58]
***E. coli* strains**		
DH5α	*recA, endA1, gyrA96, hsdR17, thi-1, supE44, relA1*, φ80, dlacZΔM15	Laboratory collection
S17-1	*thi pro hsdR recA chr*::RP4-2	[59]
Plasmids		
pUCPSK	AmpR; *E. coli-P. aeruginosa* shuttle vector	[58]
pUCP*chpC*	AmpR; A 740 bp PCR amplicon containing *chpC* inserted against P*lac* into *Eco*RI site of pUCPSK	This study
pFlp2	AmpR; encodes FLP recombinase and *sacB*.	[60]
pGEMTeasy	AmpR; *E. coli* cloning vector	Promega
pGemCLF	AmpR; A 1149 bp PCR amplicon from the 5′ end of *chpC* (CLF) and inserted into pGEMTeasy	This study
pGemCRF	AmpR; A 1238 bp fragment PCR amplicon from the 3′ end of *chpC* (CRF) and inserted into pGEMTeasy	This study
pCRFV	KanR; *Bam*HI-*Kpn*I fragment from pGEMCRF inserted into pOK12	This study
pOKCDEL	Kan, TcR; The concatamerized fragment including the *Bgl*II-*Kpn*I fragment from pGEMCLF and the 1638 bp KpnI fragment from pLM1 with a *frt* flanked TcR cassette, inserted into the *Kpn*I and *Bam*HI sites of pCRFV fragment;	This study
pRIC380	AmpR; *P. aeruginosa* suicide vector	[58]
pCDEL	AmpR, TcR; A 4008 bp *Spe*I fragment from pOKCDEL containing the *chpC* deletion construct inserted into the *Spe*I site in pRIC380 against *sacB*	This study

$MgSO_4.7H_2O$ (8 mM), citric acid (anhydrous) (9.6 mM), K_2HPO_4 (1.7 mM), $NaNH_5PO_4.4H_2O$ (22.7 mM), pH 7, and filter sterilized) with 1.5% (w/v) agar containing tetracycline (200 µg ml^{-1}) for allelic exchange mutagenesis. Counter selection and curing of pFlp2 was achieved on LB agar with 5% (w/v) sucrose.

The PAK genome (NCBI number accession number LR657304 [48];) was used to search for methyl-accepting chemotaxis protein (MCP) and solute-binding protein (SBP) orthologs. The Pseudomonas.com website was also used to identify *P. aeruginosa* PA14/PAO1 orthologs [49].

Recombinant DNA techniques

The preparation of plasmid DNA (Qiagen, Valencia, CA), restriction endonuclease digestion (New England Biolabs, Ipswich, MA), and ligation reactions (Promega, Madison, WI, and New England Biolabs) were carried out using standard protocols [50]. Oligonucleotides used in the current study are listed in Table 2. The complementation plasmid pUCP*chpC* was generated by amplification of *chpC* with primers chpCF and chpcR and cloning the amplicon via pGEMT*easy* into pUCPKS. The preparation of *E. coli* competent cells and transformations were performed as previously described [50]. *P. aeruginosa* competent cells were prepared by $MgCl_2$ treatment and transformed as previously described [51]. *P. aeruginosa* cells were prepared by sucrose treatment for electroporation and electroporated as previously described [41].

Deletion mutagenesis

To generate the in-frame *chpC* deletion construct, 1 kb of sequence on both the 5′ flank of *chpC* (CLF) and the 3′ flank of *chpC* (CRF) were amplified by PCR with primer pairs chpCLFF / chpCLFR (fragment CLF) and chpCRFF/ chpCRFR (fragment CRF) from PAO1 genomic DNA. The fragment CRF was inserted into pOK12 resulting in the construct pCRFV. The fragment CLF was then inserted into pCRFV to generate pOKCDEL. The *chpC* deletion construct fragment was then sub-cloned from pOKCDEL into the suicide vector pRIC380 to generate pCDEL. *chpC* in-frame

Table 2. Oligonucleotides used in the current study

Primer	Sequence (5′–3′)	Binding position in genome	Purpose
chpC-LFF	AGATCTGCAAGCCTTACCAGGAAACCG	1085 bp 5′of the *chpC* start codon	*chpC* deletion
chpC-LFR	GGTACCTTGGCTGGGGTGTTCCG	52 bp 3′ of the *chpC* start codon	*chpC* deletion
chpC-RFF	GGTACCGGACATCGGCGGCGAAAC	79 bp 5′ of the *chpC* stop codon	*chpC* deletion
chpC-RFR	GGATCCCCGCACCTGCTCGTAGCC	147 bp 3′ of the *chpC* stop codon	*chpC* deletion
chpCF	TACAGCAGTTTCAATGCCAGCCCC	89 bp 5′ of *chpC* start site	*chpC* complementation
chpCR	CAGACATCTTCCAATCCGCAGAG	144 bp 3′ of *chpC* stop codon	*chpC* complementation

deletion mutants were made in *P. aeruginosa* strains PAK and PA14 by allelic exchange recombination followed by FRT-mediated deletion of the resistance cassette using previously published methods [52, 53].

Phenotypic assays

Interstitial biofilm expansion was assayed using a modification of the subsurface twitching motility stab assay described previously [54]. Briefly, the *P. aeruginosa* strain to be tested was stab inoculated through an agar plate and cultured for 24 h at 37 °C. The longest (a) and shortest (b) diameters of each interstitial biofilm at the agar and petri dish interface were measured and the surface area calculated using the formula: area=abπ. Intracellular cAMP assays were conducted as described previously [41] using cells grown on base medium supplemented with or without BSA (0.1% (w/v)), mucin (0.05% (w/v)) or tryptone (3% (w/v)).

Growth experiments

Growth of *P. aeruginosa* strains was followed by recording changes in OD_{595nm} over a 20 h period. Cells were grown in microtitre plates or in glass flasks (experiments for +/- protease inhibitor), and incubated at 37 °C, shaking at 250 r.p.m. Base medium supplemented with and without GlcNAc (50 mM), BSA (0.1% (w/v)), mucin (0.05% (w/v)), tryptone (3% (w/v)), or casamino acids (3% (w/v)) in the presence or absence of protease inhibitor cocktail (4% (w/v)) was used in growth assays.

PilA immunoblotting

Detection of cell-associated pilin was performed as described previously [14] with cells being harvested from plates grown at 37 °C for 20 h on base medium or base medium supplemented with BSA (0.1% (w/v)), mucin (0.05% (w/v)) and tryptone (3% (w/v)).

PilA ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed as described previously [14] with cells harvested from plates grown at 37 °C for 20 h on base medium or base medium supplemented with BSA (0.1% (w/v)), mucin (0.05% (w/v)) or tryptone (3% (w/v)). ELISAs of cells harvested from tryptone plates were treated with 100 Kunitz units per mL DNaseI (D5025, Sigma Aldrich) for 1 h statically at 37 °C, then washed three times with PBS, prior to use in the assay.

RESULTS

ChpC is involved in the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone

BSA, mucin and tryptone have previously been shown to stimulate *P. aeruginosa* twitching motility-mediated interstitial biofilm expansion with the effect of mucin and tryptone, but not BSA, being regulated to some extent by FimX [42]. However, as some stimulation of a *fimX* mutant is still observed, this suggests that other components must also be involved in mediating the twitching motility response to these host-derived signals. The *P. aeruginosa* Chp system is a putative chemosensory system that is thought to regulate twitching motility in response to environmental signals [14]. We hypothesised that the second CheW homolog of this system ChpC, which is not essential for twitching motility, may serve to modulate twitching motility in response to environmental cues. We were therefore interested in investigating the role of ChpC in controlling twitching motility in response to BSA, mucin and tryptone, to determine if this pathway plays a role in controlling twitching motility in response to these environmental cues.

To investigate the role of ChpC in stimulating twitching motility in response to BSA, mucin or tryptone, we first generated in-frame deletion mutants of *chpC* in *P. aeruginosa* strains PAK and PA14 and transformed the wildtype and isogenic Δ*chpC* strains with the *chpC* complementation plasmid (pUCP*chpC*) and empty vector control (pUCPSK). Interstitial biofilm expansion assays were then performed with these strains in base medium agar or base medium agar supplemented with BSA, mucin or tryptone. In both PAK and PA14, twitching motility-mediated interstitial biofilm expansion was significantly increased in the presence of BSA, mucin and tryptone compared to base medium (Fig. 1a, b). This corresponded to a relative change in the area of the interstitial biofilm for PAK of approx. five-fold for BSA and mucin and approx. eight-fold for tryptone compared to base medium (Fig. 1a), whereas due to the impact of larger interstitial biofilms on base medium, PA14 showed smaller relative changes in the area of interstitial biofilm of approx. two-fold for BSA, mucin and tryptone compared to base medium

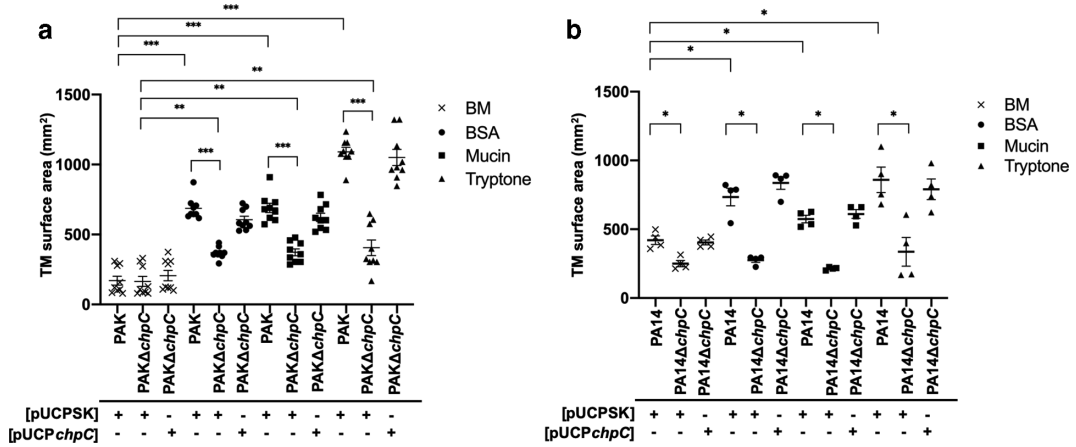

Fig. 1. ChpC is involved in the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone. Subsurface twitching motility (TM)-mediated biofilm expansion of PAK and PAKΔ*chpC* (A) or PA14 and PA14Δ*chpC* (B) containing pUCPSK or pUCP*chpC* at the agar/plastic interstitial space after 24 h at 37 °C in base medium (BM), or BM supplemented with BSA (0.1%), mucin (0.05%) or tryptone (3%). The mean of each set of technical triplicates was calculated to give an *n*=9 for PAK and *n*=4 for PA14, which is presented as mean±SEM (* *P*<.05 ** *P*<.005 *** *P*<.0001; Mann-Whitney *U*-test comparing wild-type [pUCPSK] or Δ*chpC* [pUCPSK] on BM to supplemented BM and wild-type [pUCPSK] to Δ*chpC* [pUCPSK] on each supplemented media. ns for wild-type [pUCPSK] compared to Δ*chpC* [pUCP*chpC*] and ns for PA14Δ*chpC* [pUCPSK] on BM compared to supplemented BM.

(Fig. 1b). Interestingly, while there was still some stimulation of twitching motility for PAKΔ*chpC* on BSA, mucin and tryptone compared to base medium (approx. three-fold on BSA and mucin and approx. four-fold on tryptone (Fig. 1a)), there was no stimulation of PA14Δ*chpC* on BSA, mucin or tryptone compared to base medium (Fig. 1b). In both PAK and PA14 the mutation in *chpC* was fully complemented by expression of ChpC *in trans* (Fig. 1a, b). To determine whether the observed stimulation of twitching motility-mediated biofilm expansion in these strains on BSA, mucin and tryptone was due to an increase in growth rate, growth assays were conducted in base medium, or base medium supplemented with BSA, mucin and tryptone. These growth curves revealed that BSA, mucin and tryptone did not affect the growth of the wildtype or its isogenic Δ*chpC* strain in either PAK or PA14 (Fig. S1).

These results demonstrate that ChpC contributes to the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone to varying extents depending on the strain background. We went on to further characterise how *P. aeruginosa* responds to these environmental signals.

The effect of BSA, mucin and tryptone on PilA and cAMP levels

Twitching motility levels can be affected by changes in expression of the T4P monomer subunit, PilA, and/or alterations in the rates of biogenesis/retraction of surface-assembled T4P. To determine if the mechanism of ChpC-mediated stimulation of twitching motility in response to BSA, mucin and tryptone involved changes in expression and/or assembly of the T4P, we performed ELISAs to measure levels of surface-assembled T4P and immunoblots to measure levels of the T4P subunit PilA in whole cells harvested from confluent lawns of PAK and PAKΔ*chpC* grown on base medium, or base medium supplemented with BSA, mucin or tryptone. The ELISAs demonstrated that both PAK and PAKΔ*chpC* grown on BSA and mucin had marginally increased levels of surface-assembled T4P compared to when grown on base medium (Figs 2a and S2a, b). We observed a significant amount of autolysis in the confluent lawns of cells cultured on tryptone as has been previously reported [39] and ELISAs of cells harvested directly from the tryptone plates were unable to detect any surface PilA in either strain, which suggested that there may be excessive amounts of extracellular DNA (eDNA) present. To remove this eDNA, cells were harvested from both base medium and tryptone plates, treated with DNaseI and then washed in PBS, prior to use in the ELISA. This allowed detection of surface-assembled T4P in these cells and revealed that both PAK and PAKΔ*chpC* cells grown in the presence of tryptone had two to three-fold higher levels of surface-assembled T4P than cells cultured on base medium (Figs 2 and S2c). Whole cell immunoblots demonstrated that there were no changes in PilA expression levels for wild-type or PAKΔ*chpC* when grown on base medium, or base medium supplemented with BSA, mucin or tryptone (Fig. 2b).

The *P. aeruginosa* Chp system has been shown to positively regulate cAMP levels via CyaB to increase levels of surface-assembled T4P [36]. To investigate the involvement of cAMP in the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone, intracellular cAMP levels were measured in cells harvested from confluent lawns of PAK and PAKΔ*chpC* grown on base medium, or base medium supplemented with BSA, mucin or tryptone. For both PAK and PAKΔ*chpC* cAMP levels were marginally increased on BSA and mucin and two to three-fold on tryptone (Fig. 2c), which reflects the changes in surface-assembled pili with these supplements (Figs 2a and S2).

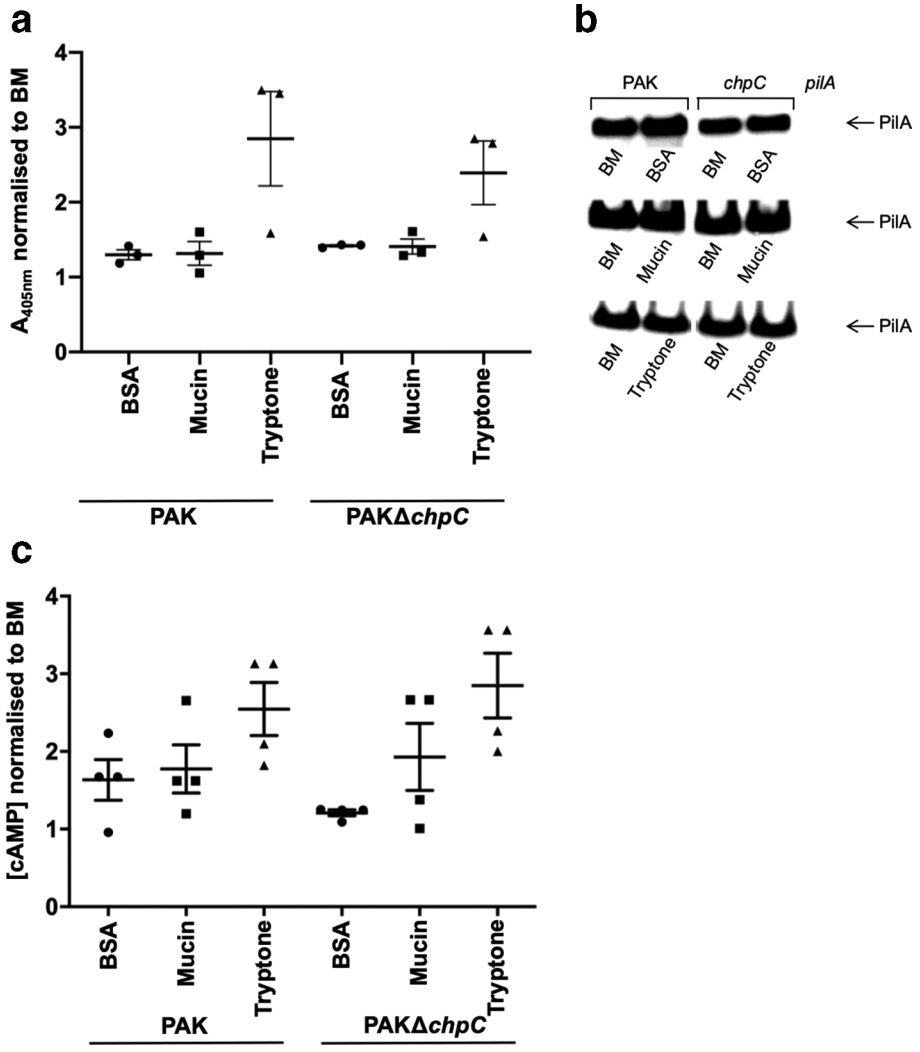

Fig. 2. The effect of BSA, mucin and tryptone on PilA and cAMP levels**.** (a) PilA ELISAs of wild-type PAK and PAKΔ*chpC* grown on base media (BM) or BM supplemented with BSA (0.1%), mucin (0.05%) or tryptone (3%). These data are based upon the raw data shown in (Fig. S2 are available with the online version of this article). Here the mean of technical triplicates for the most concentrated sample for PAK or PAKΔ*chpC* on BM for each biological replicate (*n*=3) was used to normalise the mean of technical triplicates of PAK and PAKΔ*chpC* on each supplement for the respective biological replicate. The data are presented as mean±SEM. Equal cell numbers were loaded. (b) PilA Western immunoblot of whole cell lysates from wild-type PAK or PAKΔ*chpC* cells grown on BM or BM supplemented with BSA (0.1%), mucin (0.05%), tryptone (3%), and PAK*pilA* cells grown on BM. Equal cell numbers were loaded. (c) Intracellular cAMP concentrations (pmol ml^{-1}) of wild-type PAK and PAKΔ*chpC* cells grown in base media (BM) or BM supplemented with BSA (0.1%), mucin (0.05%) or tryptone (3%). The mean of technical triplicates for PAK or PAKΔ*chpC* on BM for each biological replicate (*n*=4) was used to normalise the mean of technical triplicates of PAK and PAKΔ*chpC* on each supplement for the respective biological replicate. The data are presented as mean±SEM. Equal cell numbers were loaded.

These observations suggest that stimulating twitching motility results in higher levels of cAMP and surface-assembled T4P in both PAK and PAKΔ*chpC* although there does not appear to be a direct correlation between these levels and rates of interstitial biofilm expansion. This is particularly evident in PAKΔ*chpC* which showed equivalent increases in interstitial biofilm sizes in BSA, mucin and tryptone (Fig. 1a) but much greater increases in the levels of cAMP and surface-assembled T4P in tryptone than in BSA or mucin (Figs 2 and S2). Thus, it appears that changes in cAMP and surface piliation levels are largely independent of ChpC but that stimulation of biofilm expansion in response to these environmental cues is modulated via ChpC.

Examination of potential chemical ligands that stimulate twitching motility

Chemosensory systems typically sense, via their MCP and SBP components, small chemical ligands such as amino acids, inorganic phosphate, chlorinated and non-chlorinated hydrocarbons, dipeptides and oligopeptides [18, 19]. We wanted to

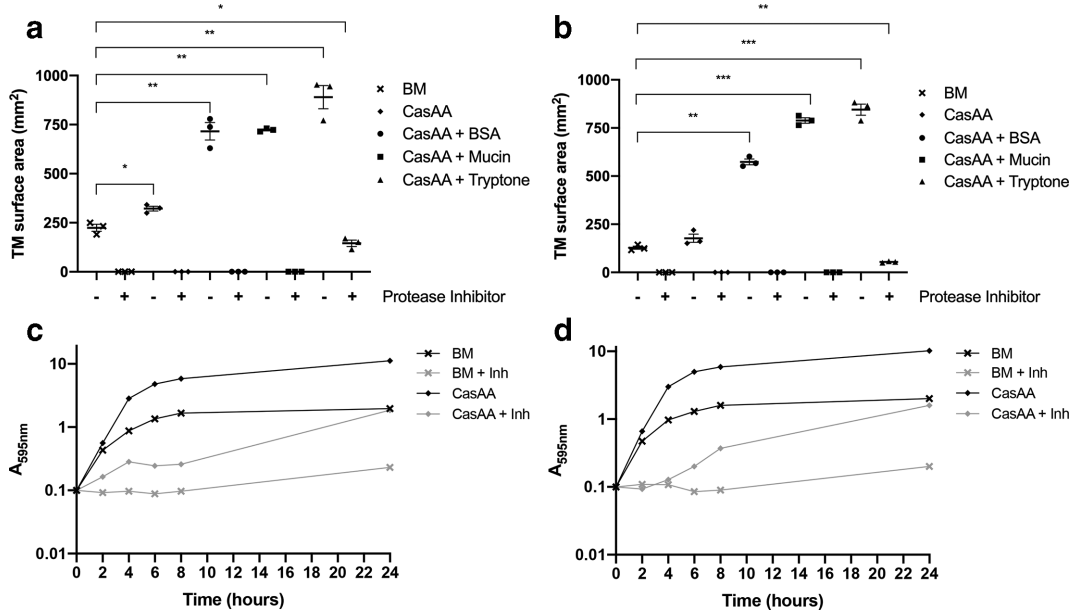

Fig. 3. Protease activity is required for the *P. aeruginosa* twitching motility response to BSA, mucin and tryptone**.** Subsurface twitching motility (TM)-mediated biofilm expansion of PAK (a) or PA14 (b) at the agar/plastic interstitial space after 24 h at 37 °C and growth assays with PAK (c) or PA14 (d) in base media (BM), or BM supplemented with casamino acids (casAA) (3%)+/-protease inhibitor cocktail (Inh) (4%). The mean of each set of technical triplicates was calculated to give an *n*=3 for PAK and PA14 in A and B, which is presented as mean±SEM (**P*<.05 ***P*<.005 ****P*<.0001; Mann-Whitney *U*-test as indicated). The mean of biological duplicates is represented for the growth assays in C and D.

determine if the twitching motility responses to BSA, mucin and tryptone were mediated by recognition of a smaller component common to each of these host-derived signals. BSA is composed of a single polypeptide chain consisting of 583 amino acid residues, the mucin used in this study is a mixture of highly glycosylated glycoproteins obtained by pepsin digestion of hog stomach, and tryptone is the assortment of oligopeptides and a few free amino acids, formed by the digestion of bovine β-casein by the protease trypsin. Therefore it is likely that the common stimulatory component is one or more oligopeptides, dipeptides or amino acids.

To determine if amino acids are the common smaller signal sensed to modulate twitching motility, we performed interstitial biofilm expansion assays with wild-type PAK and PA14 strains on base medium supplemented with casamino acids, which are produced by acid hydrolysis of bovine β-casein, resulting in mainly free amino acids, and a few oligopeptides. Casamino acids did not stimulate twitching motility in PA14 and had only a slight stimulatory effect on twitching motility for PAK (Fig. 3a, b), which suggests that amino acids are not the smaller common component of BSA, mucin and tryptone that is sensed by *P. aeruginosa* to stimulate twitching motility.

It is possible that individual amino acids could have stimulatory or inhibitory effects that would be masked when in the casamino acids mixture. To investigate the twitching motility response to individual amino acids we performed interstitial biofilm expansion assays with base medium supplemented with 17 of the 21 common amino acids (Fig. S3a; note cysteine, glutamic acid, selenocysteine and tyrosine were insoluble in base medium). None of the amino acids tested significantly affected twitching motility of PA14 when compared to base medium (Fig. S3a). Twitching motility of PAK was significantly decreased by tryptophan (Trp) and significantly increased by glutamine (Gln) (Fig. S3a). As bovine β-casein contains 20 Gln residues and one trptophan residue [55] this suggests that Gln may account for some of the observed stimulation of twitching motility in PAK by casamino acids.

To determine if extracellular protease activity would contribute to the observed increase in twitching motility levels in response to serum albumin, mucin or tryptone, a protease inhibitor cocktail was added to supplemented base medium and interstitial biofilm expansion assays performed with PAK and PA14. Biofilm expansion was completely abolished in the presence of protease inhibitor (Fig. 3a, b). However, we noted that the protease inhibitor cocktail also completely inhibited growth resulting in no colony growth at the site of inoculation. We confirmed that the presence of the protease inhibitor cocktail inhibited growth by performing growth curve assays in base medium broth supplemented with protease inhibitor (Fig. 3c, d). To determine if the growth inhibition by the protease inhibitor cocktail was due to deprivation of amino acids derived from proteins in the media as a nutrient source, we supplemented the base medium with 3% casamino acids. In the absence of protease inhibitor casamino acids stimulated growth in both PAK and PA14 and partially

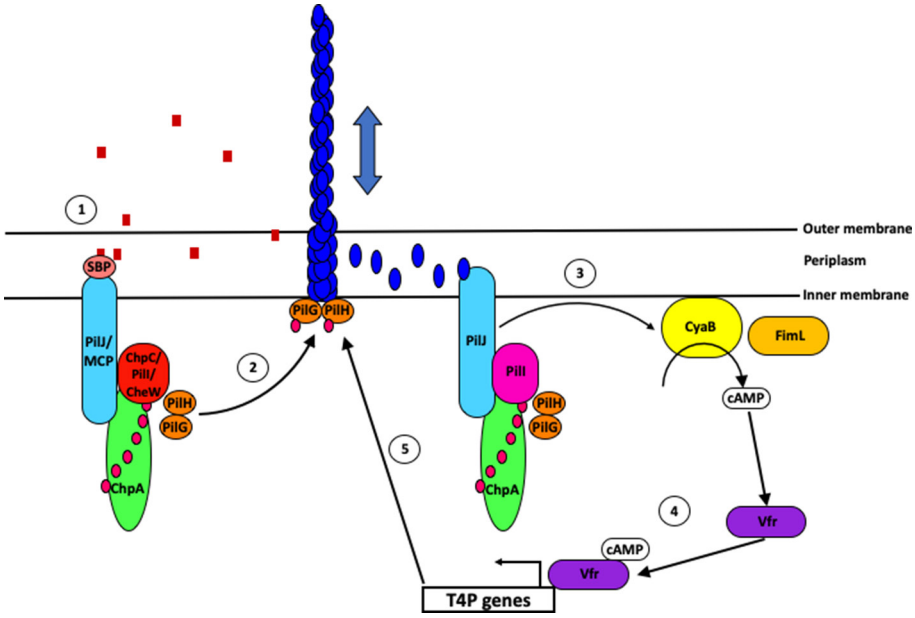

Fig. 4. Chp system sensing of environmental signals and modulation of twitching motility and T4P levels. (1) Environmental signal/ligand (red squares) is sensed by a SBP, which is likely linked to the MCP PilJ, that transfers the signal via ChpC/PilI/CheW to ChpA. (2) A twitching motility response is mediated by extension and retraction of the T4P. (3) PilJ senses the levels of retracted T4P in the periplasm and stimulates the generation of cAMP via CyaB. FimL affects CyaB activity. (4) cAMP-Vfr activates expression of T4P components. (5) These increase levels of assembled T4P to allow the cell to continue to undergo twitching motility in response to the environmental signal.

restored growth in the presence of protease inhibitor cocktail in broth (Fig. 3c, d).

Therefore, to determine if secreted protease activity was involved in producing twitching motility stimulatory signals, we performed interstital biofilm expansion assays using base medium containing 3% casamino acids supplemented with mucin, BSA, or tryptone and examined the influence of incorporating the protease inhibitor cocktail. Growth in broth medium was also examined (Fig. S3b, c). Consistent with our earlier observations (Fig. 1a, b), the addition of mucin, BSA or tryptone significantly stimulated twitching motility when added to base medium containing casamino acids but did not enhance growth in broth. The inclusion of protease inhibitor cocktail completely abbrogated twitching motility in base medium containing casamino acids and also in this medium supplemented with BSA or mucin. Interestingly, twitching motility was stimulated to some extent when tryptone was added (Fig. 3a, b). These observations suggest that secreted protease activity may be required for liberating components from mucin, BSA and tryptone that stimulate twitching motility and that a small quantity of this component is present in tryptone that does not require protease activity to liberate. Together, the data in Figs 3 and S3 suggests that oligopeptides stimulate twitching motility in both PAK and PA14.

Mucin is a highly glycosylated glycoprotein with up to 90% of its molecular weight due to O- and N-linked oligosaccharides. As N-acetylglucosamine (GlcNAc) is a common sidechain of mucin and is present in CF sputum at mM concentrations [44], we were interested in determining if GlcNAc might also stimulate *P. aeruginosa* twitching motility. We therefore tested the twitching motility response of PAK and PA14 at a GlcNAc concentration within this range (50 mM). Whilst there was no significant increase in PAK twitching motility in GlcNAc compared to base medium (Fig. S4), PA14 was significantly stimulated by GlcNAc resulting in an approx. two-fold increase in interstitial biofilm area relative to base medium (Fig. S4). Growth assays with PA14 in base medium supplemented with GlcNAc confirmed that the observed stimulation of twitching motility was not simply due to an increased growth rate, as cells grew at the same rate in base medium as in base medium supplemented with GlcNAc (Fig. S1e). As ChpC appears to be necessary for the twitching motility response to mucin by strain PA14 (Fig. 1b), we examined the twitching motility response of PA14Δ*chpC* to GlcNAc and found that PA14Δ*chpC* was stimulated by GlcNAc to the same extent as wild-type PA14 (Fig. S4). This indicates that while twitching motility of *P. aeruginosa* is stimulated by GlcNAc in strain PA14, this is not the component of mucin that is being sensed to modulate twitching motility via ChpC.

DISCUSSION

As *P. aeruginosa* possesses a large number of regulatory systems that intersect to control the biogenesis and function of T4P and twitching motility [56], it is clear that the regulation of twitching motility is complex. In this study we have

investigated how *P. aeruginosa* modulates twitching motility-mediated biofilm expansion in response to the host-derived signals serum albumin (in the form of BSA), mucin, and oligopeptides (in the form of tryptone). We found that the Chp chemosensory system appears to be involved in regulating twitching motility in response to these signals and that this occurs, at least in part, via ChpC. We found that each of these leads to an increase in the levels of surface-assembled T4P and intracellular cAMP to varying extents but that this is independent of ChpC. Interestingly, our observations indicate that there is not a direct relationship between the levels of surface assembled T4P, cAMP and rates of interstitial biofilm expansion and that simply increasing T4P levels is not sufficient to increase the rate of twitching motility expansion, but that ChpC is also required to direct this expansion.

Our data suggests that protease activity is required for twitching motility, presumably to liberate smaller components of albumin, mucin and tryptone, such as Gln, and di- or oligo-petides that are then sensed either directly via an MCP that feeds into the Chp system via ChpC or indirectly via one or more of the 98 SBPs possessed by *P. aeruginosa* [19]. PilJ is the only MCP shown to be associated with the Chp system [12]. However ChpA, the histidine kinase for the Chp system, is predicted to interact with 17 of the 26 *P. aeruginosa* MCPs [23] suggesting that other MCPs may also link environmental signals to the Chp system. While PilI and ChpC are the only CheW adapters predicted to interact with PilJ [23], given that PilJ is also predicted to interact with CheW from the Che I chemosensory cluster [23] suggests it is also possible that this CheW adapter feeds environmental signals to ChpA (Fig. 4). This proposes a model (Fig. 4) whereby environmental signals that modulate twitching motility are sensed via a SBP linked to an MCP/PilJ, which is linked to ChpA by PilI/ChpC/CheW. This twitching motility response generates retracted T4P in the periplasm, which are sensed by PilJ to modulate levels of T4P component expression, in a cAMP-dependent manner, via the CheW adapter PilI. Overall this study advances our understanding of how the Chp system controls T4P biogenesis and twitching motility in response to environmental signals.

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Author contributions
L.M.N., L.C.M., J.M. and C.B.W. conducted experiments. L.M.N and C.B.W. analysed results. C.B.W. and L.T. provided project supervision. C.B.W. provided project administration and funding. L.M.N. and C.B.W. wrote the manuscript.

Conflicts of interest
The authors declare no conflicts of interest.

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