PAAR proteins act as the ‘sorting hat’ of the type VI secretion system

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INTRODUCTION

Bacteria must influence their surroundings and compete with other micro-organisms for nutrients and space. The deployment of protein secretion systems such as the type VI secretion system (T6SS) facilitates the elimination of competitors and enables bacteria to establish a foothold within a niche. The T6SS is a prevalent Gram-negative bacterial virulence factor and antibacterial apparatus, delivering effector proteins directly into target cells or into the local environment [1–3]. Many effector proteins exhibit antibacterial activities, such as nuclease, phospholipase and peptidoglycan hydrolases, to induce stasis or lysis of the target bacterium [4]. The T6SS is a contractile apparatus that propels a lance-like structure decorated with effectors into neighbouring bacteria in a contact-dependent manner. The lance is composed of a tube of stacked haemolysin-coregulated protein (Hcp) rings capped by a spike complex, consisting of a trimer of valine–glycine repeat protein G (VgrG) proteins on which sits a conical proline–alanine–alanine–arginine repeat (PAAR) tip protein [5–7]. Effectors exist as either domains covalently linked to the lance constituents, called ‘evolved’ structural components, or cargo proteins that bind Hcp, VgrG or PAAR proteins in a non-covalent but specific manner [7].

We have previously proposed an ‘à la carte’ delivery mechanism for the T6SS whereby effectors are recruited to the apparatus for secretion by specific spike components, a concept corroborated by others [8, 9]. Our understanding of the relationship between PAAR, VgrG and effector proteins has only recently developed through dissection of the effector repertoires of bacteria harbouring a single T6SS, namely Vibrio cholerae, Serratia marcescens and Agrobacterium tumefaciens [10–12]. Pseudomonas aeruginosa possesses three T6SSs, designated H1-, H2- and H3-T6SS, with antibacterial activity attributed to each [13–15]. Although the associations between evolved PAAR proteins and their cognate VgrGs are well established for the H1-T6SS, our knowledge of the make-up of the H2-T6SS spike complex is lacking [8, 9, 16]. No genes encoding spike proteins have been identified within the H2-T6SS locus in P. aeruginosa PA01, although many satellite islands containing vgrG and PAAR genes are located distally on the chromosome (Fig. 1) [17]. Several VgrG proteins, including VgrG2a, VgrG2b, VgrG4b, and VgrG6, have been functionally associated with the H2-T6SS, with only VgrG4b having been characterised as being associated with a specific cargo effector, PldA [18–20]. Recently, Burkinshaw and co-workers determined that the PAAR4 tip protein is the cognate delivery device for the TseT nuclease effector of the H2-T6SS, which associates with VgrG4b or VgrG6 in the spike complex [19]. However, our understanding of the repertoire of VgrG–PAAR effector assemblies for the H2-T6SS is still in its infancy.

Here, we characterise a new cargo effector protein, Tle3, and identify the VgrG–PAAR subassembly responsible for its delivery by the H2-T6SS. Bioinformatic analysis of the P. aeruginosa genome also uncovers an unannotated open reading frame (ORF) encoding a PAAR protein bearing homology to the H2-T6SS-associated tip proteins. Use of bacterial competition assays and secretion assays dissects the capacity of the H2-T6SS to deliver multiple effector proteins and reveals a preferential delivery of the VgrG2b–PAAR3 spike subassembly. These data demonstrate that competition between spike complexes defines the effector payload of the H2-T6SS.

RESULTS

Bioinformatics analysis of P. aeruginosa PAAR proteins

Effector delivery by the T6SS requires a functional spike complex, composed of a trimer of VgrG proteins capped by of effectors for certain spike components. Pseudomonas aeruginosa encodes several PAAR proteins, whose roles have been poorly investigated. Here we describe a phospholipase family antibacterial effector immunity pair from Pseudomonas aeruginosa and demonstrate that a specific PAAR protein is necessary for the delivery of the effector and its cognate VgrG. Furthermore, the PAAR protein appears to restrict the delivery of other phospholipase effectors that utilise distinct VgrG proteins. We provide further evidence for competition for PAAR protein recruitment to the T6SS apparatus, which determines the identities of the delivered effectors.
a PAAR protein [7, 21]. Seven proteins with PAAR or PAAR-like domains have been described in *P. aeruginosa* PAO1, four of which are canonical PAAR proteins (PAAR2–4 and PA2375) with no fused effector domains [7–9, 19]. Three effectors containing PAAR or PAAR-like domains (Tse5, Tse6 and Tse7) have been functionally associated with the H1-T6SS, while the *PA2375* gene, found in the H3-T6SS locus, encodes a member of the DUF4280 family, shown to be structural homologues of PAAR proteins [8, 9, 16, 22, 23].

In *silico* analysis of the *P. aeruginosa* PAO1 genome using a tblastn search with PAAR protein queries identified an unannotated ORF, designated with the locus tag *PA1659.1*, between the *tseE2* and *tseF2* genes within the H2-T6SS locus (Fig. 2a). The putative product of this gene is a polypeptide of 10.8 kD, comprising a single PAAR_CT_1 domain sharing 55% sequence identity over 97 residues with the PAAR4 protein. Expanding this analysis to 89 complete *P. aeruginosa* genomes identified this ORF within the H2-T6SS locus in all strains except *P. aeruginosa* PA7 (Table S1, available in the online version of this article). We henceforth refer to this conserved putative tip protein as PAAR5, in line with the PAAR nomenclature recently put forth by Burkinshaw and colleagues [19].

Analysis of the phylogeny of all PAAR domains encoded in *P. aeruginosa* PAO1 revealed that PAAR5 clusters with PAAR4 and PAAR2 (Fig. 2b), both of which have recently been functionally associated with the H2-T6SS [19]. Since PAAR5 is encoded within the H2-T6SS locus, it is likely that the tip protein is a component of this specific T6SS machinery. The PAAR3 protein, encoded by *PA1508*, is in the *vgrG2a* satellite island along with the Tle4–Tli4 effector immunity pair and an Hcp2 paralogue, HcpA. The genetic linkage of PAAR3 with H2-T6SS-associated genes led us to hypothesise that PAAR3 is also functionally linked to the H2-T6SS (Fig. 1). Our laboratory amongst others has begun to identify the key residues at the PAAR–VgrG interface, determining the specificity of the PAAR–VgrG interaction [7, 8, 16, 19]. PAAR proteins display a conserved conical tertiary structure despite generally exhibiting low similarity at the amino acid

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**Fig. 1.** Schematic of the satellite vgrG and PAAR islands of *P. aeruginosa* PAO1. The vgrG and PAAR islands distal to the core T6SS gene clusters also encode characterised or putative effector–immunity pairs. Genes encoding PAARs are in red, those encoding VgrG are in yellow, Hcp are in purple, effectors are in orange, immunity proteins are in green, chaperones are in dark blue and hypothetical proteins are in grey. The locus tag of each vgrG and PAAR gene is shown in parentheses. Scale bar shows 5 kb.
level, yet alignment of PAAR2, PAAR3, PAAR4 and PAAR5 reveals high sequence identity (Fig. 2c). Since the residues of PAAR2, PAAR4 and PAAR5 that are predicted to lie at the VgrG interface are well conserved, this supports the notion that they form subassemblies with closely related VgrG proteins and furthermore are functionally associated with the H2-T6SS (Fig. 2c).

**PAAR3 is required for VgrG2b secretion**

Amongst the four PAAR proteins that we propose to be associated the H2-T6SS, PAAR3 and PAAR5 are completely uncharacterised. To initiate their characterisation, we constructed strains lacking the \( \text{PAAR3} \) or \( \text{PAAR5} \) genes and probed the activity of the H2-T6SS in these genetic backgrounds. We have previously shown that Hcp2 and VgrG4b are secreted by the H2-T6SS of \( P. \ aeruginosa \) and thus monitored their secretion, and that of VgrG2a and VgrG2b, in the presence and absence of PAAR3 and PAAR5 [18]. In PAO1, HcpA, HcpB and HcpC are identical proteins encoded in the satellite islands of \( \text{vgrG2a} \), \( \text{vgrG6} \) and \( \text{vgrG2b} \), respectively (Fig. 1), and we refer to these tube proteins collectively as Hcp2, since the native proteins are indistinguishable. Secretion of Hcp2 is unaffected by the deletion of either \( \text{vgrG2a} \), \( \text{vgrG2b} \), \( \text{vgrG4b} \) or \( \text{PAAR3} \) or \( \text{PAAR5} \), indicating that individually none of these spike and tip proteins is indispensable for H2-T6SS function (Fig. 3a). VgrG4b also appears to be secreted independently of this subset of VgrG and PAAR proteins, as none of \( \text{vgrG2a} \), \( \text{vgrG2b} \), \( \text{PAAR3} \) or \( \text{PAAR5} \) is essential for its secretion by the H2-T6SS (Fig. 3a). Likewise, VgrG2a is secreted independently of \( \text{vgrG2b} \), \( \text{vgrG4b} \), \( \text{PAAR3} \) or \( \text{PAAR5} \), although its levels are slightly lowered in the absence of either PAAR protein. However, deletion of \( \text{PAAR3} \) does prevent secretion of VgrG2b, whereas this protein can still be detected in the supernatant of a \( \Delta \text{PAAR5} \) strain (Fig. 3a).
Global analysis of complete *P. aeruginosa* genomes revealed that all strains encoding VgrG2b also contain the vgrG2a cluster in which PAAR3 is encoded, apart from *P. aeruginosa* PA7 (Table S1). This taxonomic outlier strain possesses few orthologues of VgrG proteins encoded by strain PAO1; however, the vgrG2b satellite island is present, albeit at a distinct chromosomal location (Fig. 3b) [24]. Since VgrG2b secretion absolutely requires PAAR3 in strain PAO1, we searched for a homologous PAAR protein within strain PA7 that would permit secretion of VgrG2b. A TBLASTN search revealed an ORF downstream of the vgrG2b cluster flanked by two putative transposase genes (Fig. 3b), which encodes...
Fig. 4. Tle3 is a periplasmic antibacterial toxin delivered by the VgrG2b-PAAR3 spike complex. (a) Domain schematic of the Tle3 protein from *P. aeruginosa* depicting the α/β-hydrolase and DUF3274 domains. A sequence logo of the predicted GXSXG consensus esterase motif of homologous proteins is shown below. (b) Heterologous production of Tle3 is toxic to *E. coli* when targeted to the periplasm. Upper panel: Serial dilutions of *E. coli* BL21 (λDE3) carrying pET28a-tle3 or pET22b-tle3 (for cytoplasmic or periplasmic effector production, respectively) or the empty vector equivalents were spotted on non-inducing (2% glucose) and inducing (100 µM IPTG) media. The range of OD<sub>600</sub> values for the inoculum is stated on the left. Lower panel: Immunoblot analysis of Tle3 production in the desired bacterial compartment. Whole cell extracts (WCE) and periplasmic fractions of *E. coli* BL21 (λDE3) pET28a-tle3 and pET22b-tle3 after growth in inducing conditions were probed using anti-His antibodies to detect the hexahistidine-tagged Tle3 protein. Antibodies against the RNA polymerase β-subunit RpoB were utilised to confirm no cytoplasmic contamination of the periplasmic fraction. Images are representative of three independent experiments. (c) Intraspecies competitive growth assay between a *P. aeruginosa* prey strain lacking the tle3-tli3 cassette and various attacker strains. The competition index indicates the change in the attacker/prey strain ratio at the end of the assay relative to the input, where a value >1 represents a growth advantage to the attacker strain. Competition between the parental strain PAO1ΔrsmA and itself, shown in grey, acts as the internal control for competitive parity. Values denote the mean of three independent experiments, with error bars displaying the s.e.m. Statistical significance of growth outcomes was determined by a one-way ANOVA followed by Dunnett’s multiple comparisons test, using the competition between the ΔrsmAΔtle3tli3 prey strain and the ΔrsmA parental strain as the comparator (** p < 0.001). (d) Immunoblot showing the production of Tle3 in *P. aeruginosa* strains. The VgrG2b-HA<sub>4</sub> construct acts as an antibody control, while RpoB is a loading control. Images are representative of three independent experiments.
a protein sharing 94% sequence identity with PAAR3. We hypothesise that the insertion of this cassette compensates for the loss of the PAAR3-encoding vgrG2a satellite island from the genome of the PA7 strain by re-enabling secretion of VgrG2b. In all, these data suggest that PAAR3 is the cognate tip component for the VgrG2b spike protein.

We have recently discovered that the C-terminal domain of the evolved VgrG2b spike protein is a metallopeptidase-like antibacterial effector, whose detrimental activity is negated by the presence of its cognate immunity protein PA0261 (Wood et al., under revision). To verify that PAAR3 is required for the delivery of VgrG2b C-ter into prey cells, we conducted a
Fig. 6. The PAAR3 spike restricts the delivery of other H2-T6SS-dependent hydrolytic effectors. Competitive growth assay between (a) PAO1ΔrsmAΔpldAtli5a or (b) PAO1ΔrsmAΔtle4tli4 and various attacker strains. Competitive index values represent the mean of at least three independent experiments, with the SEM shown by error bars. A competition assay between the PAO1ΔrsmA parental strain and itself acts as the internal control for competitive parity. Competitive growth outcomes that were statistically significant from the comparator competition between the parental strain and the prey strain lacking the effector–immunity pair of interest were determined using a one-way ANOVA with Dunnett’s multiple comparisons test and are denoted with asterisks (** p < 0.01; * p < 0.05; ns, not significant).
Tle3 is an antibacterial effector delivered by the VgrG2b–PAAR3 spike complex

The T6SS delivers a vast repertoire of antibacterial toxins, including nucleases, NAD(P)⁺ hydrolases, pore-forming effectors, peptidoglycan hydrolases and phospholipases [12, 25, 26] [15, 27]. Phospholipase toxins form the type VI lipase effector (Tle) superfamily, composed of Tle1-5 [15]. The characterised T6SS-associated phospholipases of P. aeruginosa PA01 are Tle1, Tle4, PldA (Tle5a) and PldB (Tle5b), all of which display antibacterial activity on membrane phospholipids within the periplasm [14, 28] [15, 29]. Our laboratory amongst others has found that effector proteins are often encoded in the same locus as the VgrG proteins responsible for their delivery [8, 9, 16, 18, 30–33]. The product of the PA0260 gene, encoded within the satellite island of the T6SS spike protein VgrG2b, is predicted to be a member of the uncharacterised Tle3 family with the consensus GXSXG catalytic motif within the α/β-hydrolase domain and a C-terminal DUF2345 domain of unknown function (Fig. 4a) [15]. Type VI effector proteins that act within the periplasmic compartment lack a native signal sequence to avoid self-intoxication, and heterologous production of Tle3 in Escherichia coli does not elicit toxicity (Fig. 4b). However, when artificially targeted to the periplasm by an N-terminal Sec-dependent signal peptide, Tle3 caused a 1000-fold decrease in cell viability.

Antibacterial toxins are invariably encoded adjacent to immunity proteins that neutralise the effector in donor and sister cells to prevent kin elimination. While upstream of tle3 lies PA0261, encoding the VgrG2b, immunity protein (Wood et al. under revision), the gene downstream (PA0259) codes for a protein of unknown function, henceforth designated Tli3 (see below), the putative immunity protein to Tle3 (Fig. 1). Since Tle3 is encoded within the vgrG2b satellite island, we reasoned that this cargo effector may be delivered by the H2–T6SS, requiring VgrG2b as its cognate spike protein. We constructed a P. aeruginosa strain lacking the tle3–tli3 cassette and assayed its contact-dependent competitive fitness with the parental strain under conditions in which the H2–T6SS is active. Indeed, the prey strain was robustly eliminated by the parent, whereas deletion of the tle3 gene from the attacker abolished its ability to kill the prey strain (Fig. 4c). Delivery of the Tle3 toxin required both the spike protein VgrG2b and a functional H2–T6SS, as deletion of vgrG2b or the baseplate component gene tssE2 rescued the survival of the prey strain. Complementation of these genes in trans partially restored killing by the attacker. We have previously defined the modular architecture of VgrG2b as consisting of the spike region (containing a gp27-like, gp5-like and DUF2345 domain), a transthyretin (TTR)-like fold and a C-terminal metallopeptidase effector domain (Wood et al., under revision). Expression of a construct encoding the N-terminal spike region of VgrG2b (gp27–gp5–DUF2345) in the ΔvgrG2b strain was unable to restore any prey killing, unlike the full-length vgrG2b gene, indicating that the C-terminal region (TTR-effector) of this evolved spike protein is necessary for Tle3 delivery (Fig. 4c). The TTR domain of VgrG1 from enterohaemorrhagic E. coli is involved in the delivery of its cognate phospholipase effector, Tle1, so we predict that the TTR domain of VgrG2b is likely responsible for Tle3 delivery [31]. In accordance with our data implicating PAAR3 in the secretion of VgrG2b, we find that Δtle3tli3 is no longer susceptible to elimination by an attacker lacking this PAAR protein, whereas a ΔPAAR5 attacker maintains a 25-fold competitive advantage (Fig. 4c). We therefore conclude that PAAR3 is required for the delivery of Tle3 by the H2–T6SS spike protein VgrG2b.

Tli3 is the cognate periplasmic immunity protein to Tle3

The cognate immunity proteins to phospholipase effectors harbour signal sequences to target them to the periplasm space; however, the annotated tli3 gene downstream of tle3 is predicted to encode a cytoplasmic protein. However, closer examination indicates that expression of tli3 from an alternative start site 150 bp upstream is predicted to produce a protein harbouring an N-terminal signal peptide (Fig. 5a). Comparison of the two putative start sites using the Kolaskar and Reddy algorithm [34] predicts that the reannotated upstream start site is more likely to initiate translation (Fig. 5a). This apparent misannotation is found in all 79 instances of the tli3 gene being identified in fully sequenced P. aeruginosa strains. Bioinformatics analyses of tle3–tli3 loci in other proteobacteria show that the effector–immunity gene juxtaposition is maintained independently of the VgrG2b_C-ter–PA0261 effector–immunity pair locus, reinforcing the notion that Tle3 is a classical cargo effector (Fig. 5b). Tli3 proteins could be identified as they bear a DUF2875 domain, which in some instances (such as in P. aeruginosa) is duplicated. Detailed inspection of the orthologous Tli3 coding sequences reveals a myriad of export signatures, such as putative lipoprotein type II signal peptides and N-terminal transmembrane helices, bolstering the predicted periplasmic localisation of the Tli3 protein family (Fig. 5b).

Heterologous expression of a construct encoding Tle3 artificially targeted to the periplasm along with Tli3 in E. coli shows that Tli3 improves the viability of bacteria producing the Tle3 effector, thus indicating that Tli3 is the cognate immunity protein of Tle3 (Fig. 5c). Moreover, while a P. aeruginosa prey strain lacking the tle3–tli3 locus is robustly eliminated by the parental strain when placed in competition on solid media, provision of the full-length tli3 gene in trans restores growth of the prey (Fig. 5d). If the sequence encoding the predicted signal peptide is omitted from the tli3 coding sequence, prey elimination remains at the level of that of the prey strain harbouring either the empty vector or the unrelated PA0261 immunity gene, demonstrating the requirement of the signal
PAAR3 restricts the delivery of other H2-T6SS-dependent effectors

Several H2-T6SS-dependent effectors besides Tle3 have been characterised, including PldA and Tle4 [14, 15]. Here, we assessed the role of PAAR3 and PAAR5 in the delivery of the phospholipase effectors PldA and Tle4 through intra-species competition assays. Elimination of the PldA-susceptible prey $\Delta$pldA$\Delta$ti$\tilde{I}$ is abolished when the attacker lacks its cognate VgrG4b delivery device or a functional H2-T6SS apparatus, as previously noted (Fig. 6a) [15, 18]. An attacker lacking PAAR5 is still able to outcompete the prey strain to a similar extent as the parental strain; however, deletion of PAAR3 greatly increases the ability of the attacker to eliminate the prey. This suggests that the presence of PAAR3 limits PldA delivery by the H2-T6SS.

Next, we demonstrated that Tle4-mediated elimination of a $\Delta$tle4 strain in an H2-T6SS-dependent manner, first shown by Jiang and co-workers, could be achieved under the contact-dependent killing conditions developed in our laboratory (Fig. 6b) [29]. Due to the tendency of effector proteins such as PldA and Tle3 to utilise the VgrG encoded immediately upstream as their cognate delivery device, we hypothesised that VgrG2a, encoded adjacent to tle4, would perform such a role (Fig. 1). Indeed, deletion of vgrG2a abrogated the competitive advantage of the attacker, which could be partially restored through complementation of the full-length vgrG2a gene in trans. VgrG2a and VgrG2b display 99.5% sequence identity across their gp27-gp5-DUF2345 spike regions, with only four residues differing between the N-terminal 757 residues of the two spike proteins. Unlike VgrG2b, VgrG2a is not considered to be an evolved VgrG and is predicted to harbour solely a C-terminal TTR domain, with just 25% sequence identity to that of VgrG2b. Since the N-terminal spike region of VgrG2b is unable to complement the vgrG2a mutant despite its homology to the equivalent region of VgrG2a (Fig. 6b), we predict that the C-terminal TTR domain of VgrG2a is required for Tle4 delivery. Finally, in a similar manner to the delivery of the VgrG4b-dependent PldA toxin, we find that deletion of PAAR5 does not hamper Tle4 delivery, prey killing is once again enhanced by the absence of PAAR3 (Fig. 6b). These data suggest that the delivery of H2-T6SS toxins by spike proteins other than VgrG2b, namely the cognate pairs VgrG4b–PldA and VgrG2a–Tle4, is restricted by the PAAR3–VgrG2a subassembly. This finding indicates that competition between PAAR–VgrG subassemblies of the H2-T6SS exists, and that at least under the conditions employed in this study, the VgrG2b–PAAR3 subassembly is able to outcompete the spike complexes formed by VgrG4b or VgrG2a. These spike complexes recruit cognate effectors to the T6SS baseplate to assemble a functional machine and therefore define the principal effectors secreted by a single apparatus.

**DISCUSSION**

In this study, we report the VgrG and PAAR dependence of a new antibacterial effector of the H2-T6SS of *P. aeruginosa* and begin to define the competition between spike subassemblies for this secretion system. The Tle3 toxin belongs to the phospholipase family of antibacterial effectors and its detrimental activity in the periplasm is neutralised by its cognate immunity protein Tli3, which is predicted to localise to this compartment. Tle3 requires the spike protein VgrG2b, encoded upstream, for its delivery and this in turn requires the PAAR3 tip protein. Intriguingly, our genetic approach reveals that the delivery of two other H2-T6SS-dependent phospholipase effectors, which utilise distinct cognate VgrG partners, is enhanced in the absence of PAAR3, therefore implying that the PAAR3-VgrG2b spike subassembly may outcompete other spike complexes for binding to the H2-T6SS apparatus.

Through a bioinformatics approach, we identify a conserved putative PAAR protein that is likely associated with the H2-T6SS based on both genetic linkage and homology to other PAAR proteins functionally linked to this system. Although this PAAR protein, which we designate PAAR5, does not appear to play a substantial role in the secretion of VgrG2a, VgrG2b or VgrG4b, several other spike proteins, namely VgrG4a, VgrG5 and VgrG6, are associated with the H2-T6SS. Recent work linked VgrG6 with PAAR4 [19]; however, the associated PAAR proteins for VgrG4a and VgrG5 remain elusive, and it is possible that PAAR5 assumes such a role. The high conservation of the predicted VgrG-binding interface of PAAR2, PAAR4 and PAAR5 may also permit functional redundancy, where non-cognate PAAR proteins may still deliver a particular spike protein with reduced efficiency.

We find that the delivery of Tle4 is VgrG2a-dependent, but that it does not require PAAR3, despite their genetic linkage. This is somewhat surprising due to their consistent synteny in *P. aeruginosa* strains, but is supported by the secretion of VgrG2a in the absence of PAAR3 or vgrG2b, and VgrG2a-independent secretion of VgrG2b, which itself associates with PAAR3. Moreover, the authors of the seminal work characterizing PAAR proteins were unable to detect an interaction between PAAR3 and the VgrG2a spike in crystallographic analyses [7]. While we cannot fully discount that a VgrG2a–PAAR3 spike subassembly may exist, our findings strongly suggest that VgrG2a associates predominantly with a distinct tip protein.

PAAR proteins appear to be extremely important for T6SS functionality, as the PAAR–VgrG complexes form the puncturing device of the nanomachine [11]. The association of specific PAAR and VgrG proteins has previously been described in several bacteria, including *Serratia marcescens*, *Agrobacterium tumefaciens* and *P. aeruginosa*, while recent work has also highlighted the direct association of cargo effectors with PAAR proteins [8–11, 16, 19, 35]. In *S. marcescens*, three VgrG–PAAR subassemblies display differing delivery efficiencies, potentially due to relative expression levels or
Table 1. Strains and plasmids used in this study

| Bacterial Strains          | Description                                      | Source                      |
|----------------------------|--------------------------------------------------|-----------------------------|
| *P. aeruginosa* PAO1       | Wild-type *P. aeruginosa* strain                 | Laboratory collection       |
| *P. aeruginosa* PAO1ΔrsmA  | Deletion of PA0905                               | [18]                        |
| *P. aeruginosa* PAO1ΔrsmADΔtssE2 | Deletion of PA0905 and PA1659            | This study                  |
| *P. aeruginosa* PAO1ΔrsmADΔvgrG2a | Deletion of PA0905 and PA3486         | This study                  |
| *P. aeruginosa* PAO1ΔrsmADΔvgrG4b | Deletion of PA0905 and PA1508          | This study                  |
| *P. aeruginosa* PAO1ΔrsmADΔPAAR3 | Deletion of PA0905 and PA1659.1        | This study                  |
| *P. aeruginosa* PAO1ΔrsmADΔvgrG2aΔtssE2 | Deletion of PA0905 and PA2626       | This study                  |
| *P. aeruginosa* PAO1ΔrsmADΔvgrG2aΔtssE2Δtli3 | Deletion of PA0905 and PA0260       | This study                  |
| *P. aeruginosa* PAO1ΔrsmADΔvgrG2aΔvgrG2b | Deletion of PA0905 and PA0260       | This study                  |
| *E. coli* DH5α             | *E. coli* DH5α                                    | Laboratory collection       |
| *E. coli* CC118Δpir         | *E. coli* CC118Δpir                              | [45]                        |
| *E. coli* Sm10Δpir          | *E. coli* Sm10Δpir                               | [46]                        |
| *E. coli* BL21 (DE3)        | *E. coli* BL21 (DE3)                             | Laboratory collection       |

**Plasmids**

| Plasmid                    | Description                                      | Source                      |
|----------------------------|--------------------------------------------------|-----------------------------|
| Mini-CTX- lacZ            | Integrative plasmid for inserting lacZ at a neutral site on the *P. aeruginosa* chromosome | [47]                        |
| pET28a                    | Expression vector, Km₈                             | Novagen                     |
| pET28a-tle3               | Expression plasmid producing Tle3 with a C-terminal hexahistidine tag, Km₈ | This study                  |
| pET22b                    | Expression vector with the PelB signal peptide to target proteins to the periplasm, Ap₈ | Novagen                     |
| pET22b-tle3               | Expression vector producing Tle3 with a C-terminal hexahistidine tag, artificially targeted to the periplasm by an N-terminal signal peptide, Ap₈ | This study                  |
| pBBR1-MCS-4               | Broad host range vector, Cb₉                       | [48]                        |
| pBBR1-MCS-4-tssE2         | Broad host range plasmid for constitutive expression of tssE2, Cb₉ | This study                  |
| pBBR1-MCS-4-vgrG2b        | Broad host range plasmid for constitutive production of VgrG2b with a C-terminal quadruple HA tag, Cb₉ | This study                  |
| pBBR1-MCS-4-vgrG2b₁₆₅₇    | Broad host range plasmid for constitutive production of the N-terminal canonical spike region of VgrG2b (1-757), Cb₉ | This study                  |
| pBBR1-MCS-4-tle4          | Broad host range plasmid for constitutive production of Tle4 with a C-terminal quadruple HA tag, Cb₉ | This study                  |
| pBBR1-MCS-4-vgrG2a        | Broad host range plasmid for constitutive production of VgrG2a with a C-terminal quadruple HA tag, Cb₉ | This study                  |
| pBBR1-MCS-4-tle3          | Broad host range plasmid for constitutive production of Tle3 with a C-terminal quadruple HA tag, Cb₉ | This study                  |
| pBBR1-MCS-5               | Broad host range vector, Gm₈                       | [48]                        |

Continued...
complex stability and prevalence [11]. The identity of the spike components appears to also influence the efficiency of the delivery of Hcp-dependent effectors; however, the precise reason for this remains elusive [9, 11].

Differential effector delivery has also been suggested within the H2-T6SS, where secretion of a PAAR4-dependent effector is enhanced in the absence of vgrG2b [19]. We further our understanding of this concept by showing that in the absence of PAAR3, which is required for VgrG2b and Tle3 secretion, the delivery of other VgrG–effector sets, namely VgrG2a–Tle4 and VgrG4b–PldA, appears to be elevated. These findings highlight that the competition for VgrG–PAAR recruitment to the T6SS apparatus defines the identity of the effector payload. Thus, the small conical PAAR ‘hat’ of the T6SS determines the identity of spike subassemblies, which recruit specific effector proteins for secretion. Comprehensive elucidation of the identities of the PAAR–VgrG–effector partnerships will enable determination of cargo delivery from amongst a broad arsenal, which could be harnessed for the selective bacterial delivery of therapeutic proteins into target cells.

METHoDGS

Bacterial Strains, growth conditions and plasmids

The strains and plasmids used in this study are listed in Table 1 and the oligonucleotides for cloning are provided in Table 2. Bacteria were cultured at 37°C in lysogeny broth (LB) under agitation or on solid LB media unless stated otherwise. Media were supplemented with antibiotics and other compounds where appropriate at the following concentrations: kanamycin (50 µg ml⁻¹), ampicillin (50 µg ml⁻¹), streptomycin (50 µg ml⁻¹), gentamicin (15 µg ml⁻¹), isopropyl-β-d-thiogalactopyranoside (IPTG) (100 µg ml⁻¹) for E. coli; carbenicillin (100 µg ml⁻¹), streptomycin (1000 µg ml⁻¹), gentamicin (50 µg ml⁻¹) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) (100 µg ml⁻¹) for P. aeruginosa. All deletion strains employed in this work are in-frame, markerless mutants and were constructed using the pKNG101 suicide vector as previously described [36]. The E. coli DH5α strain was used as the generic cloning host, while CC118λpir and Sm10λpir were used to mobilise the integrative plasmids pKNG101 and Mini-CTX into P. aeruginosa by three- and two-partner conjugation, respectively. E. coli BL21 (λDE3) was used for heterologous expression experiments.

Secretion assays and immunoblot analysis

Overnight cultures of P. aeruginosa cultures were diluted to OD₆₀₀ 0.1 in 25 ml tryptic soy broth (TSB) and grown at 25°C in TSB for 8 h with agitation. To recover secreted bacterial proteins, cultures were centrifuged at 4000 g at 4°C for 10 min three times successively, carrying forward the uppermost supernatant each time to eliminate cellular contamination. Proteins were precipitated with 10% trichloroacetic acid supplemented with 0.03% sodium deoxycholate overnight at 4°C. The precipitate was pelleted by centrifugation at 16 000 g for 30 min at 4°C before washing.
Table 2. Oligonucleotides used in this study

| Primer Name | Purpose | Sequence |
|-------------|---------|----------|
| OAL3632     | tle3-His_F | AGAGGATCCGGCAAGGAGAAGCCATG |
| OAL3628     | tle3-His_R | GTAGAATTCTAGTGGTGGTGGTGGTGGATTTGTCACCCACAAAGCG |
| OAL1265     | vgrG2b_F | AAAGGATCCATGCGTCAAGGGACCTG |
| OAL2923     | vgrG2b_Hs_R | TACAGCCTTTACGCGGACAT |
| OAL4192     | Ha_F | CAAGAATTCGCTAGCAGCGGAGATCGATGTATATGC |
| OAL4098     | tle4-Ha_F | ATAGGGGCTCTCAACACGACGACAGGAG |
| OAL4099     | tle4-Ha_R | TCAAGATTCGCTAGCCTCCCTTGGTCACTAGGAT |
| OAL790      | tssE2-His_F | CGGAATTCGTGGTCACGGGTCAAGCG |
| OAL3508     | tssE2-His_R | TTAGACGTCTCACTGGTGTTGGTGCGGTGCCACCTACCTGCCG |
| OAL4095     | vgrG2a/b-Ha_F | ATAGGGGCTCCGAAAGAACAGCAT |
| OAL4096     | vgrG2a-Ha_R | TCAAGATTCGCTAGCCTCCCTTGGTCACTAGGAT |
| OAL4097     | vgrG2b-Ha_R | TCAAGATTCGCTAGCCTCCCTTGGTCACTAGGAT |
| OAL3333     | li3-Ha_F | TACAGCCTTTACGCGGACAT |
| OAL3334     | li3-Ha_R | GTTAAGCTTTAGACCGACGCTAGCCTCCCTTGGTCACTAGGAT |
| OAL5193     | li3(no SP)-Ha_F | ATAGGGGCTCCGAAAGAACAGCAT |
| OAL2452     | tle3_vector_F | TTTGCGCCGACACATCAACG |
| OAL5194     | tle3-Ha_R | GTAGCGCTCCGCAAGCCTCCCTTGGTCACTAGGAT |
| OAL2575     | pKNG101-(ΔPAAR3)Up_F | AAAGAATTCCTTTGTCAG |
| OAL2576     | pKNG101-(ΔPAAR3)Up_R | TCGAGTTTTCGATCACCCAGC |
| OAL2577     | pKNG101-(ΔPAAR3)Dn_F | TTTGCGCCGACACATCAACG |
| OAL2578     | pKNG101-(ΔPAAR3)Dn_R | TCGAGTTTTCGATCACCCAGC |
| OAL2579     | pKNG101-(ΔPAAR3)Ext_F | TCGAGTTTTCGATCACCCAGC |
| OAL2580     | pKNG101-(ΔPAAR3)Ext_R | TCGAGTTTTCGATCACCCAGC |
| OAL3366     | pKNG101-(ΔPAAR5)Up_F | TCGAGTTTTCGATCACCCAGC |
| OAL3367     | pKNG101-(ΔPAAR5)Up_R | TCGAGTTTTCGATCACCCAGC |
| OAL3368     | pKNG101-(ΔPAAR5)Dn_F | TCGAGTTTTCGATCACCCAGC |
| OAL3369     | pKNG101-(ΔPAAR5)Ext_F | TCGAGTTTTCGATCACCCAGC |
| OAL3370     | pKNG101-(ΔPAAR5)Ext_R | TCGAGTTTTCGATCACCCAGC |
| OAL3289     | pKNG101-(Δtle3 / Δtle3/tli3)Up_F | CATTCTAGACACCAGGGATAGCTACATCAATGAA |

Continued
| Primer Name       | Purpose                          | Sequence                                      |
|-------------------|----------------------------------|-----------------------------------------------|
| OAL290 pKNG101-(Δile3 / Δile3tli3)Up_R | GAGGCTGCTATCGTCTATGCGCTTCTTCCTTGC             |
| OAL291 pKNG101-(Δile3tli3)Dn_F | ATGAAACGATAGCAGCCTCAAAACCCAGC               |
| OAL292 pKNG101-(Δile3tli3)Dn_R | TGAACAGATGAGCTCTATGCGCTTCTTGG                |
| OAL293 pKNG101-(Δile3 / Δile3tli3)Ext_F | CCGGAAAGACGTTGAGGA                          |
| OAL294 pKNG101-(Δile3tli3)Ext_R | GTAGGGTCCGATGGCGGTAG                         |
| OAL2049 pKNG101-(Δile4 / Δile4tli4)Up_F | GCCGGGCAGAGGTGTTGACAC                         |
| OAL2050 pKNG101-(Δile4)Up_R | TCCTTTGCTCTGCTATGTTGACGCTTGG                |
| OAL2051 pKNG101-(Δile4)Dn_F | ATGAGCACGCAAAACGGAGACGACATG                 |
| OAL2052 pKNG101-(Δile4)Dn_R | AGCCCTAGATATCTCCACAGCAGATGT                 |
| OAL2053 pKNG101-(Δile4 / Δile4tli4)Ext_F | CCGCGAGCAAAACCCCAAC                        |
| OAL2054 pKNG101-(Δile4)Ext_R | AATGTTTCCATACCTGCAACGCTG                  |
| OAL2587 pKNG101-(Δile4tli4)Up_R | CTATATGCGCTCTGCTATGTTGACGCTTGG             |
| OAL2588 pKNG101-(Δile4tli4)Dn_F | ATGAGCACGCAAAACGGAGACGACATG                 |
| OAL2584 pKNG101-(Δile4tli4)Dn_R | ATCAGCCATGGCTCTTGT                         |
| OAL2586 pKNG101-(Δile4tli4)Ext_R | GTTTTCAGCAGAACCCTCACCT                   |
| OAL3360 pKNG101-(ΔtssE2)Up_F / (ΔPAAR5)Ext_F | TGATCTAGAATCGACGACGACAGATCAGCCCC          |
| OAL3361 pKNG101-(ΔtssE2)Up_R | ACCTTTACCTTGCCTGTAATCCATATG                |
| OAL3362 pKNG101-(ΔtssE2)Dn_F | ATACGCGGAGGCTGAAGTTGACGAGCTAAGGA           |
| OAL3363 pKNG101-(ΔtssE2)Dn_R | TCAACTAGTAACCTGCTACGCTTGACGCTCCTG          |
| OAL3364 pKNG101-(ΔtssE2)Ext_F | CTTCGCCAAGTATGCTGG                         |
| OAL3365 pKNG101-(ΔtssE2)Ext_R / (ΔPAAR5)Dn_R | TCAACTAGTCAGCAGGACAGGTAGAGC               |
| OAL2631 pKNG101-(ΔvgrG2a)Up_R | TGATGGCCAGGGCTTCAAC                      |
| OAL2632 pKNG101-(ΔvgrG2a)Up_R | CGCTTTGGTGGTGAGCTGAGCAGCATCGTGTC          |
| OAL2633 pKNG101-(ΔvgrG2a)Dn_F | ATGAGCTACACACGACGACGAGCAGTAGGAA           |
| OAL2137 pKNG101-(ΔvgrG2a)Dn_R | GCTGACGATGATCCATGCTTC                    |
| OAL2634 pKNG101-(ΔvgrG2a)Ext_F | ACTTCTCTTCACCCCTTGGCC                    |
| OAL2590 pKNG101-(ΔvgrG2a)Ext_R | CACCAAGCGTCCCTCCATGCGTAGG                 |
| OAL2486 pKNG101-(ΔvgrG2b)Up_R | TGACCTCCGCGACGGAG                       |
| OAL2487 pKNG101-(ΔvgrG2b)Up_R | TCAGTATCCATGACGCTACGCTGCTTGTCWG           |
| OAL2488 pKNG101-(ΔvgrG2b)Dn_F | ATGCGCTACAGCATGACGCTACGCTGCTTG           |
| OAL2489 pKNG101-(ΔvgrG2b)Dn_R | GTCGACGACGTACGCTGCTGCG                    |
| OAL2490 pKNG101-(ΔvgrG2b)Ext_F | CCAGGACACCTCAGGAGA                      |
| OAL2491 pKNG101-(ΔvgrG2b)Ext_R | CTCTCCCTGCGCGCTCTTC                    |

F, forward primer; R, reverse primer. Restriction enzyme sites are underlined.
with cold 90% acetone and centrifugation once more. The washed pellet was air-dried and resuspended in Laemmli buffer to an equivalent of OD₆₀₀ of 20. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting was conducted as previously described [37]. Here, polyclonal rabbit anti-VgrG2a [20], anti-VgrG2b [18], anti-VgrG4b [18], anti-Hcp2 [38] and anti-LasB (gift from Romé Vouhoux) antibodies were used at a 1:1000 dilution, along with monoclonal mouse anti-RpoB (1:5000, Neoclide), anti-HA (1:1000, Biolegend) and anti-His₆ (1:1000, Sigma) antibodies.

**Periplasmic fractionation**

E. coli cultures producing Tle3 or its periplasm-targeted fusion were grown to an OD₆₀₀ of 0.6 before the induction of construct expression with 100 µM IPTG. Three hours post-induction, cells were harvested by centrifugation at 8000 g at 4°C. Pellets were normalised to an OD₆₀₀ of 20, resuspended in 200 µl spheroplast buffer (200 mM Tris-HCl pH 8.0, 500 µM EDTA and 500 mM sucrose) containing 50 µg hen egg-white lysozyme (Sigma) and incubated on ice for 15 min. Then, 720 µl spheroplast buffer was diluted 1:1 with distilled water and added to the spheroplasts. The spheroplasts were separated from the periplasmic fraction by centrifugation at 5000 g for 10 min at 4°C and the isolated periplasmic fraction was centrifuged once more to remove residual spheroplasts. The periplasmic fraction was adjusted to the equivalent of an OD₆₀₀ of 10 in Laemmli buffer.

**Bacterial toxicity and competition assays**

E. coli cells producing Tle3 and Tli3 constructs were grown overnight and normalised to an OD₆₀₀ of 1 before serial dilution in phosphate-buffered saline (PBS). Dilutions were spotted on solid media containing the appropriate antibiotics and inducer (100 µM IPTG) or repressor (2% glucose) compounds before being air-dried and grown at 37°C for 24 h. For P. aeruginosa intra-species competition assays, overnight cultures were normalised to an OD₆₀₀ of 3 and washed with PBS. Attacker and prey strains were mixed at a 1:1 ratio, centrifuged at 8000 g for 3 min and resuspended in 100 µl PBS. Five microlitres of the competition mixtures was spotted on low salt LB agar (10 g bactopeptone, 5 g yeast extract, 30 g agar l⁻¹), dried and incubated at 25°C for 24 h. Spots were scraped up into 1 ml PBS and serially diluted for plating on LB agar containing 100 µg ml⁻¹ X-gal for the discrimination of attacker and prey strains, since the latter harbour mini-CTX-lacZ on the chromosome.

**In silico analyses**

Nucleotide sequences for the P. aeruginosa genome were retrieved from [www.pseudomonas.com](http://www.pseudomonas.com) [39] and queried using BLASTN and tblASTN analyses [40]. MAFFT [41] was used for all amino acid sequence alignments and the phylogeny of PAAR domains was analysed with MEGA7 [42] using the maximum-likelihood method of tree generation with 1000 bootstrap replicates. The sequence logo for the consensus catalytic motif of Tle3 homologues was generated using the WebLogo server [43] and SignalP 4.1 was used for the prediction of signal peptides [44]. All statistical analyses described in this work were conducted with Prism 8.0 software (GraphPad).

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**Author contributions**

T. E. W. and A. F. designed the experiments and wrote the manuscript and T. E. W., S. A. H. and S. W. conducted the experimental work.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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