An *rhs* Gene Linked to the Second Type VI Secretion Cluster Is a Feature of the *Pseudomonas aeruginosa* Strain PA14

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The type VI secretion system (T6SS) of Gram-negative bacteria has been involved in various processes, notably bacterial competition and eukaryotic cell subversion. Most *Pseudomonas aeruginosa* strains possess three T6SS gene clusters, but only the function of the first T6SS (H1-T6SS) has been clearly elucidated. It is involved in the secretion of three toxins (Tse1 to -3) that target bacterial competitors. In the case of the H2- and H3-T6SS, no clear function has been assigned, and only one effector has been associated with these systems. Yet the H2-T6SS was proposed to promote *P. aeruginosa* internalization in nonphagocytic epithelial cells. Although the H2-T6SS genetic organization is conserved across *P. aeruginosa* isolates, one feature is the presence of an additional transcriptional unit in the PA14 strain H2-T6SS cluster, which is divergent from the core H2-T6SS genes. A specific set of four genes encodes an Hcp protein (Hcp2), a VgrG protein (VgrG14), an Rhs element (PA14_43100 or RhsP2), and a protein with no homologies with previously characterized proteins (PA14_43090). In this study, we engineered a *P. aeruginosa* PA14 strain carrying an arabinose-inducible H2-T6SS on the chromosome. We showed that arabinose induction readily promotes assembly of the H2-T6SS, as seen by monitoring Hcp2 secretion. We further studied the secretion fate of VgrG14 and RhsP2, but these were not detectable in the extracellular medium. We finally investigated whether activation of the PA14 H2-T6SS gene cluster could influence phenotypic traits such as internalization in eukaryotic cells, and we reported noteworthy differences compared to strain PAO1, which may be accounted for by the described genetic differences.

*Pseudomonas aeruginosa* is a Gram-negative bacterium that is an opportunistic pathogen equipped with a wide range of protein secretion systems (1). These systems are named by type, i.e., the type I (T1SS) to type VI (T6SS) secretion systems. All of these systems, in some cases in more than one copy, are found encoded in the genomes of all sequenced *P. aeruginosa* isolates ([www.pseudomonas.com](http://www.pseudomonas.com)), with the exception of the type 4 secretion system (T4SS). This combination of secretion nanomachines is dedicated to the release of enzymes and toxins, which are involved, for example, in the degradation of complex carbon sources (2), the acquisition of iron (3), the degradation of host tissues (4, 5), the subversion of eukaryotic host cell signaling (6), and even motility (7, 8).

The T6SS of *P. aeruginosa* was discovered in 2006 (9). This resulted in rejuvenation of the field by bringing in novel and important concepts. It was already noteworthy that several secretion systems coevolved with machines involved in the assembly of extracellular appendages (10). For example, the type II secretion system (T2SS) is similar to the type IV pilus assembly machine (11), the type III secretion system (T3SS) has similarity with the basal body of flagella (12), and the T4SS has similarity with conjugative pili (13). In contrast, the T6SS is similar to the contractile tail of bacteriophages (14–16). One remarkable feature is the tube formed by hexameric rings of the Hcp protein (9, 17), a structural homologue of the gp19 component of the bacteriophage T4 tail tube (18). Another striking protein is VgrG, which resembles the heterotrimetric gp27-gp53 complex of the phage (18–21). In this complex, the gp5 protein forms a rigid helix, made of regularly spaced series of β-strands, which acts as a needle to puncture the bacterial cell envelope (22). In VgrG proteins associated with the T6SS, the C-terminal domain is similar to gp5, whereas the N terminus is similar to gp27. A further observation is the conservation in the T6SS of a sheath-like structure which is contractile and made of the gp18 protein of the T4 phage (15, 23). In the T6SS, this sheath structure is seen as long tubules by electron microscopy, but in cross section it forms cogwheel-like structures. Whereas the bacteriophage sheath contains a single protein, the T6SS counterpart is made of two interacting proteins: VipA-VipB in the case of *Vibrio cholerae* (24) and HsiB-HsiC in the case of *P. aeruginosa* (25). The T6SS is thus considered an inverted bacteriophage tail whose contraction will result in breaching of the bacterial cell envelope, allowing secretion of proteins/effectors.

Until recently, only a few T6SS substrates were described. One important example is the VgrG1 protein, which is an evolved puncturing device from *V. cholerae*, since it has a C-terminal extension consisting of an actin cross-linking domain (21, 26). Remarkably, this domain is translocated into the cytosol of macrophages (27, 28). While a few examples of this type were studied experimentally, it quickly became evident that one main function of the T6SS is not to subvert the host cell but to transport toxins into bacterial competitors and kill them (29). The toxin-encoding genes are not necessarily linked with the T6SS cluster, e.g., as is the case with the first *P. aeruginosa* T6SS (H1-T6SS) and the three associated pairs of toxin-antitoxin (Tse1 to -3 and Tsi1 to -3). These are encoded on distinct loci but are coregulated with the T6SS genes via the RetS/Gac/Rsm signaling pathway (29, 30). Since the discovery of these toxins, similar examples have been...
found in a number of bacterial species, including *V. cholerae*, *Serratia marcescens*, and *Burkholderia* species (31–34). In several cases, these toxins have been shown to degrade the peptidoglycan of the target bacterial cells, which results in rounding and lysis (35).

Whereas the *hcp* and *vgrG* genes encode core components of the T6SS machine, genomic analysis indicated that several of these genes are located distantly from any T6SS gene clusters (36). Importantly, it has been proposed that genes located downstream of the *vgrG* loci encode potential effectors, many of which are described as phospholipases (37, 38). The *P. aeruginosa* genome carries three T6SS clusters (39) and 10 *vgrG* genes, in the case of strain PAO1 (19). Interestingly, no *vgrG* gene could be found in the vicinity of the *H2*-T6SS gene cluster in the PAO1 genome. Instead, it was recently proposed that in PAO1, the *H2*-T6SS is involved in the secretion of a bacterial toxin with phospholipase activity (37). The toxin has been called Tle5, and the corresponding gene is found adjacent to a *vgrG* gene which is distal from the *H2*-T6SS locus. However, in the case of the virulent *P. aeruginosa* strain PA14 (40), the *H2*-T6SS cluster is linked with an 11th *vgrG* gene and a gene encoding a protein from the Rhs family (41). In the present study, we investigated the assembly of the PA14 *H2*-T6SS and whether the distinct genetic organization is relevant to the difference in behavior of the PAO1 and PA14 strains.

**MATERIALS AND METHODS**

*Strains, plasmids, and growth conditions.* The strains and plasmids used in this study are described in Table 1. *P. aeruginosa* strains were grown in tryptone soy broth (TSB) at 37°C with agitation. *Escherichia coli* strains were grown in LB broth at 37°C with shaking. The pKNG101 suicide vector was used to generate deletion mutants or chromosomal insertions into the *H2*-T6SS genes. For induction of the arabinose-inducible promoters, 2% arabinose was added into the growth medium.
Bioinformatic analysis. Amino acid sequences of *P. aeruginosa* proteins were obtained from [www.pseudomonas.com](http://www.pseudomonas.com) (42). Secondary structure predictions were made using the online Psipred service (43), and phylogenetic analysis was performed at [www.phylogeny.fr](http://www.phylogeny.fr), using the “one-click” option (44).

Engineering arabinose-inducible H2-T6SS genes into PA14 strains. A PA14 strain carrying inducible H2-T6SS genes was generated by the introduction of arabinose-inducible pBAD promoters and the regulatory *araC* gene at the H2-T6SS chromosomal location. This was achieved by manipulating the existing *araC* pBAD arrangement found in arabinose-inducible promoters, e.g., pN105 (45). This promoter region was subcloned into pCR2.1, and the pBAD promoter region was amplified using primers shown in Table 2 and, following restriction digestion, inserted in the opposite orientation upstream of the *araC* gene, flanked by Nhel and Ndel restriction sites.

To manipulate the H2-T6SS promoter region, 500-bp regions upstream and downstream of the intergenic region were cloned using primers defined in Table 2. A total of 50 bp upstream of the *hisA2* and *hcp2* open reading frames were conserved. The 500-bp regions were fused by overlap extension PCR, incorporating Nhel and Ndel restriction sites into the overlap region. This generated an ~1-kb mutator fragment, which was cloned into the pCR2.1 cloning vector.

This mutator plasmid was subsequently modified by introduction of the divergent pBAD construct within the overlap region by restriction digestion. This construct was confirmed by PCR, subcloned into the pKNG101 suicide vector, maintained in *E. coli* CC118 Aø, and mobilized into *P. aeruginosa* by three-partner conjugation. Double-recombination events resulting in the exchange of the native promoter region with the divergent promoter construct were selected on sucrose plates, generating the PA14-DP (divergent promoter) strain, and confirmed by PCR using external primers and an internal primer specific to the pBAD region (Table 2).

Construction of clean deletion mutants. Deletion mutants were generated in *P. aeruginosa* by using the pKNG101 suicide vector as previously described (46). Primers used are described in Table 2. Deletion of the H2-T6SS cluster in *P. aeruginosa* PA14 and PA01 was achieved by deletion of a region spanning from mid-*hisA2* to mid-*clpV2*. Deletion of *pscC* was achieved by deletion of the PA14_43250 open reading frame. The *vgrG14* to *rshP2* cluster was disrupted by deleting a region spanning from the beginning of *vgrG14* (PA14_43080) to the end of *rshP2* (PA14_43100), including the PA14_43090 open reading frame. pKNG mutator plasmids were maintained in *E. coli* CC118 Aø and mobilized into *P. aeruginosa* by three-partner conjugation. Double-recombination events resulting in the deletion of the required chromosomal regions were selected on sucrose plates and verified by PCR using external primers (Table 2).

Construction of PA14 strain chromosomally encoding V5-tagged VgrG14 and RhsP2 proteins. The PA14-DP strain was engineered to allow production of a C-terminally V5-tagged version of either VgrG14 or RhsP2 to allow detection of the protein. This was achieved by exchange of the native stop codon of either vgrG14 or rhsP2 with the sequence encoding the V5 and His tags, followed by a new stop codon. The sequence encoding the V5-His5-stop sequence was amplified from pDEST42, flanked by restriction sites engineered into primers described in Table 2, and cloned into the pCR2.1 vector.

To disrupt the desired chromosomal location, 500-bp regions upstream and downstream of the native stop codon of the gene of interest were amplified using primers defined in Table 2 and fused by overlap extension PCR, incorporating a restriction site into the overlap region. This generated the mutator fragment, which was cloned into the pCR2.1 cloning vector.

The mutator fragment was subsequently modified by insertion of the V5-His5-stop sequence between the two 500-bp regions by restriction digestion. The modified mutator fragment was subcloned into the pKNG101 suicide vector, maintained in *E. coli* CC118 Aø, and mobilized into *P. aeruginosa* by three-partner conjugation. Double-recombination events resulting in the insertion of the sequence encoding the V5-His5 tag at the stop codon of the required gene were selected on sucrose plates and verified by PCR.

qRT-PCR analysis. Overnight cultures were subcultured in TSB, grown to mid-exponential phase, and harvested into RNAlater (Ambion). RNA extraction, reverse transcription (RT), and quantitative RT-PCR (qRT-PCR) were carried out as previously described (47). The primers used for amplification are shown in Table 3. Gene expression was normalized to expression of *rpod* and expressed as a ratio relative to the value for the PA14 wild-type strain, which was set to 1.

Production of antibodies against Hcp2 and HsiB2. The gene encoding an *hcp2* homolog (PA1512) was transferred by LR recombination (Invitrogen) from the pDONR shuttle vector to the pETDEST-42 expression vector. *hsiB2* was cloned into pET28a by using primers described in Table 2.

For each construct, protein expression was induced at an optical density at 600 nm (OD600) of 0.6 by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. Following induction, cultures were incubated at 18°C overnight with agitation. Cells were harvested and resuspended in 50 mM HEPES, 500 mM NaCl, 200 mM imidazole, and Complete EDTA-free protease inhibitors, pH 7.2, and then lysed by use of a French press. The lysate was centrifuged at 3,000 × g for 15 min, and the supernatant was applied to His-trap columns (GE Health Care) for purification by use of imidazole gradients. Protein was subsequently purified by size-exclusion chromatography under final buffer conditions of 50 mM Tris, 250 mM NaCl, pH 8. Purified protein was concentrated and sent for antibody production in rabbits (Eurogentec).

Protein secretion assay. T6SS secretion assays were performed following growth with or without 2% arabinose in the culture medium to induce the arabinose promoters. Culture supernatants were harvested and set to 1.0 OD600 unit per 10 μl of Laemmli buffer. Cell extracts were prepared by harvesting 1 ml of bacterial culture by centrifugation and were resuspended to 0.01 OD600 unit per 10 μl of Laemmli buffer. Samples were analyzed by SDS-PAGE and Western blotting. T6SS secretion assays were performed as described for T6SS secretion, but calcium chelation was used to induce the T3SS machinery. This was achieved by supplementing cultures with 5 mM EDTA and 20 mM MgCl2 prior to incubation, as previously described (48).

SDS-PAGE and Western blotting. Cell extracts and supernatant samples were boiled at 95°C for 10 min prior to separation by SDS-PAGE. Cell extracts were loaded at an equivalent of 0.1 OD600 unit per well, and supernatants at 1.0 OD600 unit per well. Following electrophoresis, proteins were transferred to nitrocellulose membranes.

Antibodies against Hcp2 and HsiB2 were generated as described above and used at a 1:1,000 dilution. Anti-RNA polymerase (Neoclone) was used at a dilution of 1:10,000. Anti-V5 antibody (Invitrogen) was used at a dilution of 1:5,000. Anti-PcrV antibody was used at 1:1,000. Primary antibodies were incubated for 1 to 2 h at room temperature, followed by 45 min of incubation with the appropriate secondary antibody (goat anti-rabbit–horseradish peroxidase [HRP] or rabbit anti-mouse–HRP) at a dilution of 1:5,000. Western blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce/Thermo Scientific) and visualized using a Las3000 Fuji imager.

Cytotoxicity of RAW macrophages monitored by LDH release assay. The cytotoxicity of the parental strain PA14 and its isogenic ∆*pscC* and ∆*pscC* ∆H2-T6SS mutants was compared to the toxicity of the PA14::*pscC* transposon mutant (49) using the murine RAW 264.7 macrophage cell line (ATCC TIB-71). Raw macrophages were routinely grown in Dulbecco modified Eagle medium, GlutaMAX I, sodium pyruvate, and phenol red (Gibco) supplemented with 10% fetal bovine serum (Gibco) and nonessential amino acids (Gibco). Macrophages were grown to 80% confluence in 96-well plates (BD Biosciences), washed with sterile phosphate-buffered saline (PBS), and incubated for 1 h prior to infection with RPMI 1640 without phenol red. Macrophages were infected with late-exponential-phase *P. aeruginosa* at a multiplicity of infection of 10 for 3 h at 37°C in the presence of 5% CO2. The macrophage infection was synchronized.
Deletion of cloning into pET28a hsiB2

Cloning of V5-His6 region

Mutation of vgrG14 stop codon

Mutation of rhsP2 stop codon

hsiB2 cloning into pET28a

Deletion of pscC

Deletion of H2-T6SS (mid-hsiA2 to mid-clpV2)

Deletion of vgrG14-rhsP2

Deletion of rhsP2

by pelleting the bacteria with a 5-min centrifugation step at 200 × g. After the infection time point, the plates were centrifuged again for 5 min at 200 × g to sediment cell debris and bacteria prior to supernatant collection. The release of cytosolic lactate dehydrogenase (LDH) into the culture supernatant was measured with the CytoTox96 nonradioactive cytotoxicity assay (Promega). Cytotoxicity was calculated relative to that of non-infected cells, set at 0%, and that of cells lysed with 1% Triton X-100, which was set at 100%.

#### TABLE 2 Cloning primers used in this work

| Primer purpose and ID | Function or target | Sequence (5′–3′) |
|-----------------------|--------------------|------------------|
| Divergent promoter engineering | pBAD region duplication | GGAATTCATATGTCGAGAAGAAGAACATCTGCGATTTG |
| OAL771 | 5′ | GGAATTCATATGTCGAGAAGAAGAACATCTGCGATTTG |
| OAL772 | 3′ | GGAATTCATATGTCGAGAAGAAGAACATCTGCGATTTG |
| OAL601 | 5′ region upstream of H2-T6SS promoter | GGGCGCATGGTACGTCTCCTG |
| OAL586 | 3′ region upstream of H2-T6SS promoter | GCAGTCTCCGCTATATGGAAGGAGTGAGTCG |
| OAL587 | 5′ region downstream of H2-T6SS promoter | AACCATATGAAAACCTAGCTCGAGGAGGTGCTGCAAC |
| OAL588 | 3′ region downstream of H2-T6SS promoter | ACTAGTTCGAGGAGGCGGAC |
| OAL589 | 5′ region outside H2-T6SS promoter | CTTCGACGGCCGAC |
| OAL590 | 3′ region outside H2-T6SS promoter | CTGCGTGGGGG |
| OAL591 | pBAD promoter screening primer | CGCGTAACAAAAAGTGT |
| Cloning of V5-His6 region | V5-His6 coding region | GCTAGGCCCATTCGAGTCTGAGGTAGCTAT |
| OAL599 | 5′ | GCTAGGCCCATTCGAGTCTGAGGTAGCTAT |
| OAL600 | 3′ | GCTAGGCCCATTCGAGTCTGAGGTAGCTAT |
| Mutation of vgrG14 stop codon | vgrG14 stop codon | CACCAAGCACGAC |
| OAL602 | 5′ region upstream of vgrG14 stop codon | TCTCTTGATTCCTGAGGAGTGAGTCG |
| OAL597 | 3′ region upstream of vgrG14 stop codon | CAAAAGCTGAACGCGCCTTTCGAGGAC |
| OAL598 | 5′ region downstream of vgrG14 stop codon | GCTACAACGAGCCGATCCAGAG |
| OAL608 | 5′ region outside vgrG14 stop codon | GCAGGTTGCTGAGGTAGCTAAG |
| OAL609 | 3′ region outside vgrG14 stop codon | CTGCGTGGGGG |
| Mutation of rhsP2 stop codon | rhsP2 stop codon | AATAAAAACTGGGTCTCCGAGGAGAAGTTG |
| OAL1136 | 5′ region upstream of rhsP2 stop codon | CATGATCTCTGCTTCTGGGAGTACATGTAGTAC |
| OAL1137 | 3′ region upstream of rhsP2 stop codon | TAGGTTCTTGTGTTG |
| OAL1138 | 5′ region downstream of rhsP2 stop codon | GAGAATAGAAGACCGCCTACTAGGAAAGAAT |
| OAL1139 | 3′ region downstream of rhsP2 stop codon | TTATAACTTGAACAG |
| OAL1160 | 5′ region outside rhsP2 stop codon | CCGAGGTACCTAGCCGGC |
| OAL1161 | 3′ region outside rhsP2 stop codon | GATGCGTGAACCTTCTGAGGAG |
| hsiB2 cloning into pET28a | hsiB2 | GCCATATGCGCAAAAGAAGGCCGCTCGAGTACC |
| OAL1171 | 5′ | GCCATATGCGCAAAAGAAGGCCGCTCGAGTACC |
| OAL1172 | 3′ | GCCATATGCGCAAAAGAAGGCCGCTCGAGTACC |
| Deletion of pscC | pscC | ACCCAACTGTCGTTGGCGCAGGAC |
| OAL1389 | 5′ region upstream of pscC | CTAAATTCCCGCGCGCATAGGGCGC |
| OAL1399 | 3′ region upstream of pscC | ATGCGGCCGCGGAAAATCTAGGAGAAGAAGCT |
| OAL1400 | 5′ region downstream of pscC | TAGGCGGCCACCTGGCG |
| OAL1401 | 3′ region downstream of pscC | GAGGTTGCTGAGGTAGCTAAG |
| OAL1484 | 5′ region outside pscC | CTTCGACGGCCGAC |
| OAL1485 | 3′ region outside pscC | CTTCGACGGCCGAC |
| Deletion of H2-T6SS (mid-hsiA2 to mid-clpV2) | H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| OAL986 | 5′ region upstream of H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| OAL997 | 3′ region upstream of H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| OAL998 | 5′ region downstream of H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| OAL999 | 3′ region downstream of H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1000 | 3′ region outside H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1001 | 5′ region outside H2-T6SS | GCTAGTCCAGAAGCTCGAGTGC |
| Deletion of vgrG14-rhsP2 | vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1900 | 5′ region upstream of vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1901 | 3′ region upstream of vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1902 | 5′ region downstream of vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1903 | 3′ region downstream of vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1984 | 5′ region outside vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL1985 | 3′ region outside vgrG14-rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| Deletion of rhsP2 | rhsP2 | GCTAGTCCAGAAGCTCGAGTGC |
| OAL346 | 5′ region upstream of rhsP2 | CTCTTGAGAACCTACATGCG |
| OAL347 | 3′ region upstream of rhsP2 | CTCTTGAGAACCTACATGCG |
| OAL348 | 5′ region downstream of rhsP2 | CTCTTGAGAACCTACATGCG |
| OAL349 | 3′ region downstream of rhsP2 | CTCTTGAGAACCTACATGCG |
| OAL350 | 5′ region outside rhsP2 | CTCTTGAGAACCTACATGCG |
| OAL351 | 3′ region outside rhsP2 | CTCTTGAGAACCTACATGCG |
TABLE 3 qPCR primers used in this work

| Primer ID | Direction | Target gene | Sequence (5’–3’) |
|-----------|-----------|-------------|------------------|
| OAL920    | Forward   | stk2        | CCGCTGTTAGCATTCAGGTCT |
| OAL921    | Reverse   | stk2        | GCCGACTCTTCATAGGACA |
| OAL922    | Forward   | his2G       | GTGTTCTGGTTGTTCTGGA |
| OAL923    | Reverse   | his2G       | GTGCTCTCCTACCCGCAAC |
| OAL924    | Forward   | his2F       | ACTACCGGTTGCACGATCTC |
| OAL925    | Reverse   | his2F       | GCCGCTACGTACGATCC |
| OAL926    | Forward   | PA14_43090  | GCCGCTACGTACGATCC |
| OAL927    | Reverse   | PA14_43090  | GCCGCTACGTACGATCC |
| OAL928    | Forward   | rhsP2       | GAAAGAGCCACACATCTC |
| OAL929    | Reverse   | rhsP2       | CAGAAGTATACGAGCTTTAGT |
| OAL931    | Forward   | vgrG2       | GGGGACGATGGGCCCC |
| OAL932    | Reverse   | vgrG2       | AACGTCGGAGATCAGT |
| OAL934    | Forward   | hcp2A/B/C   | CAAGGCTGAGATCCAGT |
| OAL935    | Reverse   | hcp2A/B/C   | GTAGTCGAGATCCAGT |
| OAL936    | Forward   | vgrG14      | TCACCCGCGCCGAT |
| OAL937    | Reverse   | vgrG14      | TGTCGACTTCGCCGAG |
| OAL938    | Forward   | rhsD        | AGGCGGAGACGGTAC |
| OAL939    | Reverse   | rhsD        | GGCTAGCGAGCTTAC |
| OAL940    | Forward   | vgrG5       | GGGCAGGAGTTCAGGAA |
| OAL941    | Reverse   | vgrG5       | TCGGCAATCTCAGTGAAT |
| OAL942    | Forward   | rhsH       | GAGGCGGAGACGGTAC |
| OAL943    | Reverse   | rhsH       | CAGGTCGGAGATCAGT |
| OAL944    | Forward   | rhsG2b      | CAGGTCGGAGATCAGT |
| OAL945    | Reverse   | rhsG2b      | GAGGCGGAGACGGTAC |
| OAL946    | Forward   | pelA        | CTCAGTCGGGAGCTTC |
| OAL947    | Reverse   | pelA        | TCGGCAATCGAGCTTAC |
| OAL948    | Forward   | vgrG5       | GGGCAGGAGTTCAGGAA |
| OAL949    | Reverse   | vgrG5       | TCGGCAATCTCAGTGAAT |
| OAL950    | Forward   | rhsP2       | GAAAGAGCCACACATCTC |
| OAL951    | Reverse   | rhsP2       | CAGAAGTATACGAGCTTTAGT |

Gentamicin protection assay and P. aeruginosa internalization in HeLa cells. P. aeruginosa internalization was assessed using the gentamicin protection assay as previously described (30), with the exception that antibiotic treatment of extracellular bacteria was performed for 75 min. The PA14-DP ΔpssC strain was grown in the presence of 2% arabinose. Arabinose (0.2%) was also added to HeLa cell culture medium during the course of the infection with the PA14-DP ΔpssC strain. Paired t tests were performed using Excel (Microsoft).

RESULTS

Comparison of P. aeruginosa H2-T6SS clusters. A growing number of P. aeruginosa isolates have had their genomes sequenced in the past few years. Eleven such genomes are available for comparison at www.pseudomonas.com. For each of these genomes, the three known P. aeruginosa T6SS clusters could readily be identified. Interestingly, it has been reported previously that the H2-T6SS from PAO1 is not physically linked to any hcp or vgrG genes (51). However, on the PA14 genome, an hcp gene and a vgrG gene are located next to hisA2 and transcribed in a divergent orientation (Fig. 1). Here these genes are called hcp2 (indicating that it is linked with the H2-T6SS) and vgrG14 (indicating that it has been found in PA14), respectively. These two genes appear to be organized in an operon with two other genes, namely, PA14_43090 and PA14_43100. Whereas BLAST analysis did not retrieve any homologous protein of known function for PA14_43090, PA14_43100 encodes a protein belonging to the Rhs (recombination hot spot) family (41, 52), which we named RhsP2 (Fig. 1). In addition to PA14, only two other P. aeruginosa genomes display a similar organization, namely, those from the PA39016 and NCGM2.51 strains. In the case of NCGM2.51, which is a highly multidrug-resistant strain (53), the rhsP2 gene is linked with a vgrG gene and an hcp gene as in PA14 (Fig. 1). In contrast, for P. aeruginosa PA39016, no hcp or vgrG gene was found at this location (data not shown). Finally,
two genes, identified as PA1654 and PA1655, are found upstream of hsiA2 on the PAO1 genome and encode a putative aminotransferase and glutathione S-transferase, respectively (Fig. 1). In PA14, PA39016, and NCGM2.S1, these genes are found downstream of the rhsP2-containing cluster, suggesting that this cluster has been lost in PAO1 or was acquired and inserted at this position in the above 3 strains.

Activation of H2-T6SS gene expression. Expression of the H2-T6SS cluster in PAO1 has been reported to be dependent on various regulatory elements, such as iron depletion and quorum sensing (50, 51). However, expression is rather low and not constitutive. We aimed at engineering a strain in which the expression of the H2-T6SS genes could be tightly controlled to ease the study of the system under laboratory conditions. Since the PA14 H2-T6SS gene cluster is organized in two transcriptionally divergent units, we constructed a strain with two inducible arabinose promoters (pBAD) inserted in divergent orientation within the intergenic region, yielding the PA14-DP strain, as described in Materials and Methods (Fig. 2A).

Production of the H2-T6SS machine. In order to evaluate whether arabinose-dependent induction of the H2-T6SS genes effectively resulted in protein production, antibodies against two components of the H2-T6SS system, HsiB2 and Hcp2, were generated. PA14 and the PA14-DP derivative were grown in LB medium containing increasing concentrations of arabinose, i.e., 0, 0.01, 0.1, 1, and 2%. Cell extracts were prepared and proteins separated by SDS-PAGE, followed by Western blot analysis (Fig. 2E). HsiB2 and Hcp2 could readily be detected with 0.1% arabinose induction and increased slightly at 1 and 2% concentrations of the inducer. In contrast, in the parental PA14 strain, no HsiB2 or Hcp2 bands could be detected, even in the presence of 2% arabinose.

For other T6SSs, it is proposed that upon assembly of the system, the associated Hcp protein forms a tubule-like structure that emerges at the cell surface (9, 17, 54). It was also demonstrated that the Hcp protein could be recovered in the extracellular medium and was secreted in a T6SS-dependent manner. In order to assess whether production of the H2-T6SS machinery could be induced upon arabinose addition, it was tested whether Hcp2 could be recovered in the supernatant fraction. PA14-DP was
grown in the presence of 2% arabinose to late exponential phase, and cells and supernatant were separated by centrifugation as described above. Whereas Hcp2 was largely detectable in the cell fraction, a significant proportion was also released into the medium (Fig. 3). A mutation in the H2-T6SS cluster of PA14-DP was then engineered to remove the DNA region carrying the hsiA2 gene down to the clpV2 gene, yielding PA14-DP AH2 (Fig. 1). In this case, whereas Hcp2 was still detected in the cells, the protein was absent from the supernatant fraction, indicating that its secretion is dependent on the H2-T6SS (Fig. 3).

Characterization of the H2-T6SS vgrG-like gene. The VgrG proteins have been proposed to form the puncturing device of the T6SS apparatus (21). The C-terminal end is a repeat of β-strands whose assembly into a trimer forms a needle-like structure (19, 21). This type of VgrG protein is called “canonical VgrG.” In some cases, the series of β-strands is followed by a large extension, such as with VgrG1 or VgrG3 in Vibrio cholerae, which appears to be the effector that is transported by the T6SS (21, 27, 55). This type of VgrG protein is called “evolved VgrG.” In total, there are 10 VgrG proteins encoded on the PAO1 genome (Fig. 4) (19), among which 1 could be identified as an evolved VgrG protein (PA0262 or VgrG2b) (21). All of these vgrG genes are also found in the PA14 genome (49), which thus has 11 copies, considering the additional (vgrG14) gene. Phylogenetic analysis suggests that VgrG proteins can be classified into groups/families (19). Here we predict that VgrG14 is tightly related to another VgrG, encoded by PA5266 (VgrG5), in PAO1 (Fig. 4). Both VgrG14 and VgrG5 are predicted to be canonical proteins, with no significant C-terminal extension following the stretch of β-strands constituting the gp5 domain (data not shown). Interestingly, VgrG5 is encoded within a gene cluster that does not include any other core T6SS genes but has an hcp gene (PA5267) and another downstream gene (PA5265), which encodes a protein of unknown function. Genes located downstream of vgrG genes, even though not clustered with other T6SS genes, have been predicted to encode potential T6SS substrates (36). For example, this is the case for PA3487, encoding a phospholipase named Tle5 (called VgrG4b here) (37), which is located next to PA3486. VgrG4b is also phylogenetically related to VgrG14, whereas the canonical VgrG1a, VgrG1b, and VgrG1c proteins, which are connected to the H1-T6SS, are more distant (19) (Fig. 4). We thus predict that VgrG14 and RhsP2 could be potential substrates for the H2-T6SS.

Analysis of VgrG14 and RhsP2 secretion. As for the Hcp proteins, VgrG proteins are released into the extracellular medium in a T6SS-dependent manner (19, 21). The fate of VgrG14 was assessed by engineering, in PA14-DP, a chromosomal vgrG14 gene encoding a V5-tagged version of the protein as described in Materials and Methods. Upon addition of arabinose, expression of the H2-T6SS genes was induced, as seen by the production of Hcp2 (Fig. 5A to C). The fate of VgrG14 was then followed by Western blotting using an anti-V5 antibody. Whereas Hcp2 clearly appeared in the supernatant, VgrG14 was seen only in the cell fraction, indicating that it was not secreted in detectable amounts (Fig. 5A). Note that production of both Hcp2 and VgrG14 was strictly dependent on addition of arabinose to the growth medium.

Only a few effectors have been characterized for T6SSs that have been studied so far, and these are mainly bacterial toxins (33). However, it was recently reported that a P. aeruginosa strain produces an RhsT protein associated with virulence and the inflammatory response, although its secretion was not clearly analyzed (36). We therefore investigated whether RhsP2 could be an H2-T6SS effector. Following a strategy similar to the one described for VgrG14, a PA14-DP strain encoding a V5-tagged version of RhsP2 was engineered. Whereas the tagged protein could readily be detected in the cell fraction upon addition of arabinose, no RhsP2 could be seen in the extracellular medium (Fig. 5B).

It has been described previously that the H1-T6SS can be assembled while not actively secreting substrate (37). The activation of the system relies on the antagonistic activity of two proteins: a serine-threonine kinase (Stk) and a serine-threonine phosphatase (Stp). The kinase promotes activity via phosphorylation of the Fha protein, whereas Stp inhibits the activity of the system by dephosphorylating Fha. As for the H1-T6SS, the H2-T6SS cluster carries fha, stk, and stp genes (Fig. 1), which are called fha2, stk2, and stp2 here to acknowledge their link with the H2-T6SS cluster. In order to investigate whether the H2-T6SS can be made secretion competent, an stp2 deletion was engineered into the PA14-DP strain. We confirmed that a mutation in stp2 did not affect the secretion of Hcp2 (Fig. 5C). However, in this case, secretion of V5-tagged VgrG14 or RhsP2 was also not detectable (Fig. 5D and E).
Does H2-T6SS induction influence internalization in nonphagocytic cells? Although neither Vrg14 nor RhsP2 could clearly be identified as an H2-T6SS substrate, we investigated whether the induction of the entire H2-T6SS in P. aeruginosa PA14 resulted in an observable phenotype, such as previously reported for PAO1 (50). In that case, it was shown that the H2-T6SS influences internalization in nonphagocytic epithelial cells. In order to investigate this aspect in PA14, a deletion of the pscC gene was engineered to inactivate the T3SS (PA14 ΔpscC). The existence of the T3SS effector ExsE, encoded on the pathogenicity island PAPI-1 of strain PA14 (40), renders this strain highly cytotoxic. We checked that the pscC mutation inactivated the T3SS and the extracellular release of PcrV (58), the T3SS tip component. Bacteria were grown under Ca²⁺-chelating conditions, and cells and supernatant were then separated by centrifugation and analyzed by Western blotting using anti-PcrV. Our data showed that in the pscC mutant, PcrV was no longer detectable in the supernatant (see Fig. S1A in the supplemental material). A cytotoxicity assay was also performed using RAW macrophages and monitoring LDH release. Upon introduction of the pscC mutation, PA14 cytotoxicity was totally abrogated, and no contribution of the H2-T6SS could be seen, since introduction of the sole H2-T6SS mutation did not alter the cytotoxic profile (see Fig. S1B).

A gentamicin protection assay was then performed as described previously, by monitoring the number of bacteria internalized in HeLa cells via counting the number of CFU. Using the PAO1 and PAO1 ΔpdrV2 strains, we confirmed previously published data (50) and showed that the H2-T6SS mutant had reduced (about 2-fold) internalization capability (Fig. 6A). We also engineered a mutant lacking most of the H2-T6SS gene cluster, PAO1 ΔH2-T6SS, and similar results were obtained (Fig. 6A). We then compared the internalization phenotypes of the PA14 ΔpscC strain and the H2-T6SS mutant derivative PA14 ΔpscC ΔH2-T6SS. In this case, and in contrast with the PAO1 situation, an increased level of internalization was observed in the absence of the H2-T6SS cluster (Fig. 6B), suggesting that the H2-T6SS prevents internalization. In this genetic context, there is no arabinose-inducible promoter, and the phenotype observed in PA14 may simply account for the induction of the H2-T6SS genes upon contact with epithelial cells. This is supported by the observation that with the PA14Δ-DP Δpsc strain, in which the original promoter was replaced by the pBAD promoter, the internalization level was similar to that observed with the H2-T6SS mutant PA14 ΔpscC ΔH2-T6SS. It suggests that, in PA14, the original promoter is needed to activate H2-T6SS genes when bacteria are in contact with epithelial cells and thus to prevent internalization in an H2-T6SS-dependent manner. Moreover, with PA14-DP Δpsc, addition of arabinose drastically reduced the level of internalization (Fig. 6C), confirming that induction of the H2-T6SS prevents internalization. We concluded that the H2-T6SS in PA14 may have a different role in bacterium-host interaction and that this may be
due to the use of a different panel of effectors, as suggested by the difference in H2-T6SS cluster organization observed between the PAO1 and PA14 strains. Interestingly, when we used a mutant deleted for the vgrG14 and rhsP2 genes in the divergent transcriptional unit (PA14 ΔpscC ΔvgrG14-rhsP2) instead of using the H2-T6SS mutant (PA14 ΔpscC ΔH2-T6SS), a similar internalization phenotype was observed (Fig. 6B), thus supporting the hypothesis that the observed H2-T6SS-dependent phenotype is also VgrG/Rhs dependent.

DISCUSSION

Previous studies have shown that the H2-T6SS from *P. aeruginosa* PAO1 can be induced under low-iron conditions or during the growth transition from planktonic to stationary growth, in a quorum sensing-dependent manner (50, 51). In the present study, we used another strain, PA14, and engineered a pBAD promoter into the chromosome, which resulted in tightly controllable expression of the H2-T6SS genes. The addition of arabinose resulted not only in transcriptional upregulation of the gene cluster but also in production of T6SS components, as seen with HsiB2.

The lack of hcp and vgrG genes linked to the PAO1 H2-T6SS genes is puzzling and suggests that independent clusters could be associated with the H2-T6SS to provide the missing hcp and vgrG genes, which are described as essential for T6SS function. In this respect, several reports have proposed that the vgrG2a and vgrG2b genes, both linked to an hcp gene, could potentially be associated with the H2 system in PAO1 (19, 36), though no data have yet supported this hypothesis. In contrast, analysis of the PA14 genome revealed that next to the genes encoding the H2-T6SS core components, and transcribed divergently, a cluster of 4 genes is found and encodes an Hcp protein (Hcp2), a VgrG protein (VgrG14), and two other proteins, one of which (PA14_43100 protein) is a putative Rhs protein (RhsP2). Interestingly, the hcp gene encodes a protein which is 100% identical to all other Hcp proteins encoded from the so-called vgrG islands (19), which are not linked to a T6SS cluster (36, 37, 55). The sequence conservation with T6SS-linked Hcp proteins, such as Hcp1, is weaker. However, the crystal structure of an Hcp protein of this subfamily shows a similar hexameric structure and only a slightly different orientation in the Hcp ring-containing nanotubes, which are stacked head to head instead of head to tail (54). Our study showed that artificial but simultaneous induction of the two gene clusters in PA14 resulted in the H2-T6SS-dependent secretion of Hcp2. This observation demonstrates that the two sets of genes are functionally linked and that VgrG14 and RhsP2 are obvious candidate H2-T6SS substrates.

VgrG proteins are definitely part of the T6SS machinery, but in some cases they have been shown to have dual functions, also acting as effectors. This is particularly well described in the case of VgrG1 and VgrG3 from *Vibrio cholerae* (21, 27, 55). VgrG1 has a C-terminal extension, described as an actin-cross-linking domain (31), which is used to impair the phagocytic activity of macrophages (27). More recently, VgrG3 was shown to carry a C-terminal extension which may act as a bacterial toxin using its hydrolase activity against the peptidoglycan (55). These two VgrG proteins are called evolved proteins, and only the *Vibrio cholerae* VgrG2 protein is canonical, i.e., it is restricted to the structure of the puncturing device of the bacteriophage (18), with no catalytic domain extension at the C terminus. In *P. aeruginosa* PAO1, there are 10 VgrG proteins, of which only VgrG2b could be predicted to have a C-terminal extension (19, 21). In PA14, the 11th VgrG protein, VgrG14, has no clear C-terminal extension, suggesting that it might not be an H2-T6SS effector *per se*. However, canonical VgrG proteins are also recovered in the culture supernatants of bacteria with active T6SS (19, 29, 59). We introduced a V5 tag at the VgrG14 C terminus to follow its fate by Western blotting, but we could not detect it in the supernatant. The lack of observed secretion can be discussed in various ways. The amount of VgrG14 protein detected in the cells was not very high, and previous studies have shown that the percentage of secreted VgrG proteins is rather low (19, 29). VgrG14 secretion could be undetected in this case. The addition of a C-terminal tag may have interfered with the puncturing device function, and therefore with H2-T6SS function. However, the observed Hcp2 secretion does not favor this hypothesis. Finally, additional genes outside the two arabinose-controlled clusters are required for VgrG secretion and were likely not expressed under our growth conditions.

Within the vgrG islands (36), additional genes could be found and happened to encode T6SS substrates. The observation that the gene downstream of *P. aeruginosa* vgrG4b, now called tle5, encodes a T6SS-dependent phospholipase with antibacterial activity is one such noteworthy example. The gene immediately downstream of vgrG14 does not display any recognizable features. However, the one next to it encodes a protein of the Rhs family (41, 52), whose role and function have yet to be understood. Interestingly, a recent study identified one such protein, called RhsT, in the *P. aeruginosa* isolate PSE9 (56). RhsT can be translocated into *J. J74* macrophage cells and kills them by influencing inflammasome signaling. However, it is not known whether RhsT is a substrate of the T6SS or any other known secretion systems. Another recent report described XadM from the plant pathogen *Xanthomonas oryzae* as a cell surface protein, a member of the Rhs family, and required for attachment to host cells, biofilm formation, and global virulence (60). We thus added a V5 tag at the RhsP2 C terminus and followed its secretion fate by Western blotting. Secretion could not be detected, but as discussed for VgrG14, several reasons may have prevented the detection of secreted RhsP2. It is interesting that in *P. aeruginosa* strain 39016, the H2-T6SS cluster does not include any vgrG or hcp genes but is associated with the rhs gene. If one considers that VgrG and Hcp are core components of the T6SS machine and that Rhs is a putative substrate, its secretion might require another vgrG/hcp subset localized elsewhere on the 39016 chromosome.

The *P. aeruginosa* PAO1 H2-T6SS has been shown to mediate bacterial internalization into nonphagocytic eukaryotic cells (50). Because PA14 is highly cytotoxic toward eukaryotic cells (40, 49), we engineered a T3SS mutant to study the impact of the PA14 H2-T6SS on internalization. Surprisingly, we observed the opposite effect compared to that with PAO1, and we concluded that in PA14 the H2-T6SS does not promote but prevents internalization. Although it is not straightforward to reconcile these data, it is clear that the function of a secretion system is given by the nature of its substrate/effectors. For example, the pathogenesis mechanisms of PAO1 and PA14 are entirely different, since one strain, PAO1, is considered invasive and the other, PA14, is cytotoxic. Both have a T3SS, but the presence of an additional effector, ExoU (61), encoded on a pathogenicity island of PA14 (40), contributes to a change in lifestyle.

Previous observations suggesting that the H2-T6SS affects virulence (51), internalization (50), and bacterial killing (37) are...
somehow puzzling and reflect the pleiotropic role of this system, and likely a broad set of associated effectors. The discovery that the phospholipase Tle5, an effector linked with the H2-T6SS (37), is involved in bacterial killing gives further support to this hypothesis. Whereas it has still to be determined whether RhsP2 is an H2-T6SS substrate, the genetic difference between the H2-T6SS gene clusters of PA01 and PA14 and the association of an rhs-like gene might account for a distinct phenotypic contribution to pathogenesis. Further analysis of the role and function of RhsP2 and the identification of the whole set of H2-T6SS-dependent effectors by secrecome analysis are needed to obtain a comprehensive vision of H2-T6SS function and to shed more light on the multiple and central roles of T6SSs in bacterial pathogenesis.

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