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Journal

Journal of Biological Chemistry, 287(32)

ISSN

0021-9258

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Publication Date

2012-08-01

DOI

10.1074/jbc.m112.376368

Peer reviewed

The Second Type VI Secretion System of *Pseudomonas aeruginosa* Strain PAO1 Is Regulated by Quorum Sensing and Fur and Modulates Internalization in Epithelial Cells^{*[5]}

Received for publication, April 26, 2012, and in revised form, June 1, 2012. Published, JBC Papers in Press, June 4, 2012, DOI 10.1074/jbc.M112.376368

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Background: Three T6SSs are present in *P. aeruginosa*. H1-T6SS secretes bacteriolytic toxins.

Results: H2-T6SS is regulated by quorum sensing and Fur and modulates internalization in epithelial cells through PI3K-Akt host pathway activation.

Conclusion: H2-T6SS plays a role in virulence.

Significance: In contrast to the anti-prokaryotic H1-T6SS, H2-T6SS targets human cells. Those T6SSs can carry out different functions important in establishing infection.

The genome of *Pseudomonas aeruginosa* PAO1 contains three type VI secretion systems (T6SSs) called H1-, H2-, and H3-T6SS. The H1-T6SS secretes three identified toxins that target other bacteria, providing a fitness advantage for *P. aeruginosa*, and likely contributes to bacterial pathogenesis in chronic infections. However, no specific substrates or defined roles have been described for the two other systems. Here, we demonstrate that the expression of H2-T6SS genes of strain PAO1 is up-regulated during the transition from exponential to stationary phase growth and regulated by the Las and Rhl quorum sensing systems. In addition, we identify two putative Fur boxes in the promoter region and find that H2-T6SS transcription is negatively regulated by iron. We also show that the H2-T6SS system enhances bacterial uptake into HeLa cells (75% decrease in internalization with a H2-T6SS mutant) and into lung epithelial cells through a phosphatidylinositol 3-kinase-dependent pathway that induces Akt activation in the host cell (50% decrease in Akt phosphorylation). Finally, we show that H2-T6SS plays a role in *P. aeruginosa* virulence in the worm model. Thus, in contrast to H1-T6SS, H2-T6SS modulates interaction with eukary-

otic host cells. Together, T6SS can carry out different functions that may be important in establishing chronic *P. aeruginosa* infections in the human host.

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections in immunocompromised humans and is responsible for chronic respiratory disease of patients with cystic fibrosis. A key factor in *P. aeruginosa* virulence is its capacity to secrete a wide range of hydrolytic enzymes and toxins to the extracellular medium as well as to inject virulence effectors directly into host cells (1, 2). Recently, a new secretion pathway, called the type VI secretion system (T6SS),⁴ that resembles an inverted phage tail has been described among Gram-negative bacteria, including *P. aeruginosa* (3–6). Substrates for this secretion system were first discovered in *Vibrio cholerae* by Pukatzki *et al.* (7), who demonstrated that secretion of proteins lacking a signal peptide, such as Hcp (hemolysin co-regulated protein) and VgrG (valine-glycine repeat), requires a functional T6SS. Gene clusters encoding T6SS machinery and putative substrates have been found in diverse animal and plant pathogens (8–10). Many bacteria encode more than one T6SS; in the case of *P. aeruginosa*, three T6SS, H1-T6SS to H3-T6SS, have been described. There is emerging evidence that secreted T6SS effectors can target either eukaryotic host cells or other bacteria (9, 10), and various T6SS within a single strain may serve different functions and/or be differentially regulated. *V. cholerae* VgrG1, which is homologous to a phage tail protein, is thought to function both as part of the secretion system and as a translocated effector (11). It encodes a C-terminal extension that can cross-link actin *in vitro* and *in vivo* and has been shown to be translocated into eukaryotic cells by a T6SS-dependent mechanism, where it is associated with cytotoxicity and virulence

* This work was supported, in whole or in part, by EuroPathoGenomics REX Grant LSHB-CT-2005512061-EPG), "Pathomics" ERA-net PATHO Grant ANR-08-PATH-004-01. This work was also supported by funds from the Royal Society Wellcome Trust Grant WT097939 (to A. F.), National Institutes of Health Grants P01 AI053194 and R01 AI065902 (to J. E.), a Ph.D. fellowship from the French Research Ministry (to T. G. S.), a post-doctoral fellowship from the French Research Minister and from EuroPathoGenomics network of excellence (to A. H.), and a Elizabeth Nash Memorial Fellowship from Cystic Fibrosis Research Inc. (to I. B.).

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[5] This article contains supplemental Figs. S1–S3.

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⁴ The abbreviations used are: T6SS, type VI secretion system; T3SS, type III secretion system; QS, quorum sensing; MEM, Eagle's minimal essential medium; C₄-HSL, N-(butanoyl)-L-HSL; LY, LY294002; TSB, Tryptic Soy Broth.

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(12, 13). Similarly, the VgrG1 protein of *Aeromonas hydrophila* contains a VIP-2 (vegetative insecticidal protein domain 2) domain with an ADP-ribosyltransferase activity capable of modifying actin *in vivo* and is targeted to the host cytosol upon infection (14).

Interestingly, gene loci encoding T6SSs are also found in nonpathogenic bacteria and symbionts, suggesting that T6SS may not be dedicated exclusively to pathogenic interactions of bacteria with eukaryotes (9, 10). Indeed, T6SS can be potent mediators of interbacterial interactions as discovered in *P. aeruginosa*, where the type VI exported effectors Tse1–3 are bacteriolytic (15, 16) and may give *P. aeruginosa* a survival benefit in a multi-bacterial environment, in biofilms, and in polymicrobial infections, such as those encountered in cystic fibrosis patient airways (3, 17, 18).

Despite the increasing information available on the H1-T6SS, very little is known about the two other T6SSs encoded by *P. aeruginosa*. The H2-T6SS locus of the PA14 strain is required for virulence in the plant model *Arabidopsis thaliana* and in the mouse model of acute infections (19). Although the H1-T6SS loci are very similar between the *P. aeruginosa* strains, PA14 and PAO1, their H2-T6SS loci differ; two putative structural components that are also exported, *hcp2* and *vgrG2*, are present in PA14 but missing in PAO1. They constitute, together with two other genes, a divergent operon to the H2-T6SS secretion machinery operon. Indeed the H2-T6SS of strain PAO1 may lack the extracytoplasmic part of the T6SS contractile phage tail-like structure that is thought to punctuate the outer membrane and host cell membrane (5). One could even ask whether the PAO1 H2-T6SS machinery can be functional. Here, we examined the regulation and function of H2-T6SS in PAO1. We find that it is subject to regulation by quorum sensing (QS) and by iron. In addition, we provide evidence that the H2 secretion machinery promotes bacterial internalization into epithelial cells, suggesting that this T6SS targets eukaryotic cells. Finally, we show that H2-T6SS plays a role in *P. aeruginosa* virulence in the worm model.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are described in Table 1. LB and TSB broths and agar were used for the growth of *P. aeruginosa* and *Escherichia coli* strains at 37 °C. The cultures were inoculated at A_{600} of 0.1 with overnight cultures, and strains were grown at 37 °C with aeration in LB or TSB. Recombinant plasmids were introduced in *P. aeruginosa* using the conjugative properties of pRK2013 (see Table 1). *P. aeruginosa* transconjugants were selected on *Pseudomonas* isolation agar (Difco Laboratories) supplemented with appropriate antibiotics. The antibiotics concentrations were as follows: for *E. coli*, ampicillin (50 $\mu\text{g ml}^{-1}$), kanamycin (25 $\mu\text{g ml}^{-1}$), tetracycline (15 $\mu\text{g ml}^{-1}$), and chloramphenicol (50 $\mu\text{g ml}^{-1}$); and for *P. aeruginosa*, carbenicillin (500 $\mu\text{g ml}^{-1}$) and tetracycline (200 $\mu\text{g ml}^{-1}$ for plates or 50 $\mu\text{g ml}^{-1}$ for liquid growth).

Construction of the *clpV2* Mutant—To generate *clpV2* deletion, 500 bp upstream and 500 bp downstream of the *clpV2* gene were amplified by overlapping PCR with High Fidelity DNA polymerase (Roche Applied Science) using TSO49,

TSO50, TSO51, and TSO52 primers (see Table 1) (20). The PCR product was cloned in pCR2.1 (TA cloning kit; Invitrogen) giving pTS24, which was then sequenced (GATC) and subcloned in pKNG101 suicide vector giving the mutator pTS27. pTS27, maintained in the *E. coli* CC118 λ pir strain, was mobilized in the wild type *P. aeruginosa* strain PAO1. The mutants, in which the double recombination events occurred that resulted in the nonpolar deletion of *clpV2* gene, were verified by PCR using external primers OA14–OA17.

***lacZ* Reporter Fusion and β -Galactosidase Assay**—The H2-T6SS-*lacZ* transcriptional fusions were constructed by PCR amplification of the 722-bp upstream DNA region from the *tssA2* gene by using TSO1 and TSO3 primers (see Table 1). PCR amplification products were directly cloned into the pMini-CTX::lacZ vector (21), yielding pTS2, or into pCR2.1 and pMP220, yielding pTS28 and pTS29, respectively. Nucleotide sequence was verified (GATC). The promoter fragment was integrated at the CTX phage attachment site in PAO1 and isogenic mutants using established protocols (21). The pTS29 was transferred into *E. coli* by conjugation.

For β -galactosidase assay, overnight bacterial culture was diluted in TSB to an A_{600} of 0.1. Growth and β -galactosidase activity were monitored by harvesting samples at different time intervals. β -Galactosidase activity was measured according to the Miller method, based on *o*-nitrophenyl- β -D-galactopyranoside hydrolysis (22), and expressed in Miller units.

RNA Extraction and Determination of the Transcription Start—PAO1 was grown in TSB at 37 °C for 5 h, and total RNA was extracted using the PureYield RNA Midiprep system (Promega). The MICROBExpress kit was used to purify mRNA (Ambion). 5' RNA ligase-mediated rapid amplification of 5' cDNA ends was performed using the GeneRacer kit (Invitrogen), starting at the TAP step on 1 μg of mRNA. Reverse transcription with Superscript III RT was performed with random oligonucleotides. TSO37, TSO38, GeneRacer 5' Nested Primer, and GeneRacer 5' Primer (see Table 1) were used for PCR amplification (high fidelity expand system; Roche Applied Science) of the first strand cDNA. The PCR product was cloned into the PCR2.1 vector using the TA cloning kit (Invitrogen) and transformed in TOP10F' competent cells. Cloning was screened using the M13 forward primer and M13 reverse primer, and the cloned products were sequenced using the M13 forward primer (GATC).

HeLa Cell Invasion, Survival, and Adhesion Assays—For invasion assay, HeLa cells (ATCC, Manassas, VA) were cultured to 70% confluence at the day of the experiment in 24-well plates in Eagle's minimal essential medium (MEM) supplemented with 10% FBS. The cells were washed twice with sterile PBS and infected with *P. aeruginosa* wild type strain and the *clpV2* mutant, grown as indicated in the text (log, transition, or stationary phase) at a multiplicity of infection of 10 in MEM with FBS at final volume of 1 ml. The plates were spun down at 1000 $\times g$ for 5 min to allow bacteria close contact with HeLa cells. After 3 h of infection at 37 °C in 5% CO₂, the cells were washed twice with sterile PBS and incubated with MEM supplemented with 300 $\mu\text{g ml}^{-1}$ of gentamicin (Sigma) for 2 h to kill extracellular bacteria. Afterward, the cells were washed three times with room temperature PBS and osmotically

shocked with ice-cold sterile water containing 0.05% Triton X-100 (Sigma) for 30 min on ice. Lysed cells were collected in Eppendorf tubes, and serial dilutions in sterile LB were plated onto *Pseudomonas* isolation agar plates. Colony-forming units were enumerated and reported as internalized bacteria.

For survival assay, HeLa cells were infected for 1.5 h and treated for 1 or 4 h with gentamicin $300 \mu\text{g ml}^{-1}$ to allow 3 h more for intracellular replication. For adhesion assays, infected cells were not incubated with gentamicin.

Calu-3 Cell Invasion Assay—Calu-3 cells, obtained from the ATCC, were maintained in MEM supplemented with 10% FBS (Invitrogen) and L-glutamate at 37°C with 5% CO_2 . For experiments, the cells were grown on 12-mm Transwell filters (0.4- μm pore size; Corning) for 2 days (1×10^6 cells/well). Calu-3 cells grown on Transwell filters were washed in serum-free MEM and preincubated with LY (Sigma-Aldrich) in serum-free MEM supplemented with L-glutamine for 1 h. Transition phase grown bacteria were added to the apical chamber of the Transwell filter for 3 h at a multiplicity of infection of 10. After incubation, the cells were washed with serum-free MEM and incubated with serum-free MEM containing $250 \mu\text{g ml}^{-1}$ gentamicin (Fisher) for 2.5 h. The cells were washed twice in PBS to remove antibiotic and lysed in 1 ml Ca^{2+} Mg^{2+} -free PBS with 0.25% Triton X-100 (Sigma-Aldrich) for 30 min. The cells were scraped off the Transwell filters, and the cell lysates were transferred to new tubes. Internalized bacteria were enumerated by plating serial dilutions of cell lysates to LB plates and counting colony-forming units. All of the assays were carried out on triplicate wells, and the results are reported as the average of three experiments \pm S.D. (standard deviation).

Akt Immunoprecipitation—Calu-3 grown on Transwell filters were washed and placed in serum-free MEM supplemented with L-glutamate for 12 h. Transition phase grown bacteria were added for 1 h at a multiplicity of infection of 100. The infected monolayers were washed with cold PBS containing 1 mM sodium orthovanadate (Sigma-Aldrich). The cells were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 50 mM NaF, 0.1 mM okadaic acid (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and proteinase inhibitor tablets (Complete; Roche Applied Science)) for 30 min. The cells were scraped off the Transwell filters, and the cell lysates were centrifuged at $16,000 \times g$ for 20 min. To preclear the cell lysate, the supernatant was mixed with 20 μl of protein G-Sepharose (4 Fast Flow; Amersham Biosciences), and the protein content was determined using protein assay reagent (bicinchoninic acid; Pierce). The cleared lysate (300–400 μg of protein) was incubated with polyclonal Akt antibody (Cell Signaling Technology) overnight at 4°C and incubated for 2 h with protein G-Sepharose. The immune complexes were washed three times with modified radioimmunoprecipitation assay buffer without deoxycholate, eluted in SDS sample buffer, electrophoresed on 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS containing 0.05% Tween 20 (PBST) and 5% nonfat milk for 1 h at room temper-

ature and then incubated overnight at 4°C with a monoclonal antibody that recognizes Akt phosphorylated on serine 473 (Cell Signaling Technology). The membranes were washed with PBST, incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature, and developed using an enhanced chemiluminescence kit (Amersham Biosciences). The membranes were then stripped and reprobed with a monoclonal antibody that recognizes all forms of Akt (Cell Signaling Technology). Primary antibodies were diluted 1:1000 and secondary antibodies were diluted 1:3000.

Caenorhabditis elegans Killing Assay—The slow killing assay was performed as described previously with modifications based on Ref. 23. Each independent assay consisted of three replicates. *E. coli* OP50 was used as a control. L4 to adult stage *C. elegans* were removed from food and placed on unseeded NGM plates for 24 h at 25°C . 50 worms were then picked onto plates containing overnight growth of each bacterial strain. The worms were evaluated for viability on a daily basis. The assay was performed three times. Animal survival was plotted using the PRISM 5.0 program. Survival curves were considered significantly different from the control when p values were <0.05 . PRISM calculated survival fractions using the product limit (Kaplan-Meier) method and compared survival curves by two methods: the log-rank test (also called the Mantel-Cox test) and the Gehan-Breslow-Wilcoxon test.

Statistical Analysis—Paired Student's t tests were performed using Excel software. In the figures, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

RESULTS

The Expression of the H2-T6SS Genes Is Controlled by Quorum Sensing—Although the H1-T6SS cluster is repressed by RetS/Gac signaling system in PAO1, the H2-T6SS and H3-T6SS are not (3, 24–26). To probe the transcriptional regulation of the H2-T6SS gene cluster, we analyzed the DNA region upstream of the first gene, *hsiA2* (PA1656), of the cluster in PAO1 (Fig. 1A). Two σ^{70} -dependent promoters were predicted in the intergenic region between the upstream gene (PA1655) and *hsiA2* (PA1656) by the BProm program (Fig. 1B). We created a *lacZ* transcriptional fusion in which the DNA region upstream of the ATG of the first gene of the H2-T6SS cluster was fused to *lacZ* and integrated at the CTX phage *attB* site as a single copy on the chromosome, yielding strain PAO1TS2. Strain PAO1Z was similarly constructed by integrating a promoter-less *lacZ* gene to serve as a negative control (Table 1). The β -galactosidase activity profile associated with the transcriptional fusion indicated that the H2-T6SS gene cluster expression was low during the exponential phase but increased at 3 h of growth in TSB medium at 37°C , during the log to stationary phase transition (Fig. 2A). The expression was at its highest (10-fold above background) in stationary phase, whereas promoter activity was undetectable in the control strain PAO1Z.

The biphasic, cell density-dependent expression profile of the reporter fusion suggested that the transcription of H2-T6SS might be regulated by QS. This could be in agreement with the regulation by LasR of the PA14 *hcp2* gene, which lacks in PAO1

TABLE 1
Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Genotype, description, or sequence	Source and/or reference
<i>E. coli</i> strains		
CG1	<i>supE, hsdΔR, thiΔ (lac-proAB), F' (traD36, proAB+, lacIq, lacZΔM15)</i>	Laboratory collection
CC118(Δpir)	(Δpir) Δ (<i>ara-leu</i>), <i>araD, ΔlacX74, galE, galK, phoA-20, thi-1, rpsE, rpoB, Arg(Am), recA1, Rfr (Δpir)</i>	Ref. 70
TOP10F'	F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ</i>	Laboratory collection
MG1655	F ⁻ λ ⁻ <i>ilvG-, rfb-50, rph-1</i>	Laboratory collection
MG1655 <i>fur</i>	MG1655 <i>fur::kan</i>	Ref. 71
<i>P. aeruginosa</i> strains		
PAO1	Wild type, prototroph, <i>chl-2</i>	B. Holloway
PAO1Z	Promoterless <i>lacZ</i> gene integrated at ctx att site in PAO1	This work
PAO1TS2	H2-T6SS promoter integrated at ctx att site in PAO1	This work
PAOR	<i>lasR</i> mutant of PAO1, CbR	Ref. 28
PAORTS2	H2-T6SS promoter integrated at ctx att site in PAOR, CbR	This work
PDO100	<i>rhlI</i> mutant of PAO1, HgR	Ref. 29
PDO100TS2	H2-T6SS promoter integrated at ctx att site in PDO100, HgR	This work
PAO1Δ <i>clpV2</i>	<i>clpV2</i> deletion mutant	This work
PAO1:: <i>gfp</i>	miniTn7 <i>gfp</i> integrated in PAO1	This work
PAO1:: <i>gfp ΔclpV2</i>	miniTn7 <i>gfp</i> integrated in PAO1Δ <i>clpV2</i>	This work
Plasmids		
pCR2.1	TA cloning, <i>lacZα</i> , ColE1, f1 ori, ApR KmR	Invitrogen
pMini-CTX:: <i>lacZ</i>	Ω-FRT-attP-MCS, ori, int, oriT, TcR	Ref. 21
pMP220	Broad host range <i>lacZ</i> transcriptional fusion, TcR	Laboratory collection
pTS2	722-bp upstream region of H2-T6SS in pMini-CTX:: <i>lacZ</i>	This work
pTS28	722-bp upstream region of H2-T6SS in pCR2.1	This work
pTS29	722-bp upstream region of H2-T6SS from pTS28 in pMP220	This work
pRK2013	Tra+, Mob+, ColE1, KmR	Ref. 72
pRK600	Tra+, OriT, OriV, Col E1, CmR	Ref. 73
pUX-BF13	Tra+, Mob+, R6K, providing Tn7 transposition functions in <i>trans</i> , ApR	Ref. 74
miniTn7 <i>gfp</i>	mob + ori; ApR GmR; miniTn7 vector containing <i>gfpmut3</i> inserted into <i>notI</i> site	Ref. 75
pKNG101	oriR6K, mobRK2, sacBR+, SmR (suicide vector)	Ref. 76
pTS24	500 bp upstream and 500 bp downstream <i>clpV2</i> in pCR2.1	This work
pTS27	<i>clpV2</i> deletion of pTS24 in pKNG101	This work
pMMB67-42	Broad host range vector adapted for the Gateway system <i>tac</i> promoter, ApR	This work
pHM49	pMM67-42 encompassing <i>clpV2-v5his6</i>	This work
pBLasR	<i>lasR</i> gene in the pBBRMCS-2, KmR	Ref. 28
pBRhIRI	<i>rhlR</i> and <i>rhlI</i> genes in the pBBRMCS-2, KmR	Laboratory collection
Oligonucleotides		
TSO1	5'-CCTAACCCCTTCAATGCACACC-3'	This work
TSO3	5'-GTCGACCTATTACGCTCTAAATCAGC-3'	This work
TSO37	5'-TAATGCGAGGAGAGCTTGCTCG-3'	This work
TSO38	5'-ATGCCGGCTGCACAGACGTTCC-3'	This work
TSO49	5'-ACGCCACCTCACATCCATCCTTAATGAATCTTG-3'	This work
TSO50	5'-AAGGATGGATGTGAGGTGGGTGCGATGTTTCAGC-3'	This work
TSO51	5'-ACTGGAACGCCTGATGCCCTACCTGG-3'	This work
TSO52	5'-CGAACGAGCCAGCTGGCCAGTGGATGG-3'	This work
OA14	5'-GGAAAGCTTTTCGCCCTCGTCGGATTG-3'	This work
OA17	5'-AAAGAATTCGAGGCGTTGCAGCAGATG-3'	This work
GeneRacer 5' primer	5'-CGACTGGAGCACGAGGACACTGA-3'	Invitrogen
GeneRacer 5' nested primer	5'-GGACACTGACATGGACTGAAGGAGTA-3'	Invitrogen

To determine whether Fur is required for H2-T6SS regulation and because Fur seems to be essential for *P. aeruginosa* viability (40), the expression of the plasmid borne transcriptional fusion was studied in an *E. coli* WT and isogenic *fur* mutant. After 4 h of growth, the activity of the fusion increased by 2.6-fold in the Fur mutant (Fig. 2E). Together, these data suggest that transcription of the H2-T6SS gene cluster is induced under iron-limiting conditions, an environment that the pathogen encounters in the human host.

H2-T6SS Contributes to *P. aeruginosa* Internalization in Non-phagocytic Cells—Although *P. aeruginosa* is considered an extracellular pathogen, it is able to measurably enter (*i.e.*, invade) normally nonphagocytic cells ((41), for a review see Ref. 77). To address the potential role of H2-T6SS in *P. aeruginosa* internalization, we constructed a nonpolar mutant in the AAA+ ATPase ClpV2 whose homologue was shown to play a crucial role in the functionality of the H1-T6SS system (3, 42). We verified that the mutation does not affect the growth of the

strain, nor the major adhesins of *P. aeruginosa* (flagellum and type IV pili), nor the secretion of ExoS, an anti-internalization factor (supplemental Fig. S1). Moreover, adhesion of the *clpV2* mutant was unaffected (supplemental Fig. S2, A and B). We then compared invasion of transition phase grown wild type PAO1 with a mutant defective in H2-T6SS (PAO1Δ*clpV2*), using a standard antibiotic protection assay adapted from Ref. 43. As shown in Fig. 3A, PAO1 was able to invade nonphagocytic cells, with an average of 1.7% of adherent bacteria internalized (supplemental Fig. S2C), whereas PAO1Δ*clpV2* entry into host cells was reduced by ~75%. Invasion was restored to wild type levels in the complemented *clpV2* mutant (Fig. 3A).

Because we previously observed a cell density expression pattern of H2-T6SS genes, we asked whether the growth phase of the bacteria impacts the internalization capacity. Although exponential grown *clpV2* mutant bacteria were less internalized (~75% reduction as with transition phase

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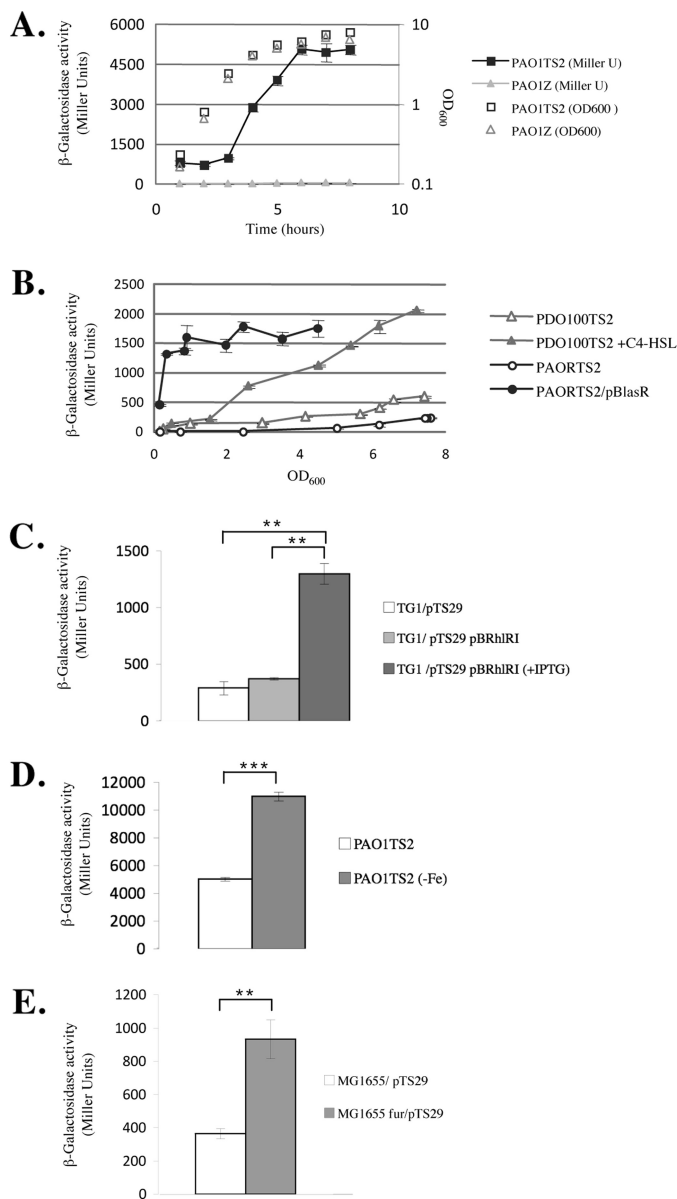


FIGURE 2. The H2-T6SS gene cluster of *P. aeruginosa* PAO1 is induced upon iron starvation and regulated by QS. A, the expression pattern of the H2-T6SS-*lacZ* transcriptional fusion from the wild type PAO1 strain (PAO1TS2) (squares) and a control strain (PAO1Z) (triangles) is given in Miller units at different time points over the growth period. The A_{600} is also presented. B, expression of the H2-T6SS-*lacZ* transcriptional fusion from the PAO1 *lasR* strain (PAORTS2) with (filled circles) or without (open circles) the pBlasR plasmid, and from the PAO1 *rhlI* strain (PDO100TS2) grown in the presence (filled triangles) or absence (open triangles) of $10 \mu\text{M}$ C₄-HSL. PAORTS2 pBlasR curve is significantly different from PAORTS2 ($p \leq 0.001$), with the last five points of the PDO100TS2 + C₄-HSL from PDO100TS2 ($p \leq 0.001$). C, expression of the H2-T6SS-*lacZ* transcriptional fusion from the pTS29 plasmid in the *E. coli* TG1 with (gray bars) or without (white bar) the pBRhIRI plasmid. Expression of *rhlR* and *rhlI* gene induced in the presence of 0.5 mM IPTG (dark gray bar) or suppressed in the absence of IPTG (light gray bar). D, expression of the H2-T6SS-*lacZ* transcriptional fusion from the PAO1 strain (PAO1TS2) grown in high (white bar) or low iron (gray bar). E, expression of the H2-T6SS-*lacZ* transcriptional fusion from the pTS29 plasmid in the *E. coli* MG1655 (white bar) and its isogenic *fur* mutant (gray bar). Each experiment was done in duplicate and independently repeated three times; the error bars indicate standard deviations, and the asterisks indicate p values.

bacteria; data not shown), stationary phase bacteria were internalized at the same level as the wild type strain (supplemental Fig. S3).

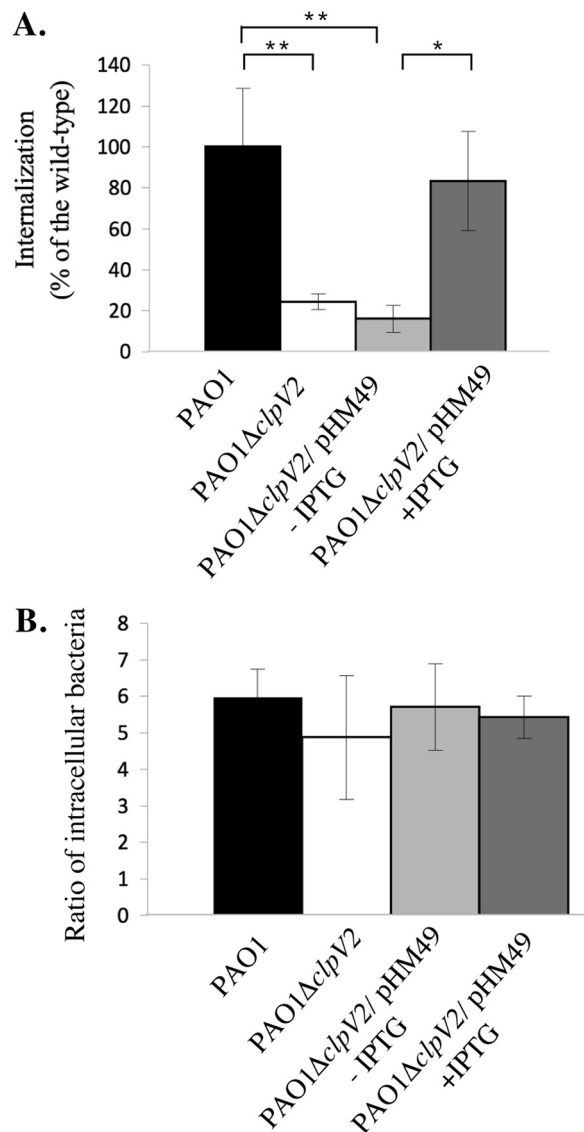


FIGURE 3. Maximal uptake of *P. aeruginosa* by HeLa cells requires the H2-T6SS apparatus. Standard bacterial invasion (A) or survival (B) assays in HeLa cells upon infection with *P. aeruginosa* strains grown at the transition phase in TSB. A, the percent invasion of PAO1 Δ clpV2 with (gray bars) or without (white bars) pHM49, normalized to PAO1 (black bars) that represents an average of 1.3×10^5 colony-forming units of internalized bacteria/well (2.5×10^5 HeLa cells). ClpV2_{V5/6His} expression is induced with 1 mM with IPTG (dark gray bars) or remains suppressed in the absence of IPTG (light gray bars). B, the ratio between the numbers of intracellular bacteria recovered after 5.5 and 2.5 h of infection (including the gentamicin treatment). Same strain color code than in A. All of the assays were performed a minimum of three times in triplicate. The error bars represent standard deviations, and the asterisks indicate p values.

Even if *P. aeruginosa* escapes the normal bactericidal mechanisms of epithelial cells, it only poorly replicates in A549 pneumocytes (41) or in corneal epithelial cells (43). Given the length of the infection in the invasion assay (5 h including the gentamicin treatment), to examine the possibility of an intracellular replication and/or persistence defect of the *clpV2* mutant, we compared the number of intracellular bacteria after 2.5 and 5.5 h of infection for each strain. After allowing 3 h for bacterial replication, we observed the same range of data (between 4.9- and 6-fold increase) for all the strains (Fig. 3B). In conclusion, the H2-T6SS mutant displays an invasion defect.

In a complementary assay, HeLa cells were infected with GFP-labeled *P. aeruginosa* and visualized by laser confocal microscopy (data not shown). Few bacteria were found in HeLa cells infected with the wild type PAO1. Conversely no bacteria were found in cells infected with the *clpV2* mutant. Together, these results support the assertion that the H2-T6SS system stimulates internalization of *P. aeruginosa* into HeLa cells.

Akt Phosphorylation upon Bacterial Invasion of Polarized Epithelial Cells Is Dependent on H2-T6SS—We further explored H2-T6SS-dependent bacterial entry into a more physiologically relevant cell line, Calu-3 cells, which are derived from human lung alveolar cells and which form polarized monolayers when grown on Transwell filters. *P. aeruginosa* binding to polarized epithelial cells activates a central host signaling molecule, PI3K, which is required for phosphatidylinositol 1,4,5-trisphosphate synthesis and for activation of a downstream effector, the protein kinase B/Akt (Akt) (44, 45). Activation of the PI3K/phosphatidylinositol 1,4,5-trisphosphate/Akt pathway was shown to be necessary and sufficient for *P. aeruginosa* entry from the apical surface (44). This event leads to a remarkable remodeling of the apical membrane in which protrusions enriched for phosphatidylinositol 1,4,5-trisphosphate and actin form at the apical surface at the site of bacterial entry (46). To determine whether the PI3K/Akt signaling pathway is required during the H2-T6SS-dependent uptake of *P. aeruginosa* by lung epithelial cells, polarized human airway epithelial Calu-3 cells were infected in the presence or absence of LY294002 (LY), a PI3K inhibitor, and adhesion, internalization, and Akt phosphorylation were determined. Although the treatment with LY did not affect bacterial adhesion, it reduced invasion of both PAO1 and the complemented PAO1 Δ *clpV2* mutant in a dose-dependent manner (Fig. 4A). Internalization levels of the *clpV2* mutant were lower than that of PAO1 or the complemented *clpV2* mutant; thus, LY treatment had little effect on the ability of the *clpV2* mutant to enter host cells (Fig. 4A).

We next determined whether Akt is activated in an H2-T6SS dependent manner, as determined by phosphorylation of Akt at serine 473. Polarized Calu-3 cells were co-cultivated with PAO1, *clpV2* mutant, or the complemented *clpV2* mutant. At 60 min after infection, Akt was immunoprecipitated followed by immunoblotting with anti-phospho-Akt^{Ser473} antibody. The ratio of phospho-Akt to total Akt was quantified and normalized to the ratio observed in untreated cells. Infection with the *clpV2* mutant resulted in ~50% decrease in Akt phosphorylation levels compared with infection with PAO1 or with the complemented mutant (Fig. 4, B and C). The magnitude of the decrease in Akt phosphorylation was similar to the magnitude of the decrease in internalization by the *clpV2* mutant (Fig. 4, B and C). LY treatment decreased Akt phosphorylation upon infection with any strain to levels observed in uninfected Calu-3 cells (Fig. 4, B and C). Together, these data suggest that H2-T6SS stimulates bacterial entry of transition phase-grown bacteria through the PI3K/Akt pathway.

H2-T6SS Contributes to *P. aeruginosa* Virulence in *C. elegans*—The involvement of H2-T6SS in *P. aeruginosa* pathogenicity led us to test its role in virulence on *C. elegans*. Previous studies

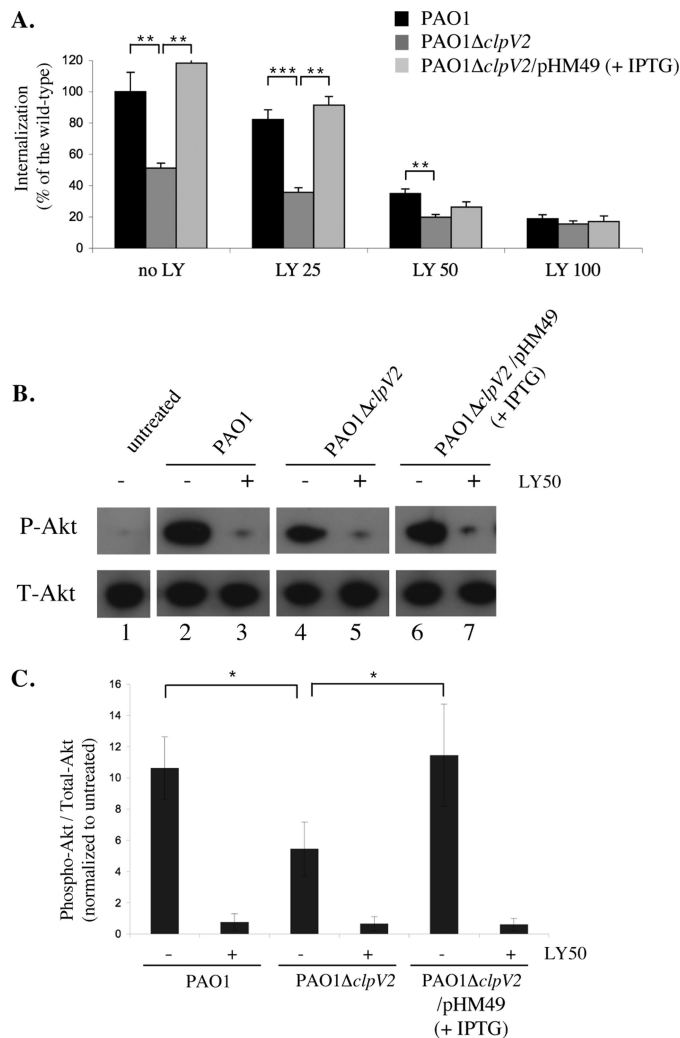


FIGURE 4. Activation of PI3K pathway in Calu-3 cells relies on H2-T6SS-dependent PAO1 invasion. A, the percent invasion of PAO1 Δ *clpV2* with (gray bars) or without (white bars) pHM49, normalized to PAO1 that represents an average of 3500 colony-forming units of internalized bacteria/well (0.8×10^6 Calu-3). When indicated, the cells were incubated with a PI3K inhibitor, LY294002, at various concentrations (25, 50, and 100 μ M) for 1 h prior to infection. The assays were performed a minimum of three times in triplicate. The error bars represent standard deviations, and the asterisks indicate *p* values. B, levels of phosphorylated Akt in Calu-3 cells infected with PAO1, PAO1 Δ *clpV2* with or without pHM49, or left uninfected (untreated). Akt phosphorylation was detected with anti-phospho-Akt antibodies (P-Akt), and total Akt with anti-Akt antibodies (T-Akt). When indicated (+), the cells were incubated with LY294002 at 50 μ M for 1 h prior to infection. C, quantification of band density by volume analysis using ChemiDoc XRS. The graph presents phospho-Akt as a percentage of Total-Akt and normalized to untreated cells from B.

have shown that *C. elegans* is a good model in which to identify *P. aeruginosa* virulence genes important for mammalian pathogenesis (47, 48). In a modified slow killing assay, we observed that the *clpV2* mutant was significantly less virulent than the PAO1 strain (Fig. 5), indicating that H2-T6SS plays a role in *P. aeruginosa* virulence.

DISCUSSION

P. aeruginosa possesses three distinct T6SS genetic loci, but the function and regulation of H2-T6SS and H3-T6SS have yet to be elucidated. Here, we show that the H2-T6SS gene cluster is up-regulated in PAO1 during the transition from exponential

P. aeruginosa H2-T6SS Regulation and Function

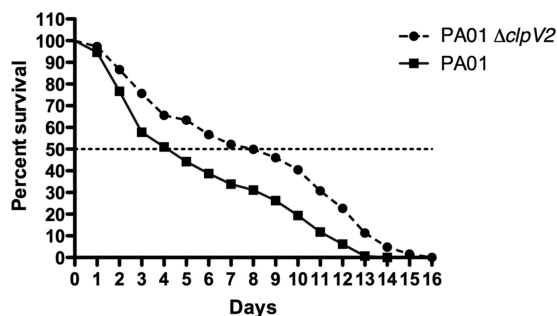


FIGURE 5. **H2-T6SS is required for virulence in *C. elegans*.** *C. elegans* was infected with PAO1 and its isogenic $\Delta clpV2$ mutant (survival curve, p value <0.0001; Prism 4 software).

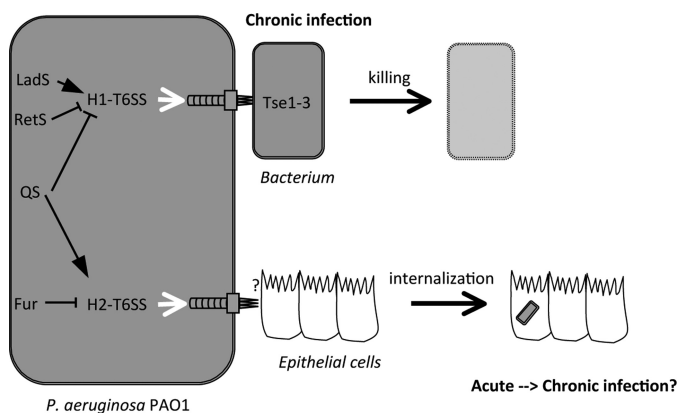


FIGURE 6. **A model of T6SS functions in *P. aeruginosa*.** H1- and H2-T6SS are differently regulated and have diverse functions. H1-T6SS targets bacteriolytic effectors in host bacteria providing a fitness advantage to *P. aeruginosa* presumably during chronic infection, whereas H2-T6SS mediates *P. aeruginosa* internalization into epithelial cells that could lead to a chronic infection.

to stationary phase growth and that its transcription is regulated by QS and induced upon iron limitation through the Fur regulator. Moreover, we demonstrate that H2-T6SS is required for PI3K/Akt-dependent internalization into epithelial cells and plays a role in *P. aeruginosa* virulence. Together, these data suggest that H2-T6SS is important in bacterial interactions with eukaryotic cells, which is quite distinct from the known functions of H1-T6SS. The latter is not expressed under laboratory conditions (3, 24, 25), secretes bacteriolytic effectors, and is primarily thought to give *P. aeruginosa* a growth advantage in mixed bacterial populations (15, 16). Thus, although the T6SS apparatus may be conserved, the diversity in secreted effectors may allow interactions with many different hosts in a broad range of habitats and conditions (Fig. 6).

Our results demonstrate that the LasR and RhlR QS regulators induce expression of genes coding the H2-T6SS machinery components in PAO1. In a different *P. aeruginosa* strain, PA14, representative gene transcripts from the three T6SS gene clusters, *hcp1*, *hcp2*, and *hcp3*, have also been reported to be regulated by LasR (19). As noted earlier, PAO1 and PA14 strains differ at the level of H2-T6SS loci, *hcp2* is absent from PAO1 locus, and the PA14 locus contains also three other putative substrate genes, *vgrG2*, PA14_43090 encoding an homologue of an *A. hydrophila* type III secretion system (T3SS) effector and PA14_43100 encoding a putative Rhs family protein (19). From our observation, it thus stands to reason to propose that the H2-T6SS machinery of PA14 is under QS control too. Collec-

tively, these findings tend to indicate that the H2-T6SS machinery and cognate substrates are co-regulated by QS, even if those divergences may even reflect a different function of the H2-T6SS machinery in these two *P. aeruginosa* strains. Indeed, QS control of the T6SS may be generally conserved, as it has also been reported for the plant pathogen *Pectobacterium atrosepticum* (49), *V. cholerae* 01 (50, 51), a clinical isolate of *A. hydrophila* (52), and in *Yersinia pseudotuberculosis* (53). This strategy may allow pathogens to coordinately regulate the T6SS with other important virulence determinants, including other secretion systems, toxins, proteases, and biofilm-promoting factors.

One challenge that many pathogens face is the low iron availability in host tissues, which sequester iron as a protective mechanism against bacterial infections. The *P. aeruginosa* Fur regulator responds to low iron conditions by de-repressing virulence factors, including siderophores, heme uptake system, exotoxin A, and proteases (54). We now show that H2-T6SS is also negatively regulated by the Fur system, which may facilitate uptake of the bacteria into epithelial cells. The co-regulation of iron acquisition genes and secretion machinery genes have been recently reported for other bacteria, including the T3SS genes of *Bordetella pertussis* (55), the T6SS of enteroaggregative *E. coli* (56), and the T6SS and T3SS genes of *Edwardsiella tarda* (57). The co-regulation of iron uptake systems and secreted virulence factors may function synergistically to improve iron availability to bacteria that are in the iron-limited environment of the host. In addition, other Fur-regulated genes may contribute to bacterial survival in the hostile environment of the host. For instance, Fur regulates the expression of stress genes involved in *Helicobacter pylori* acid resistance response during the gut infection (58), helping the bacteria to adapt and to adjust to the acidic conditions encountered in the stomach.

Our studies reveal that H2-T6SS mediates interactions with mammalian host cells. Interestingly, phylogenetic analysis demonstrates that the *P. aeruginosa* H2-T6SS is most closely related to the eukaryotic-cell targeting T6SSs of *V. cholerae*, *A. hydrophila*, or *H. hepaticus* (59–61). Consistent with the notion that H2-T6SS targets eukaryotic cells, we find that H2-T6SS plays a role in PI3K/Akt-dependent bacterial entry into epithelial cells; inactivation of the ATPase ClpV2 results in an ~50% decrease in bacterial internalization and a corresponding decrease in Akt phosphorylation. This raises an interesting question about the mechanism of action of H2-T6SS in facilitating the uptake of bacteria by mammalian cells. Internalization promoting factors could act from the cellular exterior in analogy with the *Listeria* internalins or *Yersinia* invasin or from the inside as the T3SS effectors of *Shigella* or *Salmonellae* (62). Because an intracellular delivery has been demonstrated for certain VgrG proteins in other Gram-negative bacteria (11, 12, 14), we would favor that one or more H2-T6SS substrates may directly activate PI3K/Akt pathway to trigger cytoskeleton rearrangement and/or to modulate the innate immune response.

In contrast to H2-T6SS, the T3SS regulon of *P. aeruginosa* is negatively controlled by QS (30) and acts as an anti-internalization factor (63). Thus, H2-T6SS and T3SS are reciprocally regulated, possibly reflecting their divergent functions at different stages of the infection process (colonization *versus* dissemina-

tion) or the infection mode (acute virulence *versus* chronic persistence). One possibility could have been a direct cross-talk between the two systems, but we did not observe a type III secretion defect in the H2-T6SS mutant (supplemental Fig. S1C). We did not monitor any difference in cell rounding and toxicity (lactate dehydrogenase release) in HeLa and macrophages infected with wild type or H2-T6SS mutant strains (data not shown). This suggests that H2-T6SS is most likely important in establishing chronic infection because it does not seem to play a role in acute toxic events like T3SS.

Taken together, our observations suggest that the H2-T6SS machinery secretes proteins that target eukaryotic host cell. Despite the lack of apparent *hcp* or *vgrG* genes within the H2-T6SS locus of PAO1, two sets of protein (Hcp2a-VgrG2a (PA1512-PA1511) and Hcp2b-VgrG2b (PA0263-PA0262)) have been proposed as putative H2-T6SS substrates (3, 4, 42, 64). One can note that neither VgrG2a nor VgrG2b is an orthologue of VgrG2 from PA14, which is homologous to VgrG6 (42). Interestingly, transcriptional analysis has revealed that these putative effectors are co-regulated with the H2-T6SS machinery in PAO1 (65–69). Future studies will be aimed at identifying the secreted substrates of the H2-T6SS machinery from PAO1 and determining whether they target the host eukaryotic cell.

Acknowledgments—We thank Paul Williams for the generous gift of C₄-HSL; Romé Voulhoux, Geneviève Ball, and Bérangère Ize for constant support; and Sana's thesis committee for fruitful discussions.

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