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ORIGINAL ARTICLE

Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae

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Bacteria and protozoa coexist in a wide range of biofilm communities of natural, technical and medical importance. Generally, this interaction is characterized by the extensive grazing activity of protozoa on bacterial prey populations. We hypothesized that the close spatial coexistence in biofilms should allow opportunistic pathogenic bacteria to utilize their eukaryote-targeting arsenal to attack and exploit protozoan host cells. Studying cocultures of the environmental pathogen *Pseudomonas aeruginosa* and the amoeba *Acanthamoeba castellanii*, we found that *P. aeruginosa* rapidly colonized and killed biofilm-associated amoebae by a quorum-sensing independent mechanism. Analysis of the amoeba-induced transcriptome indicated the involvement of the *P. aeruginosa* type III secretion system (T3SS) in this interaction. A comparison of mutants with specific defects in the T3SS demonstrated the use of the secretion apparatus and the effectors ExoU, ExoS and ExoT in the killing process, of which ExoU had the greatest impact. T3SS-mediated virulence towards *A. castellanii* was found to be controlled by the global regulators RpoN and RpoS and through modulation of cAMP and alginate biosynthesis. Our findings suggest that conserved virulence pathways and specifically the T3SS play a central role in bacteria–protozoa interactions in biofilms and may be instrumental for the environmental persistence and evolution of opportunistic bacterial pathogens.

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Introduction

Microbial biofilms constitute the major proportion of bacterial biomass and activity in many natural and man-made systems. At the same time, biofilms serve as important environmental reservoirs for pathogenic bacteria (Flanders and Yildiz, 2004) and are the causative agents for many persistent bacterial infections (Costerton *et al.*, 1999). Synergistic and antagonistic interactions among microorganisms are predicted to be central to the structure, composition and function of biofilm communities (Hassell *et al.*, 1994; Kerr *et al.*, 2002; Battin *et al.*, 2003). Despite the consensus that biofilms represent mixed microbial communities, studies to date have addressed the physiology and regulation of biofilm bacteria almost to the exclusion of the other major players.

One group of microorganisms that lives in close association with bacterial biofilms are phagotrophic protists, the protozoa. Predation by protozoa is considered to be the major source of mortality for bacterial populations in many ecosystems (Sherr and Sherr, 2002). Protozoa, free-living amoeba in particular, are associated with bacterial biofilms in a wide range of habitats, including rivers, activated sludge, water pipes and filters, as well as dental unit waterlines and the oral cavity (Arndt *et al.*, 2003; Marciano-Cabral and Cabral, 2003; Parry, 2004). The close association of bacteria and protozoa in biofilms and their long co-evolutionary history are thought to give rise to a series of bacterial adaptations ensuring survival and coexistence (Matz and Kjelleberg, 2005). Several bacteria, among them

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many facultative intracellular pathogens, have developed mechanisms to survive and replicate inside free-living amoebae, such as *Legionella pneumophila*, *Francisella tularensis*, many *Mycobacterium* spp. and *Escherichia coli* O157 (Molmeret *et al*., 2005). Besides being able to grow and survive intracellularly, bacteria may develop protective and exploitative mechanisms that act before the internalization by protozoa (Matz and Kjelleberg, 2005). The formation of biofilms and matrix-encased microcolonies has recently been reported to enhance the persistence of *Vibrio cholerae* and *Pseudomonas aeruginosa* in the presence of protozoa. This occurs because localized high bacterial densities allow the synchronized secretion of antiprotozoal compounds via quorum-sensing signals (Matz *et al*., 2004, 2005).

Although biofilms play roles in ensuring the persistence of human pathogens in the environment, little is known about the specific ways pathogens benefit from these associations. *P. aeruginosa* is a versatile gram-negative bacterium that is ubiquitous in soil, freshwater and marine habitats (Botzenhart and Döring, 1993; Kimata *et al*., 2004; Pirnay *et al*., 2005). At the same time, it is one of the most widespread opportunistic pathogens causing infections in a variety of hosts, including insects, plants, animals and humans (Bodey *et al*., 1993; Rahme *et al*., 1995; Tan *et al*., 1999; Plotnikova *et al*., 2000; Lau *et al*., 2003; Miyata *et al*., 2003; He *et al*., 2004). We have recently demonstrated that biofilms of *P. aeruginosa* are effectively defended from grazing by the amoeba *Acanthamoeba* sp. (Weitere *et al*., 2005). In the present study we investigated the molecular mechanisms by which *P. aeruginosa* responds to the presence of *Acanthamoeba castellanii*. We hypothesized that the coexistence of bacteria and protozoa in biofilms should induce either defensive or exploitative behaviour by the opportunistic pathogen *P. aeruginosa*.

Table 1 Strains and plasmids used in this study

Strain or plasmid	*Relevant characteristic*	*Reference*
Bacteria		
Pseudomonas aeruginosa		
PAO1	Wild-type ATCC 15962	American Type Culture Collection
rhlR/lasR	PAO1 *rhlR*::Tc^r *lasR*::Gm^r	Rasmussen *et al*., 2005
vfr	PAO1Δ*vfr*	University of Washington Genome Center
rpoS	PAO1Δ*rpoS*	University of Washington Genome Center
rpoN	PAO1 *rpoN*::Gm^r	Thompson *et al*., 2003
mucA22	Point mutation in *mucA* of PAO1	Mathee *et al*., 1999
PA99	Clinical isolate	Feltman *et al*., 2001
PA99S	PA99Δ*exoTexo*U	Shaver and Hauser, 2004
PA99T	PA99*null* complemented with mini-CTX1-ExoT	Shaver and Hauser, 2004
PA99U	PA99Δ*exoSexoT*	Shaver and Hauser, 2004
PA99secr$^-$	PA99Δ*pscJ*	Shaver and Hauser, 2004
Plasmids		
pMF230	pMF36 containing *gfp mut2*; Cb^r	Franklin and Ohman, 1993
mini-CTX1-ExoT	*exoT* in mini-CTX1; Tet^r	Shaver and Hauser, 2004
Protozoa		
Acanthamoeba castellanii	Wild-type ATCC 30234	American Type Culture Collection

Material and methods

Organisms and growth conditions

Bacterial strains and mutants used in this study are listed in Table 1. The *P. aeruginosa* PAO1 wild type used was obtained from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001). This PAO1 isolate has served as DNA source for the *Pseudomonas* Genome Project (www.pseudomonas.com) and, subsequently, as template for design of the *P. aeruginosa* GeneChip (Affymetrix Inc., Santa Clara, CA, USA). *P. aeruginosa* PAO1 transposon insertion mutants were obtained from the University of Washington Genome Center (Jacobs *et al*., 2003). Transposon type, insertion, location and orientation were confirmed for each of the mutants. The isogenic *rhlR/lasR* QS mutant was constructed by Rasmussen *et al*. (2005). To study the components of the type III secretion system (T3SS), we used *P. aeruginosa* PA99, a well-characterized clinical isolate that naturally carries the *exoU*, *exoS* and *exoT* genes, but lacks *exoY* (Feltman *et al*., 2001). Bacterial strains were routinely grown on Luria–Bertani agar, and, prior to the experiments, on a 10% dilution of M9 medium (Sambrook *et al*., 1989) supplemented with 20 mM glucose. Axenic cultures of *A. castellanii* ATCC 30234 were maintained as confluent monolayers in tissue culture flasks on M9-based peptone/yeast extract/glucose (PYG) medium at 30 °C. For the experiments, trophozoites were suspended from the flask bottom (incubation on ice for 30 min), washed twice by centrifugation (500 *g*, 5 min) and quantified photometrically by OD_{600} measurements.

Flow-cell experiments

Flow-cell experiments were performed as described previously (Weitere *et al*., 2005) at a constant

temperature of 22 °C. Flow channels were inoculated with overnight cultures of the *P. aeruginosa* PAO1 wild type and the isogenic *lasR/rhlR* mutant and incubated without flow for 1 h to allow bacteria to attach. The biofilms were grown without amoebae for 3 days in 10% M9 medium (supplemented with 0.3 mM glucose). On day 4, washed cells of *A. castellanii* were resuspended in 10% M9 medium at a final concentration of 10^3 cells per ml and injected into three replicate flow chambers. Amoebae numbers and biofilm structures were monitored daily by phase contrast and scanning confocal laser microscopy, respectively. In each flow chamber, amoebae were counted in 15 randomly distributed *x*–*y* grids. The experiment was repeated for the *P. aeruginosa* PA99 wild type and the isogenic PA99secr$^-$ mutant under identical conditions.

Transcriptome profiling of P. aeruginosa *biofilms*
The transcriptional response of *P. aeruginosa* biofilms to *A. castellanii* was profiled by using PAO1 Affymetrix GeneChips (Affymetrix Inc., Santa Clara, CA, USA). Biofilms were grown at 22 °C for 3 days in continuous-culture silicon tubing (length: 12 cm; inner diameter: 0.8 cm; total volume: 6 ml). Washed cells of *A. castellanii* were resuspended in 6 ml of 10% M9 medium at a final concentration of 10^6 cells per ml and injected into the flow chamber. After 2 h of co-incubation, the excess fluid inside the tube was discarded and the biofilm cells were harvested mechanically and collected in two volumes of RNA*later* (Qiagen GmbH, Hilden, Germany) to be stored at −80 °C. The RNA*later*-suspended biofilms were sonicated to dissolve clumped cells. Amoebae and bacteria were separated by centrifugation. RNA was extracted from the harvested bacterial cells with the RNeasy Mini Purification kit (Qiagen GmbH) and contaminating chromosomal DNA was removed by RQ1 RNase-free DNase treatment. RNA was converted to cDNA (Random primers, RT SuperScript III, Invitrogen Corporation, Carlsbad, CA, USA) and fragmented. The fragments were labelled with Biotin-ddUTP (EnzoBioArray terminal labelling kit; Invitrogen Corporation) and hybridized to the GeneChip *P. aeruginosa* Genome Array (Affymetrix Inc.). Microarray data were analysed with Affymterix GeneChip Operating Software and Microsoft Excel. To identify a common set of amoeba-responsive genes, the experiment was repeated twice. Those genes that showed a statistically significant ($P<0.05$) change in expression and a more than threefold change in magnitude when grown in the presence of *A. castellanii* relative to M9 medium alone were regarded as significant. The less stringent cut-off value was chosen because of the reported heterogeneous nature of biofilms to allow gene expression patterns of subpopulation to be recorded in the analysis.

Coculture batch experiments
Batch experiments of *A. castellanii* and *P. aeruginosa* were conducted in flat bottom, tissue culture-treated 96-well plates (Corning Incorporated). Tissue culture plates containing M9-based PYG medium were inoculated with *A. castellanii* and incubated at 30 °C for 2 days. Prior to the experiment, the PYG medium was carefully removed from the wells and the cells were gently washed with 10% M9 medium. Cultures of *P. aeruginosa* were grown on 10% M9 medium supplemented with 0.2% glucose to an OD ≈ 0.1. For the experiment, bacterial suspensions were adjusted to OD = 0.025 by diluting the culture with the filtered culture supernatant. Wells containing washed *A. castellanii* were then inoculated in the following arrangement per *P. aeruginosa* strain: three replicate wells with 75 µl of the bacterial culture of OD = 0.025 (treatment wells), three replicate wells with 75 µl of 10% M9 medium without glucose (medium-only control) and three replicate wells with 75 µl of bacteria-free supernatant (supernatant control). The plates were incubated at 30 °C for 24 h. Fixing was done by adding 75 µl of 4% glutaraldehyde to each well to get a final concentration of 2% glutaraldehyde. The plates were sealed with Parafilm and stored at 4 °C until they were examined using an inverted light microscope (Olympus). The number of amoeba was obtained by counting the numbers of cells in one transect per well using a magnification of × 30.

Statistical analysis
Changes in amoebae numbers over time were tested for significance with repeated measures ANOVA. Survival percentages were arcsine-square root transformed. Pairwise comparisons of means were done by Student's *t*-tests.

Results

QS-independent killing of A. castellanii *in* P. aeruginosa *biofilms*
In flow-cell experiments, we observed that the amoeba *A. castellanii* was rapidly killed when colonizing pre-grown biofilms of *P. aeruginosa* PAO1. After settling on *P. aeruginosa* biofilms, amoeba numbers declined within 48 h from 80.2 ± 7.8 to 30.6 ± 4.1 cells per mm^2 (Figure 1a). Microscopic inspection revealed that the lysis of *A. castellanii* trophozoites coincided with amoebae being colonized by *P. aeruginosa*. Both colonization and lysis of amoebal cells were also observed in biofilms of the QS-deficient *rhlR/lasR* mutant (Figure 1a). Differences between the wild-type and the *rhlR/lasR* mutants were not significant ($P>0.1$), indicating that the killing of amoebae was QS independent.

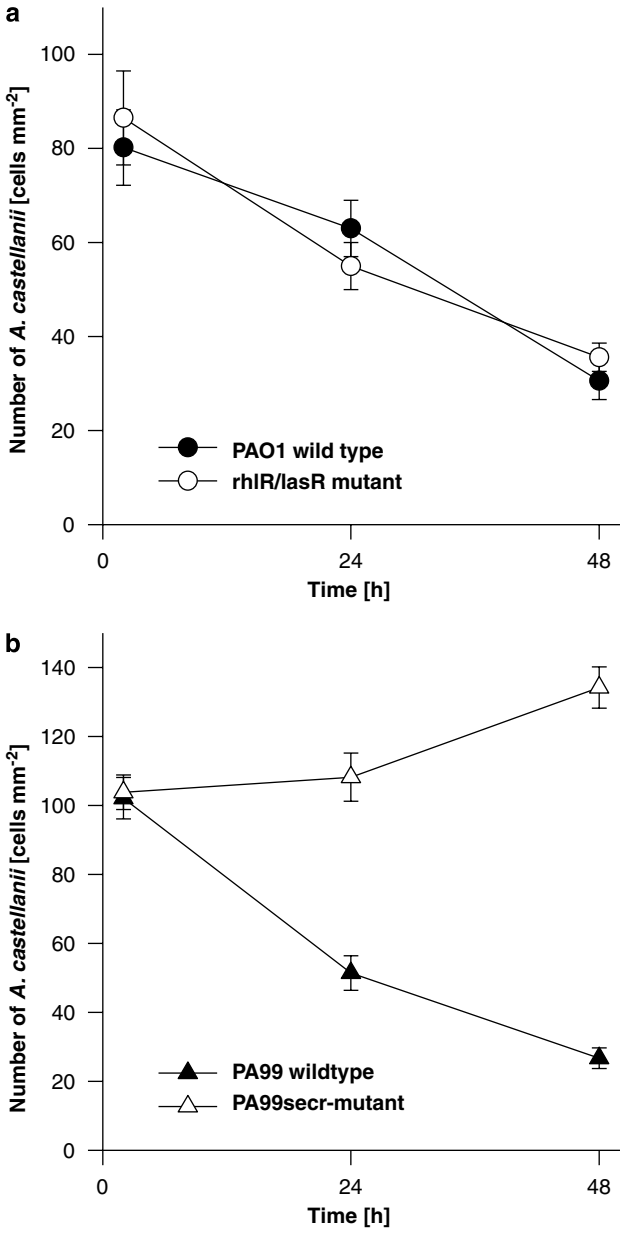

Figure 1 Lysis of *Acanthamoeba castellanii* settling on biofilms of *Pseudomonas aeruginosa*. Amoeba survival on (**a**) biofilms of *P. aeruginosa* PAO1 wild type versus QS-deficient *rhlR/lasR* mutant, and (**b**) biofilms of *P. aeruginosa* PA99 wild type versus T3SS-deficient PA99secr$^-$ mutant. Biofilms were pre-grown in flow-cell systems for 3 days prior to the addition of *A. castellanii*. Amoeba numbers include only structurally intact cells and are given as means ± standard deviation ($n = 3$).

Amoeba-induced expression of T3SS-related genes

To identify the underlying mechanism, we performed a transcriptomic analysis of *P. aeruginosa* PAO1 biofilms responding to *A. castellanii* cells. Analysis of the transcriptome profiles showed that the expression of the number of genes was significantly altered ($P < 0.05$) in response to *A. castellanii* when compared with the unexposed control biofilm. A total of 379 genes representing about 7% of the genome were differentially expressed (⩾3-fold change in all three experiments) with approximately 70% of genes being upregulated upon exposure to *A. castellanii*. Among the upregulated QS-independent genes with known function we found genes encoding proteins involved in the type III secretion, translocation and regulation machinery (Table 2): the genes *pscTSQ* of the operon PA1690-PA1697 (*pscUTSRQPON*), the gene *psrD* of the operon PA1698-PA1704 (*popN-pcr1234DR*), the gene *pcrH* of the operon PA1705-PA1709 (*pcrGVH-popBD*), the genes *exsCA* of the operon PA1710-PA1713 (*exsCEBA*) and the gene *pscF* of the operon PA1714-PA1725 (*exsD-pscBCDEFGHIJKL*). The amoeba-induced transcriptome did not indicate the involvement of QS-controlled virulence factors in the killing process (see Supplementary Information). Instead, two genes encoding enzymes with acyl-homoserine lactone acylase activity, *quiP* and *pvdQ*, were induced 16.4- and 4.6-fold, respectively, suggesting signal degradation rather than production. Exposure to amoebal attack also caused 3.9- to 16.6-fold enhanced expression levels in genes encoding nitric oxide reductases (*norC* and *norB*), which may contribute to the detoxification of amoeba-derived reactive nitrogen intermediates. Moreover, *P. aeruginosa* cells coexisting with amoebae in biofilms showed a 6.4-fold up-regulation of *fliO*, a gene involved in flagellar biosynthesis and adherence to eukaryotic cells.

Table 2 Expression of T3SS-related genes in *Pseudomonas aeruginosa* biofilms in response to the amoeba *Acanthamoeba castellanii* (⩾3-fold change in all three experiments)

ID no.	*Gene*	*Product function/ description*	*Fold change*
PA1691	*pscT*	Translocation protein in T3SS	4.3
PA1692	*pscS*	Probable translocation protein in T3SS	3.8
PA1694	*pscQ*	Translocation protein in T3SS	4.0
PA1703	*pcrD*	T3SS apparatus protein	3.8
PA1707	*pcrH*	Regulatory protein	4.7
PA1710	*exsC*	ExsC	4.4
PA1713	*exsA*	Transcriptional regulator	6.4
PA1719	*pscF*	T3SS export protein	4.9

Killing is cell contact-dependent and requires a functional T3SS

To test for the involvement of T3SS components in the killing of biofilm-associated amoebae, we used previously characterized mutants of the clinical strain *P. aeruginosa* PA99, which secretes ExoS,

ExoT and ExoU (Feltman *et al.*, 2001; Shaver and Hauser, 2004; Table 1). In flow-cell experiments, we found that *A. castellanii* associated with biofilms of the *P. aeruginosa* PA99 wild type were lysed at a significantly higher rate than on biofilms of *P. aeruginosa* PAO1 (75.8 ± 5.1% decline within 48 h, $P<0.01$, Figure 1b). In contrast, numbers of *A. castellanii* remained stable and even increased by about 30% when colonizing biofilms of the PA99secr$^-$ mutant, which does not produce any of the T3S proteins and is secretion apparatus defective.

On the basis of our observation that amoebae settling on *P. aeruginosa* biofilms become rapidly surrounded by highly motile planktonic bacteria before being colonized and eventually killed, we performed defined batch experiments, where *P. aeruginosa* suspensions were added to surface-attached amoebae. Upon adding the *P. aeruginosa* PA99 wild type to *A. castellanii* in batch culture, we observed that highly motile cells of *P. aeruginosa* colonized the amoebae within 2–6 h to form dense biofilms surrounding the amoebal cells (Figure 2). After 24 h of incubation, about 97% of *A. castellanii* cells were colonized by the *P. aeruginosa* PA99 wild type (Figure 2a), which was followed by a substantial decline of amoebae numbers (12.7 ± 3.2% amoeba survival, Figure 2b). The PA99secr$^-$ strain, which does not produce any of the T3S proteins and is secretion apparatus defective, colonized *A. castellanii* cells to a similar extent (about 90% of the amoebae were colonized, $P=0.12$; Figure 2a) but did not cause a significant decrease in amoeba cell numbers (Figure 2b). Testing the effect of cell-free supernatant of *P. aeruginosa*, PA99 and PAO1 cultures revealed low cytotoxicity for both strains (88.1 ± 5.2% and 96.4 ± 8.4% amoeba survival with PA99 and PAO1, respectively), which indicates that the killing of amoebae requires direct contact with bacterial cells.

T3SS-mediated cytotoxicity in *P. aeruginosa* is elicited by the combined action of secreted effector proteins, ExoS, ExoT, ExoU and ExoY. To determine the relative contribution of the type III effector proteins of *P. aeruginosa* to the killing of *A. castellanii,* isogenic mutants were screened, which secrete only one of the T3S effectors, ExoS, ExoT and ExoU (designated PA99S, PA99T and PA99U; Table 1). The ExoU-secreting strain PA99U caused significantly lower but still considerable mortality in *A. castellanii* populations (80.4 ± 3.2% compared to 87.3 ± 3.1% in the wild type, $P=0.002$, Figure 3). Secretion of ExoS alone was also sufficient to kill 63.8 ± 4.5% of the amoebae ($P<0.001$). The double knock-out mutant PA99T, which secretes only ExoT, caused the greatest drop in virulence compared to the PA99 wild type resulting in 42.9 ± 2.9% amoeba mortality ($P<0.001$). On the basis of these data, we conclude that all three effector proteins contribute to the ability of *P. aeruginosa* to kill the amoeba *A. castellanii*.

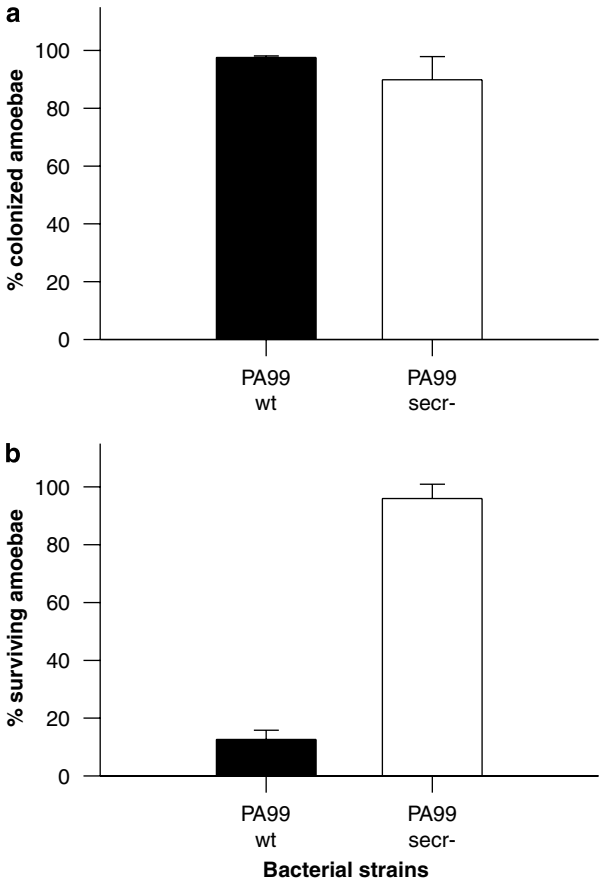

Figure 2 Colonization and killing of *Acanthamoeba castellanii* by *Pseudomonas aeruginosa*. (**a**) Colonization of *A. castellanii* by *P. aeruginosa* PA99 wild type and the T3SS-deficient mutant PA99secr$^-$ given as percentage of the total amoeba number after 24 h. (**b**) Survival of *A. castellanii* after 24 h exposure to *P. aeruginosa* PA99 wild type and the T3SS-deficient mutant PA99secr$^-$ given as percentage of the initial amoeba number. Error bars represent standard deviations ($n=5$).

Regulation of T3SS-mediated cytotoxicity towards amoebae

Given the involvement of exopolymers, cell-to-cell communication and metabolic stresses in *P. aeruginosa* cytotoxicity and biofilm development, we examined regulator mutants defective in alginate biosynthesis, quorum sensing, adenosine 3′,5′-cyclic monophosphate (cAMP) signalling, and stationary phase sigma factors (Figure 4). As found in the flow-cell studies, the QS-deficient *rhlR/lasR* mutant only showed a marginal difference to the PAO1 wild type in the batch experiments (37.0 ± 5.1% compared to 28.1 ± 3.4%, $P=0.04$). The stationary phase sigma factor RpoS seems to be involved in the repression of the T3SS-mediated killing of *A. castellanii* as the *rpoS* mutant significantly increased amoebae mortality ($P=0.02$). Modulation of intracellular cAMP levels in response to environmental signals is known to be another important mechanism controlling T3SS expression (Rietsch and Mekalanos, 2006; Wolfgang *et al.*, 2003). Accordingly, we found that a

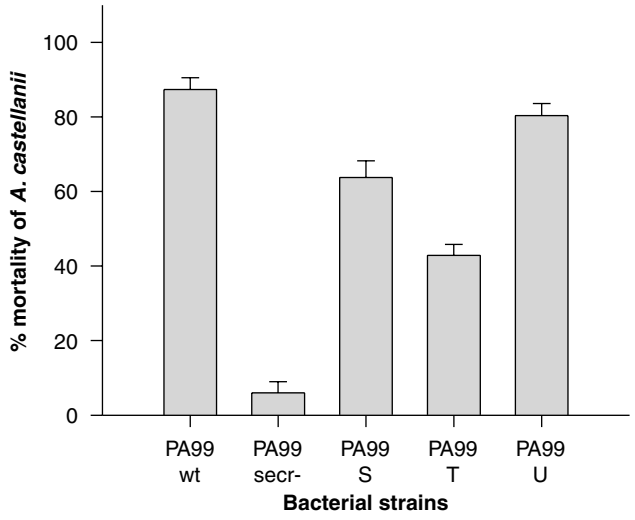

Figure 3 Contribution of type III secreted effectors to the killing of *Acanthamoeba castellanii*. Amoeba lysis is given as relative reduction of initial cell numbers after 24 h of co-incubation with *Pseudomonas aeruginosa* PA99 wild type and the T3SS-deficient mutant PA99secr$^-$, and mutants expressing only one of the three effectors, ExoS (PA99S), ExoT (PA99T) or ExoU (PA99U). Error bars represent standard deviations ($n=5$).

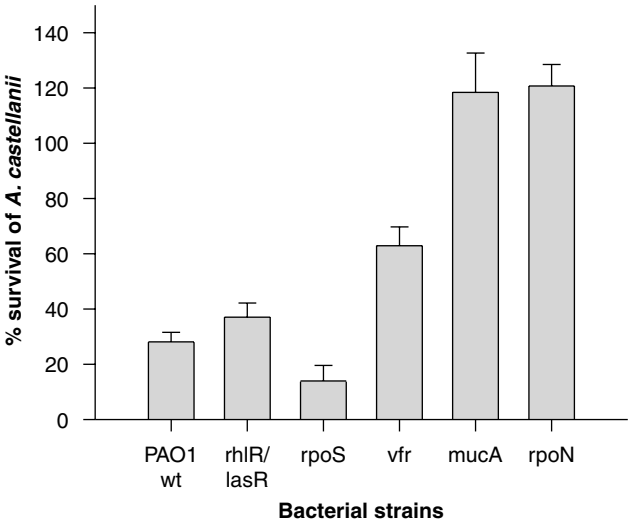

Figure 4 Contribution of global gene regulators to T3SS-mediated killing of *Acanthamoeba castellanii*. Amoeba survival is given as relative change of initial cell numbers after 24 h of co-incubation with *Pseudomonas aeruginosa* PAO1 wild type and mutants defective in *rhlR/lasR*, *rpoS*, *vfr*, *mucA*, *rpoN*. Error bars represent standard deviations ($n=5$).

mutant defective in the cAMP receptor protein Vfr caused substantially lower mortality in populations of *A. castellanii* ($63.1 \pm 6.8\%$, $P<0.001$). A complete loss of cytotoxicity was found in strain *mucA22*, lacking the functional anti-sigma factor MucA, which is involved in the repression of alginate biosynthesis. Strain *mucA22* even caused a significant increase in amoebae numbers ($118.5 \pm 14.2\%$, $P<0.001$), indicating the lack of toxicity and the feeding activity of *A. castellanii* on *P. aeruginosa* cells. Similarly, a mutant defective in the alternate sigma factor rpoN showed an increase in amoebae numbers ($120.8 \pm 7.8\%$, $P<0.001$), suggesting a central role of RpoN in the cytotoxicity towards amoebae.

Discussion

Our cocultivation experiments demonstrate that biofilms of *P. aeruginosa* respond to the colonization by environmental amoebae by upregulating genes of T3SS. Interestingly, recent studies have linked negative regulation of T3SS with factors thought to contribute to biofilm formation (Kuchma *et al.*, 2005; Furukawa *et al.*, 2006; Kulasakara *et al.*, 2006; Laskowski and Kazmierczak, 2006; Ventre *et al.*, 2006). This view is supported by our observation that amoebae settling on *P. aeruginosa* biofilms become rapidly surrounded by highly motile planktonic bacteria before being colonized and eventually killed (Figure 2). One global network affecting the reciprocal regulation of T3SS and biofilm formation is controlled by the RetS and LadS sensor proteins (Laskowski and Kazmierczak, 2006; Ventre *et al.*, 2006). RetS is required for expression of the T3SS genes and for repression of exopolysaccharide biosynthesis, whereas LadS has the opposite effect. The exopolysaccharide alginate is further regulated by the alternative sigma factor AlgU, which is normally antagonized by the MucA anti-sigma factor. Mutation in the mucA gene is known to cause alginate overproduction, resulting in the mucoid phenotype. A recent study comparing global gene expression of *mucA* mutant versus wild type under T3SS-inducing conditions confirmed the downregulation of T3SS genes and upregulation of genes involved in alginate biosynthesis (Wu *et al.*, 2004). In accordance with this, we found no decline in cell numbers of *A. castellanii* when cocultured with a *mucA* mutant (Figure 4), suggesting that matrix-encased cells do not colonize and kill the amoebae but a mobile subpopulation of bacteria which is transiently released from *P. aeruginosa* biofilms.

A recent study examining the contribution of the transcriptional anti-anti-activator ExsC to host contact-dependent T3SS gene expression found induction of the T3SS genes to be highly dependent on undefined host-cell factors (Dasgupta *et al.*, 2006). Among the T3SS-related genes induced by *A. castellanii* we found a significant upregulation of ExsC (Table 2), which supports the notion that T3SS-mediated cytotoxicity to host cells other than mammalian cells is regulated through a generic ExsC-dependent pathway (Yahr and Wolfgang, 2006).

We have previously reported that biofilms of *P. aeruginosa* are protected against protozoan grazing through a QS-controlled inhibitory

mechanism (Matz *et al.*, 2004). However, indications for QS-independent response of *P. aeruginosa* were presented in an agar-plate based study on *P. aeruginosa* and the social amoeba *Dictyostelium discoideum* (Pukatzki *et al.*, 2002). Besides the involvement of LasR in growth inhibition of the amoeba, the study described the lytic impact of the phospholipase ExoU on *D. discoideum*, suggesting the existence of two independent mechanisms acting in parallel. LasR is known to be without direct influence on the expression of T3SS genes (Dasgupta *et al.*, 2003; Hogardt *et al.*, 2004; Bleves *et al.*, 2005). Mutants lacking the Rhl quorum-sensing system, however, show increased expression of T3SS genes and secretion of ExoS at an earlier stage during exponential growth (Hogardt *et al.*, 2004; Bleves *et al.*, 2005). We found that a double knock out in the *rhl/las* system was without significant effect on cytotoxicity, suggesting a minor role of QS in this interaction. Notably, cocultivation of *P. aeruginosa* and amoebae in flow cells over relatively long time periods (10 days) leads to significant recovery in amoeba cell numbers associated with *lasR/rhlR* biofilms compared to wild-type biofilms (Matz *et al.*, unpublished data). It appears that *P. aeruginosa* shows an instant response to the presence of amoeba by the active colonization and killing of amoebae and that QS-controlled mechanisms gain more significance during longer term coexistence at higher cell densities.

The differential significance of QS-dependent and independent mechanisms may find support in the emerging concept that T3SS is under strong transcriptional control of metabolic states (Dacheux *et al.*, 2002; Wolfgang *et al.*, 2003; Rietsch *et al.*, 2004; Rietsch and Mekalanos, 2006). Growth conditions, cell densities and biofilm physiology may thus have a direct impact on which of the two pathways dominates. One transcriptional regulator that is induced at the transition from logarithmic to stationary growth phase is the sigma factor RpoS. About 770 genes are regulated by RpoS in the stationary phase of growth (Schuster *et al.*, 2004). We found that an *rpoS* mutant had increased cytotoxicity against *A. castellanii* (Figure 4), which could be explained by the negative regulatory effect on T3SS expression reported for RpoS (Hogardt *et al.*, 2004). Moreover, our transcriptome data reveal that 15% of the amoeba-induced genes are regulated by RpoS, among which are genes encoding T3SS components (ExsC, PcrH, PscF), two-component response regulators and chemotaxis transducer genes. Interestingly, algR transcription and alginate production have been shown to be RpoS-dependent (Suh *et al.*, 1999; Schuster *et al.*, 2004). As RpoS also regulates the Rhl system (Whiteley *et al.*, 2000), transcriptional regulation by RpoS could be central to the timing of QS-independent and dependent mechanisms in the interaction of *P. aeruginosa* with biofilm-associated amoebae.

Modulation of intracellular cAMP levels in response to environmental signals is another important mechanism controlling T3SS expression. Studies using whole-genome microarray analyses revealed that mutants lacking cAMP or the cAMP receptor protein Vfr exhibited reduced expression of nearly 200 genes, including those involved in T3SS (Wolfgang *et al.*, 2003). In our experiments, the *vfr* mutant was attenuated in killing *A. castellanii* (Figure 4), which supports the view that intracellular cAMP levels and the metabolic state of cells set the scene for *P. aeruginosa* to attack *A. castellanii*. Among the Vfr-regulated genes in the amoeba-induced transcriptome (9% of total), we found genes encoding T3SS components (PcrH, PscF, PscQ, PscS, ExsA, ExsC) and the chemosensory signal transduction protein ChpA.

Interactions of *P. aeruginosa* with the fungus *Candida albicans* share some features such as the active colonization of filamentous cells and the formation of biofilms culminating in the lysis of *C. albicans* (Hogan and Kolter, 2002). The killing was reported to be multifactorial and under the control of the alternative sigma factor RpoN. Although T3SS components were not crucial to *Pseudomonas–Candida* interactions (R Kolter and D Hogan, personal communication), our data clearly indicate the central role of RpoN in the regulation of T3SS-mediated killing of *A. castellanii* (Figure 4). Our transcriptome data reveal that 12% of the amoeba-induced genes are regulated by RpoN (cf Dasgupta *et al.*, 2003), but mostly encode hypothetical proteins with unknown function. Among the RpoN-regulated genes we find no evidence for their effect on flagella and pili biosynthesis. Interestingly, genes of the *pel* gene cluster, which is involved in polysaccharide biogenesis and early biofilm formation, show higher expression levels in an rpoN mutant (Dasgupta *et al.*, 2003) and are found to be downregulated upon contact with *A. castellanii*. Assuming that higher biofilm-forming capacity is reciprocally linked to cytotoxicity, this could explain the higher survival and even growth of *A. castellanii* on biofilms of the rpoN mutant (Figure 4). *P. aeruginosa* mutants defective in *rpoN* are also significantly attenuated in other virulence models (Rahme *et al.*, 1995; Tan *et al.*, 1999; Hendrickson *et al.*, 2001). However, although RpoN is known to be absolutely required for the expression of *hrp* genes encoding the components of a T3SS in *Pseudomonas syringae* (Hendrickson *et al.*, 2000a, b), its role in the regulation of cytotoxicity is less clear in *P. aeruginosa* and awaits further elucidation.

To date, four type III effector proteins have been described to be injected by *P. aeruginosa* into the cytoplasm of eukaryotic cells: ExoU is a potent cytotoxin with phospholipase activity. ExoS and ExoT are bifunctional enzymes that have a small GTPase-activating protein domain and an ADP-ribosyltransferase domain, and inhibit phagocytosis

by disrupting the actin cytoskeleton. ExoY is an adenylate cyclase causing elevated levels of cellular cAMP. Our experiments revealed that each one of ExoS, ExoT and ExoU contributes to amoebae killing (Figure 3). ExoU, however, appears to be the most toxic effector, which is confirmed by a study on *P. aeruginosa* virulence towards the social amoeba *D. discoideum* (Pukatzki *et al.*, 2002), reporting loss of virulence in *exoU* and *pscJ* mutants and restored cytotoxicity in a complemented *exoU* mutant. Strains of *P. aeruginosa* have different combinations of genes encoding T3SS effectors. The lack of *exoU* in *P. aeruginosa* PAO1 (containing *exoS*, *exoT* and *exoY*) may explain the slightly lower cytotoxicity compared to that of strain PA99 (containing *exoS*, *exoT* and *exoU*; Figure 1).

The recent sequencing of many bacterial genomes has shown that T3SS are quite common among proteobacteria and *Chlamydiae*. *Chlamydiae* are notorious human pathogens with an obligate intracellular lifestyle but also include species living as endosymbionts of environmental amoebae (Wagner and Horn, 2006). Interestingly, genome analyses of the *Acanthamoeba* sp. endosymbiont *Protochlamydia amoebophila* UWE25 revealed that the common ancestor of pathogenic and symbiotic *Chlamydiae* has been intracellular for several hundreds of millions of years and contained a complete T3SS (Horn *et al.*, 2004). Phylogenetic analysis of T3SS key components suggest that the T3SS evolved in the chlamydial lineage more than 700 million years ago in the interaction with early eukaryotes, which strengthens the notion that T3SS constitute an important module in past and present interactions between bacteria and protozoa.

As environmental pathogens like *P. aeruginosa* are integral members of natural microbial communities, it is anticipated that some virulence factors studied in the context of human disease may have their functional origin in the interaction with coexisting microbes. Toxins secreted by the *P. aeruginosa* T3SS have profound effects on the progression and severity of pneumonia in humans. Yet the pressures driving evolution of these toxins have been unclear, because humans are predominantly an accidental host of *P. aeruginosa*. Our findings suggest that the natural targets of this bacterium's type III secretion may be environmental protozoa. It seems that protozoa, particularly biofilm-associated amoeba, can induce exploitative behaviour in opportunistic bacterial pathogens and thus contribute to their pathogenic potential. Although it becomes obvious that multiple pathways exist in this interaction, future studies need to focus on the timing and regulatory fine tuning as well as the impact of environmental and metabolic conditions. Knowledge on the switch between persistence and aggression—or from a medical perspective between chronic and acute infection—is central to understand the opportunistic nature of pathogens like *P. aeruginosa* and, more generally, the functional role of natural biofilm communities as potential hotspots for eukaryote-targeting lifestyles.

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