Expression of *Pseudomonas aeruginosa* exoS is controlled by quorum sensing and RpoS

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In *Pseudomonas aeruginosa*, virulence determinants and biofilm formation are coordinated via a hierarchical quorum sensing cascade, which involves the transcriptional regulators LasR and RhlR and their cognate homoserine lactone activators C12-HSL [N-(3-oxododecanoyl)-L-homoserine lactone] and C4-HSL (N-butanoyl-L-homoserine lactone), which are produced by LasI and RhlI, respectively. The exoenzyme S regulon of *P. aeruginosa*, comprises genes for a type III secretion system and for four anti-host effector proteins (ExoS, T, U and Y), which are translocated into host cells. It is a reasonable assumption that this ExoS regulon should be downregulated in the biofilm growth state and thus should also be under the regulatory control of the Las/Rhl system. Therefore, an exoS5-gfp reporter construct was used, and the influence of the Las and Rhl quorum sensing systems and the effect of the stationary-phase sigma factor RpoS on regulation of the exoS gene was examined. Evidence is provided for downregulation of exoS during biofilm formation of *P. aeruginosa* PAO1. The rhl mutant PDO100 and rhlR mutant PDO111, but not the lasl mutant PDO-JP1, showed approximately twofold upregulation of the exoS5-gfp reporter in comparison to PAO1. Upregulation of exoS5-gfp in the PDO100 mutant could be repressed to normal level by adding C4-HSL autoinducer, indicating a negative regulatory effect of RhlR/C4-HSL on exoS expression. As RhlR/C4-HSL is also involved in regulation of RpoS, the *P. aeruginosa* rpoS mutant SS24 was examined and the exoS5-gfp reporter was found to be fivefold upregulated in comparison to PAO1. For the first time evidence is reported for a regulatory cascade linking RhlR/RhlI and RpoS with the expression of the anti-host effector ExoS, part of the exoenzyme S regulon. Moreover, these data suggest that the exoenzyme S regulon may be downregulated in *P. aeruginosa* biofilms.

INTRODUCTION

*Pseudomonas aeruginosa*, an increasingly prevalent opportunistic human pathogen, is the most common Gram-negative bacterium found in infections of immuno-compromised patients and of individuals suffering from cystic fibrosis (CF). Once *P. aeruginosa* has taken residence in the lungs of CF patients, it contributes substantially to morbidity and mortality in this population. The pseudomonads, which are seldom eradicated by antibacterial chemotherapy, chronically colonize the bronchoalveolar lumen of CF lungs and lead to recurrent pulmonary exacerbations, resulting in a progressive decline in lung function. Various virulence determinants have been shown to play a role in the pathogenesis of *P. aeruginosa*. The production of multiple of these virulence factors, including elastase, alkaline protease, LasA protease, phospholipase C, exotoxin A, rhamnolipid and pyocyanin relies on a cell-to-cell communication system known as quorum sensing (van Delden & Iglewski, 1998; Winzer & Williams, 2001). These regulatory systems enable *P. aeruginosa* to produce virulence factors in a coordinated, cell-density-dependent manner. *P. aeruginosa* contains two separate quorum sensing systems, termed Las and Rhl (Latifi et al., 1996; Pearson et al., 1997). Each of these systems possesses a homoserine lactone synthase (LasI or RhlI, respectively), a regulator protein (LasR or RhlR, respectively) that modulates gene transcription, and their cognate activators, N-(3-oxododecanoyl)-L-homoserine lactone (C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL). Moreover, the *P. aeruginosa* genome encodes a type III secretion system (TTSS) similar to that found on the virulence plasmid

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Abbreviations: AHL, N-acylhomoserine lactones; C4-HSL, N-butanoyl-L-homoserine lactone; C12-HSL, N-(3-oxododecanoyl)-L-homoserine lactone; CF, cystic fibrosis; GFP, green fluorescent protein; SM, stimulation medium; TTSS, type III secretion system; VB, Vogel–Bonner.
of *Yersinia* (Frank, 1997; Hueck, 1998). The TTSS of *P. aeruginosa*, also known as the exoenzyme S regulon, includes four secreted effectors: ExoS, ExoT, ExoU and ExoY. Exoenzyme S (ExoS) and exoenzyme T (ExoT) have ADP-ribosylating activity toward low-molecular-mass GTP-binding proteins of the Ras family (McGuffie et al., 1998). Exoenzyme Y (ExoY) is an adenylyl cyclase whose activity is associated with profound morphological changes in epithelial cells (Yahr et al., 1998). Exoenzyme U (ExoU), which displays lipase activity *in vitro* (Sato et al., 2003), is responsible for the acute cytotoxicity of *P. aeruginosa* toward epithelial cells (Finck-Barbancon et al., 1997) and macrophages (Hauser & Engel, 1999). Expression of these exoenzymes is primarily regulated by a transcriptional activator, ExsA, in response to various environmental signals, including low calcium and direct contact with tissue culture cells (Yahr & Frank, 1994; Yahr et al., 1995, 1996; Hornef et al., 2000).

Despite this capacity to produce an arsenal of multiple exoproduct virulence determinants *P. aeruginosa* is able to grow in biofilms which also exist in the lungs of CF patients (Singh et al., 2000; Costerton, 2001). *P. aeruginosa* biofilms consist of bacterial communities embedded in an exopolysaccharide matrix. Interestingly, bacteria growing in biofilms possess characteristics distinct from their planktonic counterparts, including increased resistance against antimicrobial agents (Whiteley et al., 2001). Moreover, recent investigations have shown that gene expression differs remarkably during various stages of biofilm formation (Whiteley et al., 2001; Sauer et al., 2002) and that both the Las and the Rhl systems are differentially expressed (Davies et al., 1998; De Kievit et al., 2001). As the exoenzyme S regulon is triggered by eukaryotic cell contact, we assume that pseudomonads embedded in bacterial biofilms should not exhibit a remarkable activity of type III effectors, which primarily are used to provoke eukaryotic cell intoxication. If this is the case, this also would raise the question if quorum sensing signals (Las and Rhl systems), which are known to coordinate biofilm formation, are involved in the downregulation of the exoenzyme S regulon, in particular of the exoS gene. To examine this hypothesis, we initially determined ExoS production in *P. aeruginosa* biofilms and in mutants of the Las and Rhl quorum sensing systems. As green fluorescent protein (GFP) reporter strategies are a valuable tool for determining the expression of type III-dependent effector proteins at the single-cell level, we used an exoS'-gfp translational reporter fusion (Jacobi et al., 1998; Hornef et al., 2000). Our experiments revealed that under inducing conditions for type III secretion the ExoS production in sessile *P. aeruginosa* cells growing in a biofilm is downregulated, but stimulated in planktonic bacteria. Moreover, we have demonstrated that ExoS production is upregulated in the rhl mutant PDO100 and the rhlR mutant PDO111, whereas the lasI mutant showed no difference in exoS'-gfp expression compared to parent strain PAO1. As exoS'-gfp upregulation in PDO100 and PDO111 was growth-phase-dependent and most notably during the stationary phase, we further tested exoS'-gfp expression in the rpoS mutant SS24. It has been reported that RpoS and the Rhl system are coordinately regulated (Latifi et al., 1996; Whiteley et al., 2000) and that RpoS is involved in biofilm formation (Xu et al., 2001; Heydorn et al., 2002). Indeed, we could show that in the rpoS mutant SS24 upregulation of exoS'-gfp was significant in comparison to strain PAO1. These data suggest a negative regulatory effect of the Rhl system and the stationary-phase sigma factor RpoS on exoS expression and possibly on the exoenzyme S regulon. In the following report we provide evidence that *P. aeruginosa* downregulates the exoS gene and possibly downregulates the exoenzyme S regulon during formation of biofilms.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** All bacterial strains and plasmids used in this study are listed in Table 1. Unless indicated otherwise, *P. aeruginosa* was routinely grown at 37 °C in Luria–Bertani (LB) broth or on LB agar plates. Standard methods were used for preparation of electrocompetent cells and for plasmid electroporation into *P. aeruginosa* (Smith & Iglewski, 1989). To study growth-phase-dependent exoS gene expression by flow cytometry, we used the recently described pExoS-gfp reporter plasmid. This plasmid carries a *Sad–BanH1* fragment from strain PAO1 comprising *orfI* and 215 codons of the exoS gene ligated to gfp (exoS'-gfp reporter). Where required *P. aeruginosa* were grown under inducing conditions for type III secretion [tryptone soy broth supplemented with 10 mM nitritolriatríc acid, 100 mM monosodium glutamate and 1 % glycerol, designated stimulation medium (SM); Yahr et al., 1997; Hornef et al., 2000]. To ensure stable carriage of plasmid pExoS-gfp, chloramphenicol (200 μg ml⁻¹) was incorporated into the growth medium. In supplementation experiments with exogenous N-acylhomoserine lactones (AHLs), *P. aeruginosa* strains were cultivated for 3 h in SM, harvested by centrifugation and resuspended in an equal volume of fresh SM supplemented with synthetic AHLs (from P. Williams, University of Nottingham). Where indicated, C12-HSL or C4-HSL was added to SM prior to inoculation, at concentrations of 4 and 5 μM, respectively. Bacterial cells were incubated for an additional 9 h and re-examined for ExoS'-GFP fluorescence by flow cytometry.

**Static biofilm experiments.** For investigation of exoS'-gfp expression during biofilm formation, *P. aeruginosa* PA01 harbouring plasmid pExoS-gfp was grown in SM-filled Petri dishes containing polypropylene slides (76 × 26 mm) cut out from square Petri-dishes (Greiner Labortechnik). An overnight culture grown in Vogel–Bonner (VB) medium (Vogel & Bonner, 2001) and diluted 1 : 100 in fresh stimulation medium was used as inoculum. Chloramphenicol (200 μg ml⁻¹) was added to the growth medium to ensure the stable carriage of plasmid pExoS-gfp. Slides with attached bacteria were removed at 24 h, washed extensively at least three times with sterile PBS and fixed with 3 % formalin for 30 min. After fixation, slides were rinsed with sterile PBS and dried at room temperature. Staining was performed with 1 μg propidium iodide ml⁻¹ for 1 min to visualize attached bacteria. Planktonic bacteria growing in the supernatant were harvested in parallel, formalin-fixed and stained with propidium iodide. Images were obtained using a Leica Aristoplan epifluorescence microscope equipped with a standard filter set (Leica Microsystems) and a SPOT digital camera system (Diagnostic Instruments).

**Flow cytometric measurements of cell-density-dependent ExoS'-GFP production.** Overnight cultures (VB medium) of *P. aeruginosa* PA01, PAO-JP1, PDO-100, PDO111 and SS24,
Table 1. Bacterial strains and plasmids used in this study

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<tr>
<th>Strain or plasmid</th>
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<td><strong>P. aeruginosa</strong></td>
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<td>PAO1 (PAO1a)</td>
<td>Wild-type strain</td>
<td>Suh et al. (1999)</td>
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<tr>
<td>PAO1 (PAO1b)</td>
<td>Wild-type strain</td>
<td>Pearson et al. (1997)</td>
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<tr>
<td>PAO-JP1 (lasI)</td>
<td>lasI mutant derived from PAO1; Tc&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Pearson et al. (1997)</td>
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<tr>
<td>PDO100 (rhlI)</td>
<td>rhlI mutant (rhlA&lt;sup&gt;™&lt;/sup&gt;;Tn501-2) derived from PAO1, Hg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Brittn &amp; Ohman (1995)</td>
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<tr>
<td>PDO111 (rhlR)</td>
<td>rhlR mutant derived from PAO1</td>
<td>Brittn &amp; Ohman (1995)</td>
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<td>SS24 (ropS)</td>
<td>ropS mutant derived from PAO1</td>
<td>Suh et al. (1999)</td>
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<td>PAO1(pExoS-gfp)</td>
<td>PAO1 with plasmid pExoS-gfp</td>
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<td>SS24(pExoS-gfp)</td>
<td>SS24 with plasmid pExoS-gfp</td>
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<td><strong>Plasmids</strong></td>
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<td>pExoS-gfp</td>
<td>pKT248 carrying the exoS&lt;sup&gt;™&lt;/sup&gt;-gfp translational fusion</td>
<td>Hornef et al. (2000)</td>
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<td>pExoU-gfp</td>
<td>pKT248 carrying the exoU&lt;sup&gt;™&lt;/sup&gt;-gfp translational fusion</td>
<td>Hornef et al. (2000)</td>
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harbouring plasmid pExoS-gfp, were diluted in fresh SM without chloramphenicol to an OD<sub>600</sub> of 0.1 and were grown at 37 °C with shaking (200 r.p.m.). Samples were taken approximately every 2 h followed by determination of OD<sub>600</sub>. Prior to ExoS<sup>™</sup>-GFP measurement, bacterial cells were washed twice with PBS and finally resuspended in 1 ml PBS. P. aeruginosa PAO1 harbouring the control plasmid pKT-gfp (pKT248 carrying the gfp gene without the exoS promoter) was used as negative control (Hornef et al., 2000). A Coulter Epics flow cytometer (Beckman Coulter) equipped with an argon 488 nm laser was used to measure the intensity of fluorescence of ExoS<sup>™</sup>-GFP-producing bacteria. In vitro stimulated bacteria were detected by side scatter as described by Russo-Marie et al. (1993). The intensity of fluorescence was determined and a gate was set corresponding to the bacterial population obtained from liquid culture. Fluorescence data and scatter data were collected for 50,000 events and mean fluorescence intensity was calculated. Flow cytometric results were verified by microscopy using a Leica Aristoplan epifluorescence microscope.

**Immunoblot analysis of ExoS<sup>™</sup>-GFP in bacterial cell lysates.** For analysis of ExoS<sup>™</sup>-GFP production via immunoblotting, pseudomonads were grown in SM to identical OD<sub>600</sub> values of about 2.0, washed twice with PBS and resuspended in Laemmli electrophoresis buffer (Laemmli, 1970). The protein concentration of cell lysates was determined using the Bio-Rad protein assay. Prior to electrophoresis, SDS, β-mercaptoethanol and bromophenol blue were added to about 10 μg protein, samples were boiled and were loaded onto SDS 12% polyacrylamide gels and either stained with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose. Blotted ExoS<sup>™</sup>-GFP was detected with monoclonal rabbit anti-GFP (BD Biosciences) and visualized on immunoblots using enhanced chemoluminescence (ECL; Amersham Pharmacia Biotech) (see Fig. 4a). This experiment was performed on each of the strains PAO1, SS24, PAO-JP1 and PDO100 with plasmid pExoU-gfp to visualize ExoU<sup>™</sup>-GFP.

**RNA isolation and Northern blot analysis.** Total RNA of P. aeruginosa PAO1, SS24, PAO-JP1 and PDO100 (all lacking plasmid pExoS-gfp) was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics). For Northern blots, RNA was isolated from stationary-phase cells (OD<sub>600</sub> of about 2.0) grown in SM, separated in a denaturating formaldehyde gel and transferred to Hybond-N<sup>™</sup> nylon membranes (Amersham Pharmacia Biotech). The exoS probe was generated by PCR using the primers exoS1 (5'-ACCCTGAC-ACCGAAGGACT-3') and exoS2 (5'-CATACCTTGCGATCATGC-3') with chromosomal DNA of PAO1 as a template. The probe was labelled with UTP using PCR procedures. Hybridization and washings were performed using the DIG Easy Hyb System (Roche Diagnostics) according to the manufacturer’s instructions. Fragment sizes were estimated by comparison with the 0.3–6.9 kb RNA ladder from Roche Diagnostics (see Fig. 4b).

**RESULTS**

**Production of ExoS<sup>™</sup>-GFP in P. aeruginosa biofilms and in planktonic growing bacteria.** P. aeruginosa PAO1 harbouring the reporter plasmid pExoS-gfp was grown at 37 °C in SM-filled Petri dishes containing polypropylene slides until a subconfluent bacterial biofilm was formed on the slides. Chloramphenicol was added to the growth medium to ensure that bacteria did not lose plasmid pExoS-gfp. At about 18 h, sessile bacteria visualized by propidium iodide staining (Fig. 1a) were distributed over the slide, partly arranged as mushroom-like structures. Sessile bacteria growing in a biofilm and planktonic bacteria remaining in the broth supernatant were examined separately for ExoS<sup>™</sup>-GFP production. As shown in Fig. 1, sessile bacteria on the biofilm did not show GFP fluorescence (Fig. 1b), whereas planktonic bacteria emitted a bright green fluorescence, indicating expression of the exoS<sup>™</sup>-gfp reporter gene (Fig. 1d). Under inducing
conditions for type III secretion, \( \text{exoS} \)-\( \text{gfp} \) expression increased with time and the difference in ExoS'-\( \text{GFP} \) fluorescence between bacteria attached to polypropylene slides and suspended cells was most pronounced after 18–24 h (Fig. 1). Covering of polypropylene slides with fresh SM led to detachment of numerous bacteria, whereas adherent bacteria showed no GFP fluorescence. These results indicate that \( P. \text{aeruginosa} \) downregulates \( \text{exoS} \) during the formation of biofilms.

**Cell-density-dependent ExoS'-\( \text{GFP} \) synthesis in mutants of the \( P. \text{aeruginosa} \) Las and Rhl systems**

To examine whether the expression of \( \text{exoS} \) in \( P. \text{aeruginosa} \) is influenced by quorum sensing regulatory pathways, we electroporated pExoS-gfp into \( P. \text{aeruginosa} \) las and rhl mutants, respectively, and determined the intensity of fluorescence of ExoS'-\( \text{GFP} \)-producing bacteria in relation to cell density. The parental PAO1 strain harbouring the plasmid pKT-gfp was used as a negative control and showed virtually no fluorescence (Fig. 2). This indicates that there was no cell-density-dependent \( \text{gfp} \) expression, possibly caused by other upstream-located promoters of the control plasmid. In contrast, \( P. \text{aeruginosa} \) PAO1 with plasmid pExoS-gfp showed a rise in GFP fluorescence per bacterial cell with increasing cell density. When compared to parental strain PAO1, the lasI mutant PAO-JP1 showed no difference in ExoS'-\( \text{GFP} \) fluorescence, whereas the rhlI mutant PDO100 exhibited a notable increase in \( \text{exoS} \)-\( \text{gfp} \) expression, particularly in the late stationary growth phase (Fig. 2). These data suggest that in \( P. \text{aeruginosa} \) a regulatory link between \( \text{exoS} \) expression and Rhl-dependent quorum sensing pathways exists. In agreement with these results, the rhlR mutant PDO111 also exhibited increased ExoS'-\( \text{GFP} \) fluorescence compared to that of PAO1 (Fig. 2). Moreover, to corroborate the influence of the Rhl system on type III secretion we analysed the expression of the \( \text{exoU} \)-\( \text{gfp} \) reporter in the quorum sensing mutant strains PDO100 and JP-1. As expected, at an \( \text{OD}_{600} \) of 1·5 we found a 2.6-fold upregulation of ExoU'-\( \text{GFP} \) in strain PDO100 (rhlI) in comparison to PAO1, but not in strain JP1 (lasI) (data not shown).

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**Fig. 1.** Propidium iodide-stained \( P. \text{aeruginosa} \) PAO1 biofilm attached to polypropylene slides (a). Planktonic bacteria growing in the supernatant (c). Synthesis of ExoS'-\( \text{GFP} \) translational fusion in sessile \( P. \text{aeruginosa} \) growing in a biofilm (b) in contrast to ExoS'-\( \text{GFP} \) expression in free-floating bacterial cells remaining in the broth supernatant (d) after incubation for 18 h in ExoS SM. Bars, 5 \( \mu \text{m} \).
ExoS'-GFP synthesis is upregulated in the rpoS mutant SS24

Previous studies reported that Rhl quorum sensing activates the expression of rpoS. RpoS, the stationary-phase sigma factor, originally identified in Escherichia coli and Salmonella typhimurium, is responsible for the transcription of a variety of genes expressed after cells enter stationary phase or during starvation and stress conditions. Expression of rpoS increases during entry into stationary phase and was found to be elevated in P. aeruginosa biofilms (Xu et al., 2001). From this we hypothesized that RpoS might be involved in the negative regulation of exoS expression. To address this issue we measured the expression of the exoS'-gfp reporter gene activity in the rpoS mutant SS24. Fig. 3 shows that strain SS24 exhibited a remarkable upregulation of exoS'-gfp in comparison to PAO1. In agreement with the data from the rhl mutants PDO100 and PDO111, the GFP fluorescence in SS24 was found to be dependent on cell density and reached its maximum during late stationary phase with approximately 2·5- and 5·0-fold higher GFP fluorescence at an OD
600
0 of 2·0 than PDO100 and PAO1, respectively (Figs 2 and 3). To verify the flow cytometric results, ExoS'-GFP production and exoS mRNA of strains PAO-JP1, PDO100, SS24 and PAO1 were detected by immunoblotting with a monoclonal GFP-specific antibody and by Northern blotting with an exoS-specific probe, respectively. Fig. 4(a) shows the immunoblot of ExoS'-GFP production of all the different strains. PAO1a (parent strain of rpoS mutant SS24) and PAO1b (parent strain of rhlI mutant PDO100) produced comparable amounts of ExoS'-GFP (only PAO1a is shown). In agreement with the cytofluorometry (fluorescence-activated cell sorter) analyses, PAO1-JP1 displayed no notable difference in ExoS'-GFP synthesis, whereas PDO100 and SS24 showed an increase in ExoS'-GFP production compared to PAO1. For exoS transcript analysis RNA was isolated from stationary-phase cells (all lacking plasmid pExoS-gfp), separated by a formaldehyde gel, transferred to nylon membrane and hybridized with an exoS probe. In spite of some degradation of the RNA, this Northern blot analysis confirmed the increased transcription of exoS in strains PDO100 and SS24 (Fig. 4b). In summary, these data demonstrate that, especially in the stationary phase, exoS expression is downregulated by both the Rhl quorum sensing system and RpoS. Interestingly, in contrast to exoS'-gfp, we found no upregulation of the exoU'-gfp reporter in strain SS24, indicating that the exoS and the
exoU genes are differentially regulated in the rpoS mutant (data not shown).

Exogenously added C4-HSL reduced the exoS'-gfp upregulation in the rhlI mutant PDO100 to wild-type level

The putative rhl-dependent downregulation of exoS expression was further confirmed by supplementation experiments with exogenous AHLs. Disruption of the autoinducer synthase gene, rhlI, abolished C4-HSL production. Therefore, we determined whether exoS'-gfp expression in PDO100 could be repressed by exogenously added C4-HSL. For this purpose, strains PAO1, PDO100 (rhlI) and PAO-JP1 (lasI) were grown in SM to mid-exponential phase and inoculated with fresh medium containing either 5 μM C4-HSL or 4 μM C12-HSL. After 9 h incubation the GFP fluorescence intensity of PDO100 was found to be similar to that of PAO1 and PAO-JP1, indicating that addition of C4-HSL represses exoS'-gfp expression in the rhlI mutant as expected (Fig. 5a and b). In contrast, addition of C12-HSL had only a slight effect on exoS'-gfp expression of the rhlI mutant. As expected, neither the addition of C4-HSL nor of C12-HSL to PAO1-JP1 (lasI) led to a change in ExoS'-GFP production (Fig. 5c). Finally, we

Fig. 4. (a) Western blot analysis of P. aeruginosa PAO1 (lane 1) and mutant strains SS24 (lane 2), PAO-JP1 (lane 3) and PDO100 (lane 4) using monoclonal anti-GFP antibody (all strains carrying pExoS-gfp). Each lane was loaded with 10 μg protein. The blot of whole-cell lysates was developed using enhanced chemiluminescence. The molecular mass standard is labelled on the left side of the figure. The relative mobility of ExoS'-GFP is indicated. (b) Northern blot analysis of exoS transcripts. RNA was isolated from strains PAO1 (lane 1), SS24 (lane 2), PAO-JP1 (lane 3) and PDO100 (lane 4) and harvested during stationary phase. RNA (1 μg) was separated in a denaturating formaldehyde gel and transferred to a Hybond-N+ nylon membrane. The exoS structural gene was amplified using primers exoS1 and exoS2 and genomic DNA of PAO1 as template. The resulting PCR product was labelled with digoxigenin (DIG)-UTP and used as a probe for hybridization.

Fig. 5. ExoS'-GFP production by parent strain PAO1 and mutants PDO100 (rhlI) and PAO1-JP1 (lasI), all harbouring plasmid pExoS-gfp in the absence of exogenously added AHLs and in the presence of 5 μM C4-HSL or 4 μM C12-HSL, respectively (indicated by arrow). Strains were grown for 3 h in SM prior to inoculation with AHLs (t=0). Bacterial cells were incubated for an additional 9 h and re-examined for ExoS expression by flow cytometry. The results represent the means of three different measured values and were performed three times for each strain.
could demonstrate that addition of C4-HSL to the liquid culture of rpoS mutant SS24 did not change the expression of the exoS\(^{-}\)-gfp reporter (data not shown), suggesting that RpoS is downstream of the Rhl-RpoS-ExoS regulatory cascade. In summary, these data provide further evidence that, especially in stationary phase, exoS expression is negatively regulated by Rhl quorum sensing and RpoS.

**DISCUSSION**

The major sets of secreted pathogenicity factors of *P. aeruginosa* can be divided into two groups. One group includes the exotoxin ExoA and diverse degrading enzymes such as proteases and lipases, which are released into the environment by the type II protein secretion system Xcp. The expression of the Xcp machinery and the cognate substrates are positively regulated by autoinducer systems (Gambello et al., 1993; Chapon-Herve et al., 1997). The second group of secreted pathogenicity factors comprises cellular modulins such as the exotoxins ExoS, T, U and Y, which are secreted and translocated into host cells through an ‘injection needle’ of the TTSS (Psc apparatus), known as the exoenzyme S regulon. Presumably, secreted proteases (e.g. LasA and LasB) impair the surface-exposed portion of the Psc apparatus and, therefore, it appears reasonable that the Xcp and the Psc systems should be coordinately, possibly reciprocally regulated. In this work, we focussed on the regulatory cascade linking quorum sensing and RpoS with the exoS gene, a representative of the exoenzyme S regulon. We have taken advantage of the exoS\(^{-}\)-gfp reporter translational fusion to determine regulation of the exoS gene at the single-cell level, which allowed direct monitoring of differential expression in planktonic and sessile bacteria. As a first approach we have demonstrated that exoS\(^{-}\)-gfp is downregulated when *P. aeruginosa* forms biofilms (Fig. 1). This appears to be reasonable, because *Pseudomonas aeruginosa* biofilms covering solid substrates do not require a sophisticated TTSS protein injection apparatus to be directed against phagocytic cells. Biofilm formation and autoinducer production has been demonstrated for *P. aeruginosa* colonizing the respiratory tract of CF patients (Erickson et al., 2002; Favre-Bonte et al., 2002). In this case exoenzyme S might not be required to protect *P. aeruginosa* against the cellular host defences (Frank, 1997) because the microbes are organized as alginate-embedded microcolonies and thus are sufficiently protected.

Next we studied the involvement of the autoinducer system in regulation of exoS expression by exoS\(^{-}\)-gfp measurement. Starting with the late-exponential growth phase (Fig. 2), ExoS\(^{-}\)-GFP fluorescence increased significantly in the rhlI and rhlR mutants. These results were confirmed by adding C4-HSL (the cognate autoinducer of RhlR) to the liquid culture of the rhlI mutant which led to an ExoS\(^{-}\)-GFP level comparable to that of parental strain PAO1 (C4-HSL-producer). Due to the Las/Rhl hierarchical relationship we would also expect upregulation of exoS\(^{-}\)-gfp in the lasI mutant in comparison to the parent strain PAO1. However, this could not be demonstrated, which underlines the complexity of cross-regulation between the Las/Rhl system and several other regulators such as the quorum sensing controlled repressor QscR and the recently reported heterodimerization between QscR and LasR and RhlR in the absence of AHLs (Ledgham et al., 2003). Furthermore, we checked the transcription of exoS (Northern blotting) and the production of ExoS\(^{-}\)-GFP (immunoblotting) of the lasI mutant PAO-JP1, the rhlI mutant PDO100, the rpoS mutant SS24 and PAO1 (Fig. 4), and can confirm the results obtained with the exoS\(^{-}\)-gfp reporter. In conclusion, we have no clear results with lasI or rhlI mutants which allows interpretation in terms of the hierarchical organization of the quorum sensing regulatory cascade. However, the results obtained from rhlI and rhlR mutants were consistent, suggesting that the Rhl system regulates the expression of the exoS gene. This finding was strengthened by the analysis of the exoU\(^{-}\)-gfp reporter, which was upregulated in the rhlI mutant as well. Moreover, we have demonstrated that the sigma factor RpoS may also be involved in exoS regulation, although there are conflicting results with respect to the link between rpoS and RhlR/C4-HSL (Latifi et al., 1996; Whiteley et al., 2000). Although the negative regulatory effect of RpoS on ExoS\(^{-}\)-GFP expression was not found for ExoU\(^{-}\)-GFP, our results have clearly shown a significant upregulation of the exoS\(^{-}\)-gfp reporter fusion (Fig. 3) and the exoS gene (Northern blot, Fig. 4b) when RpoS is lacking. The difference in the expression of ExoS\(^{-}\)-GFP and ExoU\(^{-}\)-GFP in strain SS24 implies that exoS and exoU, which do not naturally coexist in the *P. aeruginosa* chromosome and which represent an ADP-ribosyltransferase and a lipase, respectively, are differentially regulated. However, there are no data which could explain a direct repressive effect of RpoS on exoS transcription.

Alternatively, it is also conceivable that RpoS induces the expression of a transcriptional repressor acting on the exoenzyme S regulon. It is known that exoS is upregulated by the transcriptional activator ExsA which is suggested to bind upstream of the exoS promoter. Moreover, ExsD functions as negative regulator or antiactivator of additional TTSS genes by interaction with ExsA (McCaw et al., 2002). Unfortunately, nothing is known about dissociation conditions of ExsD/ExsA heterodimers. Recently, it was shown that the cAMP-binding protein Vfr, which also acts on the transcriptional control of the Las system (Albus et al., 1997), positively regulates the *P. aeruginosa* TTSS, including effector genes, genes of the Psc apparatus, and the regulatory exsC-exsA and exsD-pscL operons (Wolfgang et al., 2003). In summary, Vfr and ExsA are central regulators of TTSS in *P. aeruginosa*. Interestingly, in *E. coli* and probably in *P. aeruginosa* Vfr is involved in RpoS repression (Venturi, 2003; Bertani et al., 2003), possibly counteracting RpoS-dependent downregulation of exoS expression. Moreover, microarray experiments have revealed that both exsC (PA1710) and exsB (PA1712) (which regulates type III-dependent ExoS secretion) are...
downregulated in *P. aeruginosa* biofilms (Whiteley et al., 2001; Wagner et al., 2003). These data fit well with the downregulation of *exoS* shown in this study.

In contrast to *P. aeruginosa* TTSS, the TTSS of the LEE pathogenicity island of enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) has been described as being upregulated by the quorum sensing regulator QseA which activates the LEE-encoded regulator Ler, and thus LEE operons (Sperandio et al., 1999, 2002a, b). In these cases EPEC and EHEC do not form typical biofilms or microcolonies, but rather form a monolayer on the surface of mucosal epithelial cells and the LEE operons which may be triggered by AHLs produced by gut bacteria. Thus, the infection strategy of these enteric *E. coli* is completely different from *Pseudomonas*.

In conclusion, our data have shown that in *P. aeruginosa* the Rhl system and RpoS exhibit an inhibitory effect (directly or indirectly) on *exoS* expression and are probably embedded in a complex regulatory pathway which controls the action of the type III secretion machinery of *P. aeruginosa*. Further studies are currently underway to analyse the role of RpoS on *exsD* and *vfr* regulation and the trigger mechanism for ExsA activation to elucidate the regulatory link between quorum sensing and TTSS.

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**REFERENCES**


Controlling P. aeruginosa exoS expression


