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The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility

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Pseudomonas aeruginosa is an important nosocomial opportunistic human pathogen and a major cause of chronic lung infections in individuals with cystic fibrosis. Serious infections by this organism are often treated with a combination of aminoglycosides and semi-synthetic penicillins. Subinhibitory concentrations of antibiotics are now being recognized for their role in microbial persistence and the development of antimicrobial resistance, two very important clinical phenomena. An extensive screen of a *P. aeruginosa* PAO1 luciferase gene fusion library was performed to identify genes that were differentially regulated during exposure to subinhibitory gentamicin. It was demonstrated that subinhibitory concentrations of gentamicin and tobramycin induced a set of genes that are likely to affect the interaction of *P. aeruginosa* with host cells, including the gene encoding Lon protease, which is known to play a major role in protein quality control. Studies with a *lon* mutant compared to its parent and a complemented strain indicated that this protein was essential for biofilm formation and motility in *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative rod, causes rapidly progressing infections in hospitalized individuals, especially those with a compromised immune system. In addition, it is the predominant respiratory pathogen in cystic fibrosis patients, causing chronic lung infections that once established are impossible to eradicate. Therapy of serious infections caused by *P. aeruginosa* often consists of the combination of a semi-synthetic penicillin and an aminoglycoside, since cross-resistance between major classes of anti-pseudomonal antibiotics is less likely to develop (Hancock & Speert, 2000). Inhaled antimicrobial agents used in patients with cystic fibrosis have included carbenicillin, gentamicin, cephaloridine, tobramycin, colistin, polymyxin B and amikacin alone or in combination with parenteral antibiotics (Hancock & Speert, 2000). However, optimization of the current therapy of *P. aeruginosa* infections presents an intimidating problem for medical practitioners since these bacteria show intrinsic resistance to a wide range of antimicrobial agents. In addition, the ability of *P. aeruginosa* to develop further resistance during antibiotic therapy is a major problem for cystic fibrosis patients, due to the repeated therapeutic courses required to control chronic infection.

Intrinsic resistance in *P. aeruginosa* has been shown to be due to restricted antibiotic uptake through the outer membrane coupled to secondary mechanisms such as active efflux and inducible antibiotic-degrading enzymes (Hancock, 1997). For aminoglycosides, uptake across the outer membrane has been shown to involve the self-promoted uptake pathway and is opposed by active efflux through the MexXY RND efflux pump in collaboration with a variety of outer-membrane channels (Aires *et al.*, 1999; Jo *et al.*, 2003; Vogne *et al.*, 2004). *P. aeruginosa* also demonstrates the ability to develop adaptive resistance to aminoglycosides (Karlowsky *et al.*, 1997) that is reversible upon removal of selective pressure. Little is known about the mechanisms of adaptive resistance in *P. aeruginosa*, although Macfarlane *et al.* (2000) demonstrated that susceptibility to aminoglycosides could be regulated by the two-component regulatory system PhoPQ, which responds to divalent cation concentrations. Furthermore it was shown that the adaptive resistance process requires the rapid production of the multidrug efflux pump MexXY and the interaction of these proteins with the constitutively produced outer-membrane component OprM (Hocquet *et al.*, 2003). Recently it was shown that subinhibitory concentrations of aminoglycoside antibiotics induce biofilm formation, which contributes to bacterial persistence in chronic infections (Hoffman *et al.*, 2005).

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A supplementary table of primers is available with the online version of this paper.

In this work global gentamicin-induced gene regulation was studied in *P. aeruginosa*. Genome-wide screening approaches to elucidate mechanisms of antibiotic action and resistance are often approached by DNA-microarray analysis (Lin *et al.*, 2005; reviewed by Brazas & Hancock, 2005) and mutant library screening (Goh *et al.*, 2002; Lewenza *et al.*, 2005). The screening of mutant libraries is of special interest and value because it combines elements of both transcriptome and proteome approaches since mutants can be generated with additional properties such as promoterless reporter genes (e.g. *lux*) to generate transcriptional or translational fusions to the inactivated gene (Lewenza *et al.*, 2005). We demonstrate that under the tested conditions a substantial set of genes are induced in *P. aeruginosa* that are favourable for this pathogen to adapt to the human host. This approach led to the discovery that the Lon protease of *P. aeruginosa* is induced by subinhibitory concentrations of aminoglycosides, and is also essential for biofilm formation and motility in this pathogen.

METHODS

Bacterial strains and media. *P. aeruginosa* PAO1 and derived Tn*5* mutants (Lewenza *et al.*, 2005) were used. All bacterial strains were grown at 37 °C in Luria–Bertani (LB) broth. Tn*5* insertions mutants were maintained on 50 μg tetracycline ml^{-1}.

Mutant library screening. A random pool of mutants (9408 individual colonies in all) from a mini-Tn*5*-*luxCDABE* mutant library in *P. aeruginosa* (Lewenza *et al.*, 2005) was screened for alterations in luminescence when grown on LB agar containing 0.25 μg gentamicin ml^{-1} (corresponding to 0.25 of the MIC on LB agar plates) compared to LB agar without antibiotic. Mutants were grown overnight in 96-well microtitre trays containing LB broth, diluted 1 : 1000 in LB broth and transferred with a custom 96-well pin device onto the surface of LB agar plates containing 1.5 % (w/v) Difco agar (Becton-Dickinson) with or without gentamicin. Gene expression was analysed by measuring luminescence as described previously (Lewenza *et al.*, 2005). The luminescence produced by the ATP-dependent luciferase system was detected and quantified using a ChemiGenius Bio-Imaging System (Syngene).

Cloning of the *lon* gene. To permit complementation of the *lon*::*lux* mutant, the ORF of the *lon* gene PA1803 was PCR-amplified from genomic DNA of PAO1 using the primers PA1803UHind and PA1803DXba (see Table S1, available with the online version of this paper, for details of primers). This amplicon was then cloned into TopoBlunt (Invitrogen). The *Hin*dIII–*Xba*I fragment containing the *lon* gene was ligated into the broad-host-range vector pBBR1-MCS5 (Kovach *et al.*, 1995), resulting in pBBR1-MCS5::lon^+, which was transformed into *Escherichia coli* XL-1 Blue and subsequently into *E. coli* S17-1 for mobilization into *P. aeruginosa* 74_D9 (*lon*::*lux*) through conjugation. This resulted in the complemented strain *lon*::*lux*/pBBR1-MCS5::lon^+, hereafter called c*lon*::*lux*.

Motility and biofilm assays. Swimming motility was evaluated on BM2 glucose plates [62 mM potassium phosphate buffer pH 7, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM FeSO4, 0.4 % (w/v) glucose] containing 0.3 % Difco agar, and swarming on modified BM2 [with 0.5 % Casamino acids substituted for 7 mM $(NH_4)_2SO_4$] glucose plates containing 0.5 % Difco agar. Swimming and swarming colony formation were evaluated by seeding with a 1 μl aliquot of an overnight culture grown on LB, and plates were incubated for 20 h at 37 °C. Twitching was measured after 24–48 h incubation at 37 °C on LB broth plates containing 1 % agar (Kohler *et al.*, 2000). For biofilm formation assays, 5×10^4 bacteria were inoculated into each well of a polystyrene 96-well microtitre plate containing 100 μl LB broth in each well. The plate was incubated for 20 h at 37 °C without shaking. Biofilms were stained with 1 % (w/v) crystal violet and absorbance measurements were taken at 595 nm (as described by Friedman & Kolter, 2004).

Growth experiment. Cells of *P. aeruginosa* PAO1 and *lon*::*lux* were grown overnight in 100 μl LB. Then 1 μl of the overnight culture was inoculated into 100 μl LB broth and growth at 37 °C was monitored using a TECAN Spectrafluor Plus by measuring the OD_{595} every 20 min without shaking.

Minimal inhibitory concentrations (MICs). MICs were assessed using standard broth microdilution procedures in LB broth (McPhee *et al.*, 2003). Growth was scored after 24 h incubation at 37 °C.

Real-time PCR. Total RNA was isolated from mid-exponential-phase *P. aeruginosa* grown in liquid LB medium with or without 0.25 MIC gentamicin (1.25 μg ml^{-1}) using RNeasy midi columns (Qiagen). Four micrograms of total RNA was combined with 750 ng random primers [random decamer $(NS)_5$, Invitrogen] and heated for 10 min at 70 °C, followed by 10 min incubation at 25 °C. Reverse transcription was done by adding 0.5 mM dNTPs, 500 U SuperRNase (Ambion) ml^{-1}, 10 μM DTT, 10 000 U SuperScriptII (Invitrogen) ml^{-1} in 1 × reaction buffer and incubating at 37 °C for 1 h followed by 2 h at 42 °C. The cDNA was centrifuged at 14 000 r.p.m. for 1 min and 500 U SuperRNase (Ambion) ml^{-1} was added before incubation at 37 °C for 45 min. The RNA was destroyed by the addition of 170 mM NaOH and incubation at 65 °C for 10 min. The reaction was neutralized by addition of 170 mM HCl. The cDNA was then used as template for real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7000 (Applied Biosystems). The real-time PCR was carried out in 1 × SYBR Green Master Mix, with 200 nM forward and reverse primers (see Table S1) and 1 μl cDNA. All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

Phage PO4 sensitivity test. Bacteria were grown overnight in LB broth at 37 °C. Cells were harvested, washed with sterile LB and diluted to OD_{600} 0.05 in LB soft agar. Ten microlitres of phage PO4 suspension was added to 2 ml of these cultures, mixed gently, and overlaid on 1.5 % (w/v) LB agar plates. Plates were incubated overnight at 37 °C and phage PO4 sensitivity was detected by plaque formation on the lawn of bacteria.

RESULTS

Identification of gentamicin-induced genes in *P. aeruginosa*

Upon treatment with tobramycin and gentamicin in aerosol form, a fraction of the infecting bacteria may inevitably be exposed to subinhibitory levels of antibiotic that select for subpopulations exhibiting adaptive resistance, especially since it is known that the exact same strain can persist for years in a single patient despite apparent therapeutic success (Doring *et al.*, 2000). To study the influence of sub-MIC gentamicin on gene transcription in *P. aeruginosa* PAO1, we screened a mini-Tn*5*-*luxCDABE* fusion library (Lewenza *et al.*, 2005) for increased luminescence when grown in the presence of 0.25 μg ml^{-1} gentamicin (0.25 MIC) compared to luminescence in the absence of antibiotic. The luminescence of 37 of the 9408 tested *lux* fusions was up-regulated

Table 1. Properties of transposon fusion strains up-regulated by gentamicin and tobramycin

Transposon insertion sites were determined using the protocol of Lewenza *et al.* (2005); * indicates intergenic fusion, and the mutant ID is the unique library identifier at http://pseudomutant.pseudomonas.com/. Pr., probable. Bioluminescence in the presence of gentamicin (Gm), tobramycin (Tb) and polymyxin B (PmB) was assessed relative to the same strain without antibiotic. The mean and standard deviation of bioluminescence was determined from three independent experiments. Shown are the ratio values comparing bioluminescence when bacteria were cultured with and without antibiotics. Selected genes were independently confirmed to demonstrate activation of gene regulation in the presence of gentamicin using real-time PCR. FC is the mean fold change of transcript upon growth in LB with 0.25 MIC gentamicin, normalized to the housekeeping gene *rpsL* and relative to the amount of transcript upon growth without gentamicin (comparative C_T method) determined by real-time PCR. Gentamicin MIC values were determined by standard broth microdilution procedures in LB broth supplemented with different gentamicin concentrations (McPhee *et al.*, 2003). Growth was scored after 24 h incubation at 37 °C. The most frequently observed MICs out of six independent measurements are shown.

Transposon insertion site	Function class	Description	Mutant ID	Fold change in luminescence at 0.25 MIC			Real-time PCR (FC)	MIC (μg ml^{-1})
				Gm	Tb	PmB		
PA0603	Transport of small molecules	Pr. ATP-binding component of ABC transporter	97_H9	3.4±0.8	2.7±0.37	1.0±0.17		
PA0605	Transport of small molecules	Pr. permease of ATP transporter	49_B7	3.1±0.58	2.4±0.48	1.3±0.03		2
PA0605	Transport of small molecules	Pr. permease of ATP transporter	93_F10	2.5±0.14	2.7±0.16	1.4±0.1		2
PA0240 (*opdF*)	Transport of small molecules	Pr. porin	42_E9	2.2±0.12	2.8±0.25	1.0±0.12		2
PA1041/PA1040*	Transport of small molecules	Pr. outer-membrane protein	75_G1	2.1±0.06	1.8±0.28	1.3±0.15		2
PA2018 (*mexY*)/*17**	Antibiotic resistance	RND multidrug efflux transporter	6_H8	7.1±0.73	13.3±5.62	1.4±0.18	26	2
PA4961	Pr. membrane protein	Unknown function	6_G1	3±0.27	1.7±0.22	1.6±0.16	3.2	1
PA4226 (*pchE*)	Transport of small molecules	Dihydroaeruginoic acid synthetase	45_F1	3.4±0.36	2.5±0.47	1.8±0.77		2
PA4225 (*pchF*)	Transport of small molecules	Pyochelin synthetase	52_F5	3.2±1.52	1.6±0.2	2.3±1.11		2
PA4220 (*fptB*)	Unknown secreted	Upstream of pyochelin receptor	65_B7	2±0.18	2.5±0.56	1.2±0.05		2
PA4554 (*pilY1*)	Motility and attachment	Type 4 fimbrial biogenesis protein PilY1	51_H7	3.9±0.81	2.8±0.88	1.2±0.26		2
PA4554 (*pilY1*)	Motility and attachment	Type 4 fimbrial biogenesis protein PilY1	76_B9	3.7±0.73	3.2±0.21	1.1±0.09		2
PA4554 (*pilY1*)	Motility and attachment	Type 4 fimbrial biogenesis protein PilY1	97_G2	3.5±0.83	2.6±0.48	1.1±0.08		2
PA4554 (*pilY1*)	Motility and attachment	Type 4 fimbrial biogenesis protein PilY1	94_F5	3.4±0.11	3.0±0.4	1.1±0.19		2
PA1080 (*flgE*)	Motility and attachment	Flagellar hook protein FlgE	51_A9	3.3±0.42	2.0±1.1	1.5±0.98	2.5	2
PA1086 (*flgK*)	Motility and attachment	Flagellar hook-associated protein 1 FlgK	24_H1	2.7±0.42	2.5±0.21	1.2±0.09		2
PA1454 (*fleN*)	Motility and attachment	Flagellar synthesis regulator FleN	71_A7	2.1±0.11	2.3±0.10	1.4±0.11	5.6	2
PA1101 (*fliF*)	Motility and attachment	Flagella M-ring outer-membrane protein	95_E2	NA	NA	NA		2
PA3352	Motility and attachment	FlgN homologue (chaperone in secretion?)	1_H3	3.7±0.39	2.2±0.64	1.3±0.05	3.5	2
PA3353	Unknown	Unknown function	9_A6	3.4±0.30	2.5±0.51	1.6±0.27		2
PA0998 (*pqsC*)	Quorum sensing	β-Keto-acyl-ACP synthase homologue	28_C6	2.6±0.13	2.4±0.74	1.1±0.16	11.3	2
PA3182 (*pgl*)	Central intermediary metabolism	6-Phosphogluconolactonase	9_A2	4±0.48	2.5±0.69	1.5±0.45	1.3	2
PA0794	Energy metabolism	Pr. aconitate hydratase	67_G8	2.6±0.42	3.0±0.69	1.1±0.16		2
PA2969 (*plsX*)	Fatty acid metabolism	Fatty acid biosynthesis protein PlsX	69_H7	3.2±0.81	1.1±0.51	1.2±0.09		2
PA3067	Transcriptional regulators	Pr. transcriptional regulator	66_C1	2.1±0.19	3±0.37	1.2±0.13		2
PA3965	Transcriptional regulators	Pr. transcriptional regulator	14_B10	2.3±0.08	3.3±0.69	1.1±0.07	6.5	2

Table 1. cont.

Transposon insertion site	Function class	Description	Mutant ID	Fold change in luminescence at 0.25 MIC			Real-time PCR (FC)	MIC (μg ml^{-1})
				Gm	Tb	PmB		
PA0294 (*aguR*)	Transcriptional regulators	Transcriptional regulator AguR	46_C9	3.8±0.34	2.8±0.13	1.3±0.22		2
PA5356 (*glcD*)/PA5355 (*glcC*)*	Transcriptional regulators	Transcriptional regulator GlcC	1_H2	3.8±0.42	2.7±0.19	1.5±0.21	8.6	2
PA3385 (*amrZ*)/PA3384 (*phnC*)*	Transcriptional regulators/transport of small molecules	Alginate and motility regulator Z/phosphonate transporter	1_F8	2.3±0.22	3.1±0.12	1.1±0.08	4.5	2
PA0933 (*ygcA*)	Post-translational modification	Pr. RNA methyltransferase	62_H10	2.0±0.51	3.0±0.38	1.0±0.15		2
PA0428 (*rhlE*)	Transcription, RNA processing and degradation	Pr. ATP-dependent RNA helicase	41_B1	2.6±0.09	2.6±0.23	1.1±0.08		2
PA1803 (*lon*)	Adaptation, translation, degradation	Lon protease	74_D9	2.3±0.04	3.6±0.45	1.2±0.10	10	1
PA0779	Putative enzymes	Pr. ATP-dependent protease	100_A2	2.5±0.37	4.1±1.09	1.2±0.18		2
PA0621	Related to phage	Conserved hypothetical protein	8_A7	9.5±3.97	3.6±0.88	1.8±0.03		2
PA1939	Unknown	Unknown function	43_C6	2.3±0.06	2.4±0.46	1.3±0.38		2
PA0318	Unknown	Unknown function	34_B3	3.2±0.66	2.0±1.3	1.2±0.04		2
PA3712	Unknown	Unknown function	24_E4	2.7±0.37	2.5±0.49	1.1±0.08		2

(⩾2-fold) in the presence of sub-MIC gentamicin. The insertion sites of all gentamicin-regulated *lux* fusions were mapped using an earlier described protocol (Lewenza *et al.*, 2005) and are listed in Table 1. Two fusions were obtained more than once, namely PA0605 and PA4554 (*pilY1*), and in three other instances we obtained gentamicin-inducible fusions to two genes in the same operon (PA0603/5, *pchEF* and *flgEK*). Among the genes in which we obtained gentamicin-induced *lux* fusions were ten genes encoding membrane and transport proteins, seven separate motility genes, predominantly influencing pili and flagella expression, one gene involved in the PQS quorum-sensing system, three genes responsible for carbon, fatty acid and energy metabolism, five genes encoding transcription regulators, three genes involved in RNA modification, adaptation and degradation, a putative enzyme, a gene from large phage-like pyocin cluster and three genes with unknown functions. Taken together the majority of the gentamicin-induced *lux* fusions mapped to genes and/or operons (based on computational prediction using operon finding software V1.2; http://v2.pseudomonas.com/) that are involved in adaptation to the environment, as well as genes involved in gene regulation and energy metabolism. Activation of gene regulation during growth on LB in the presence or absence of 0.25 MIC gentamicin was independently confirmed using real-time PCR on 12 selected genes (Table 1).

Gene activation of the gentamicin-induced *lux* fusions is largely aminoglycoside specific

We previously demonstrated that polymyxin B, but not aminoglycosides, regulated the *pmrAB* (polymyxin resistance) two-component regulator operon (McPhee *et al.*, 2003). To determine the specificity of induction, we quantified the luminescence of the 37 *lux* fusions when grown in the presence and absence of subinhibitory (0.25 MIC) concentrations of gentamicin, tobramycin or the positively charged antimicrobial lipopeptide polymyxin B. Table 1 shows the luminescence ratios representing gene transcription during growth with and without added antibiotic. The transcription of all genes was similarly activated during growth in the presence of the two aminoglycosides tested, except for PA4961::*lux*, PA4225::*lux* and *plsX*::*lux*, which showed stronger activation when grown in the presence of gentamicin than tobramycin. Only five *lux* fusions from our mutant pool (PA4961::*lux*, PA4226::*lux*, PA4225::*lux*, PA3353::*lux* and PA0621::*lux*) were slightly up-regulated in the presence of polymyxin B. These results suggest that transcriptional activation of mutants belonging to the gentamicin-induced mutant pool was predominantly aminoglycoside specific.

Mutants carrying gentamicin-inducible *lux* fusions are not universally more susceptible to aminoglycosides

Aminoglycoside resistance in *P. aeruginosa* typically results from drug inactivation by plasmid- or chromosomally

encoded enzymes, or defects in the uptake or net accumulation (due to enhanced efflux) of the drug (Poole, 2005a, b). One possible explanation for the induction of these genes would be as a countermeasure by *P. aeruginosa* to prevent the lethal action of the aminoglycoside; such mutants that lack important resistance genes would be expected to be more susceptible to aminoglycosides than their parent strain, although mutants deficient in host adaptation genes would not necessarily show increased susceptibility *in vitro*. Indeed, as expected, the majority of the mutants had wild-type susceptibility to gentamicin (MIC 2 μg ml^{-1}). Only two mutants PA4961::*lux* (encoding an unknown cytoplasmic membrane protein) and *lon*::*lux* (see below) had a small but consistent lowering in MIC (Table 1), showing a mean MIC value of 0.67 ± 0.28 μg ml^{-1} for PA4961::*lux* and 1.00 ± 0.00 μg ml^{-1} for *lon*::*lux* in comparison to 2.00 ± 0.00 for PAO1.

lux fusions mapping within *lon* are impaired in biofilm formation

P. aeruginosa is present in the soil, where it naturally encounters aminoglycoside-producing bacteria such as *Streptomyces tenebrarius*; therefore it has been suggested that adaptive resistance may be an evolutionary adaptation that occurred prior to the clinical use of antibiotics (Hoffman *et al.*, 2005). One such adaptive response, the formation of biofilms, was recently demonstrated to be induced in *P. aeruginosa* when grown in sub-MIC tobramycin (Hoffman *et al.*, 2005). Biofilms resist antibiotic treatment and contribute to bacterial persistence in chronic infections (Mah *et al.*, 2003; Whiteley *et al.*, 2001). Since the transcription of the majority of the genes belonging to our mutant pool was also induced in the presence of tobramycin, we screened the entire mutant pool for *lux* fusions showing reduced biofilm formation in comparison to the parent strain PAO1 when grown in LB medium as well as in subinhibitory concentrations of gentamicin (Fig. 1a).

When grown in LB medium, seven *lux* fusions from our mutant pool showed impaired biofilm formation compared to the parent PAO1 strain (Fig. 1a, black bars). Five of these *lux* fusions mapped within genes that are involved in flagella synthesis, two were undesignated genes (although PA3352 is homologous to *E. coli flgN*, a putative chaperone involved in flagella synthesis), while the most substantially affected mutant was PA1803, which is annotated as the Lon protease in *P. aeruginosa*. All mutants belonging to our gentamicin-induced mutant pool were also tested for biofilm formation when grown in the presence of sub-MIC gentamicin. Besides the *lux* fusions that already showed an impaired biofilm formation phenotype during growth in LB we found 11 additional *lux* fusions that were only impaired in the gentamicin induction of biofilm formation (Fig. 1a, white bars). Again, *lon*::*lux* showed the weakest biofilm formation phenotype among the tested mutants. Interestingly there was no obvious biofilm defect when grown in LB alone in the *pilY1* mutant, which encodes a tip-associated (adhesin-like) component of type IV pili, despite the known role of pili in biofilm formation.

To independently confirm the crippled biofilm formation nature of *lon*::*lux* fusions, we tested the biofilm formation phenotype of two further *lon*::*lux* fusions from our PAO1mini-Tn5-*luxCDABE* transposon mutant library (http://pseudomutant.pseudomonas.com/view.html; 31_C9 and 24_A2). The same, biofilm-impaired phenotype was observed (data not shown). In addition, complementation of *lon*::*lux* with the entire ORF of PA1803 (designated c*lon*::*lux*) restored the biofilm formation phenotype to wild-type levels (data not shown). To rule out the possibility of impaired growth of *lon*::*lux* resulting in the observed phenotype, we monitored the growth of *lon*::*lux* and its parental strain PAO1 under the growth conditions used for the biofilm studies above. No obvious growth defect of *lon*::*lux* was detected (Fig. 1b).

The Lon protease of *P. aeruginosa* is involved in swimming, swarming and twitching motility

Important factors for biofilm formation by *P. aeruginosa* are its ability to swim and twitch (Ramsey & Whiteley, 2004). Loss of surface motility influences phage uptake via type IV pili, conferring phage sensitivity to *P. aeruginosa*. To determine whether *lon*::*lux* was impaired in type IV pili formation, a phage overlay assay was performed to compare phage sensitivity of *lon*::*lux* with its parental strain PAO1 as well as with the pilus-deficient strain *pilR*::*lux* (strain 80_E5 from our mutant library). Pilus-specific bacteriophage PO4 formed plaques on strain *lon*::*lux* to the same extent as on PAO1, but not on the negative control strain *pilR*::*lux*, indicating the normal presence of pili in *lon*::*lux* (data not shown). Since crippled pili may retain enough function to induce phage uptake but not enough to drive coordinated cell motility, we tested *lon*::*lux* for twitching and swarming as well as for the flagellum-based movement of swimming. The *lon*::*lux* mutant showed dramatically reduced swarming motility, a moderate change in twitching motility and a small impairment in swimming motility compared to the parent strain PAO1, while this phenotype was restored in the PA1803 complemented c*lon*::*lux* construct (Fig. 2).

DISCUSSION

In this report we show that the transcription of 33 genes in *P. aeruginosa* is activated when cells are cultured in the presence of sub-MIC aminoglycosides on LB agar plates. Most of these *lux* fusions map within genes that are not apparently involved in aminoglycoside resistance mechanisms but rather in host adaptation processes. This is supported by our finding that the majority of the gentamicin-induced *lux* fusions listed in Table 1 showed the same MIC values for gentamicin as did the parental strain PAO1 (Table 1). Since aminoglycosides induce biofilm formation in *P. aeruginosa* (Hoffman *et al.*, 2005), we tested our gentamicin-induced *lux* fusions for possible involvement in this phenotype. In addition to mutants that

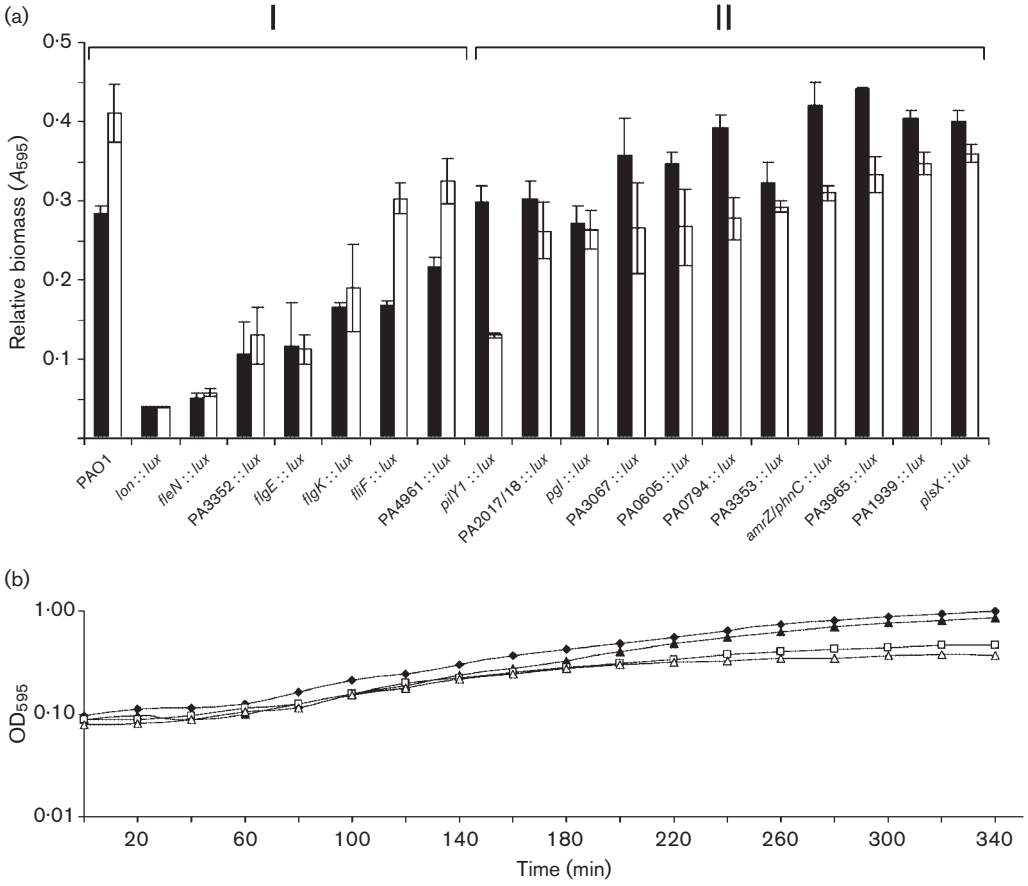

Fig. 1. (a) Biofilm formation by *P. aeruginosa* PAO1 gentamicin-induced *lux* fusions. Bacteria were grown for 24 h at 37 °C in 96-well polystyrene microtitre plates containing LB (black bars) or LB supplemented with 0.5 μg gentamicin ml^{-1} (white bars). I, Biofilm-repressed phenotype under both growth conditions; II, gentamicin-mediated biofilm repression. Surface-associated biofilm formation was analysed by crystal violet staining of the adherent biofilm followed by the extraction of the crystal violet with ethanol and measurement of A_{595}. Means ± SD of three independent experiments are plotted. Changes were statistically significant ($P<0.05$) compared to the parental strain PAO1. (b) Growth of *P. aeruginosa* PAO1 (◆, □) in comparison to *lon*::*lux* (▲, △) in LB (◆, ▲) and LB supplemented with 0.5 μg gentamicin ml^{-1} (□, △). Growth was measured every 20 min during incubation at 37 °C using a TECAN Spectrafluor Plus.

were deficient in functional flagella synthesis, we found *lon* mutants to be totally deficient in biofilm formation (Fig. 1a).

PA1803 encodes a protein that is 84 % similar to Lon protease (http://v2.pseudomonas.com/). Lon belongs to a class of ATP-dependent proteases, conserved in bacteria and even in bovine mitochondria (recently reviewed by Tsilibaris *et al.*, 2006). It is known to be involved in the degradation of abnormal proteins, e.g. during heat shock, and in protein quality control (Tsilibaris *et al.*, 2006) but also influences certain short-lived regulatory proteins. It is well known that such proteases are essential for virulence gene regulation (Robertson *et al.*, 2000; Summers *et al.*, 2000).

Biofilm formation by opportunistic pathogens is devastating because the bacteria in these structured communities can withstand host immune responses, and are resistant to the highest deliverable doses of antibiotics (Stewart & Costerton, 2001). One mechanism that explains this tolerance is that bacteria in a biofilm experience nutrient limitation that leads to a localized slow growth (Brown *et al.*, 1988; Xu *et al.*, 2000). Many antibiotics are known to be less effective against non-growing cells than against rapidly growing cells. During amino acid starvation, protein degradation by ATP-dependent proteases is stimulated in order to increase the amino acid pool that can be used for the synthesis of specific enzymes required for adaptation to this condition (Miller, 1996). Upon nutritional downshift and at the onset of starvation, *E. coli* accumulates poly-P, a linear polymer of orthophosphate residues. Poly-P binds to Lon and redirects its activity towards free ribosomal proteins (Kuroda *et al.*, 2001). Thus, in the centre of a biofilm, *P. aeruginosa* cells could recover from amino acid starvation by stimulating Lon-mediated turnover of free ribosomal proteins.

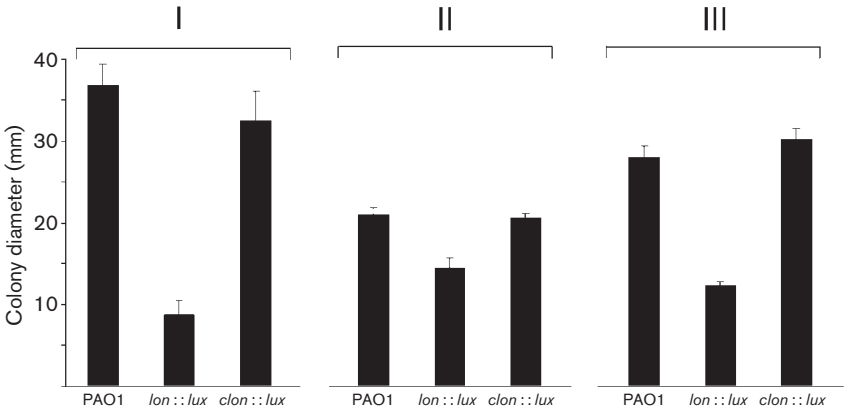

Fig. 2. Swarming (I), swimming (II) and twitching (III) motility of *P. aeruginosa* PAO1, *lon*::*lux* and c*lon*::*lux*. For swarming, bacteria were spot-inoculated onto BM2 swarm plates containing 0.5 % agar. Swimming motility was evaluated on BM2 plates containing 0.3 % agar. Colony diameters of swarming and swimming colonies were measured after 20 h incubation at 37 °C. Twitching was measured after 24–48 h incubation at 37 °C on LB broth plates containing 1 % agar. Means ± SD of four independent experiments are plotted.

Recent evidence indicates that *P. aeruginosa* residing as biofilms in airway mucus of cystic fibrosis patients is undergoing anaerobic metabolism (Hassett *et al.*, 2002). In *E. coli*, the SoxRS regulon is induced under aerobic growth conditions. Once the inducing oxygen stress has subsided, the Lon protease was found to be responsible for degrading the *de novo*-synthesized regulator SoxS (Griffith *et al.*, 2004). We thus speculate that the Lon protease in *P. aeruginosa* might have a function in the early onset of biofilm formation.

To maximize movement and colonization in a viscous environment and on solid surfaces, numerous bacteria differentiate into swarmer cells, which may be a prerequisite to biofilm formation. Swarmer cell differentiation in peritrichous bacteria involves upregulation of the number of flagella otherwise used to swim. In polarly flagellated bacteria, swarmer cell differentiation involves the induction of an alternate flagella system. Interestingly, Lon appears to be involved in the control of both types of swarmer cell differentiation (Tsilibaris *et al.*, 2006). We assessed the influence of *lon*::*lux* on motility using soft BM2 agar for swimming and swarming as well as 1 % LB agar plates for twitching motility assays. The *lon*::*lux* mutant showed impaired motility compared to PAO1. The defect in biofilm formation of *lon* mutants could thus be in part due to impaired motility, although the defect in biofilm formation seems to correlate more with the drastic defect on swarming than the rather more modest change in twitching (~60 %) and swimming (~30 %) motility. A swimming-impaired phenotype of a *lon* mutant has also been observed for *Agrobacterium tumefaciens* (Su *et al.*, 2006).

Emphasizing the importance of motility in the presence of gentamicin, our screen for gentamicin-induced gene expression also revealed seven *lux* fusions where the transposon insertion site was located within motility genes such as *fliF*, which was shown to play a role in bacterial adherence to mucin (Arora *et al.*, 1996), and PA4554, a gonococcal PilC homologue involved in adhesion (Alm *et al.*, 1996). In this context it is interesting that we observed aminoglycoside regulation of transcription of the *lux* fusion mapping immediately upstream of *amrZ*. AmrZ is required for proper surface localization of type IV pili, and it was previously shown that an *amrZ* mutant is twitching negative (Baynham *et al.*, 2006). Independent of its role in twitching, *amrZ* is also essential for alginate production (Baynham *et al.*, 1999). Cystic fibrosis patients are highly susceptible to chronic pulmonary disease caused by mucoid *P. aeruginosa* strains that overproduce the exopolysaccharide alginate (Silo-Suh *et al.*, 2005). Interestingly we also found two PA3182::*lux* fusions to be up-regulated under the tested aminoglycoside induction conditions. PA3182 (*pgl*) is included in the same operon as *zwf*, which encodes glucose-6-phosphate dehydrogenase. A mutation in *zwf* leads to a 90 % reduction in alginate production in the mucoid cystic fibrosis isolate *P. aeruginosa* FRD1 (Silo-Suh *et al.*, 2005). In addition both, *pgl* and *zwf* are more highly expressed in the Liverpool epidemic strain LES400 as well as in the strain FRD1 cystic fibrosis isolate (Silo-Suh *et al.*, 2005), compared to strain PAO1 (Salunkhe *et al.*, 2005; Silo-Suh *et al.*, 2005).

Our screen for gentamicin-induced genes revealed three encoding transport systems. The MexXY proteins form a functional tripartite efflux machinery with outer-membrane component OprM (Mine *et al.*, 1999; Aires *et al.*, 1999). In addition to its ability to accommodate a wide range of antibiotics the MexXY system has the distinctive property of being able to export aminoglycosides (Masuda *et al.*, 2000a, b; Aires *et al.*, 1999) and was shown to be necessary for adaptive resistance of *P. aeruginosa* to aminoglycosides (Hocquet *et al.*, 2003). The PA4226 to PA4220 operon, which encodes the receptor and biosynthesis enzymes for the siderophore pyochelin, was also aminoglycoside regulated. Interestingly, this system is known to be involved in *P. aeruginosa* pathogenesis in that pyochelin stimulates bacterial growth during murine infections (Cox, 1982), reverses iron deprivation caused by human serum and transferrin (Ankenbauer *et al.*, 1985) and efficiently removes iron from transferrin (Sriyosachati & Cox, 1986) while mutants with defects in pyochelin-mediated Fe(III) transport are less virulent than wild-type strains of *P. aeruginosa* (Sokol, 1987). Pyochelin is induced by the respiratory mucus derived *P. aeruginosa* strains from cystic fibrosis

patients (Palmer *et al.*, 2005; Wang *et al.*, 1996). We also found a putative ABC transporter (PA0603–PA0606) with an unknown substrate to be inducible by gentamicin and tobramicin.

The *Pseudomonas* quinolone signal (PQS) was shown to be a quorum-sensing regulatory network in *P. aeruginosa* (Pesci *et al.*, 1999). PQS is required for phenazine production in *P. aeruginosa* (Mavrodi *et al.*, 2001; McKnight *et al.*, 2000). PA0998 (*pqsC*), which was shown in our screen to be up-regulated in the presence of aminoglycosides, and PA0999 are required for PQS production (Gallagher *et al.*, 2002). Emphasizing the role of *pqsC* in infection, the Liverpool epidemic strain LES431 expresses *pqsC* to a higher extent than PAO1 (Salunkhe *et al.*, 2005) and it was shown that the production of PQS is increased and dysregulated in *P. aeruginosa* isolates from infant patients with cystic fibrosis (Guina *et al.*, 2003).

This study has strengthened our understanding of the potential diversity of impacts of subinhibitory concentrations of aminoglycosides on microbial persistence in *P. aeruginosa*. Further studies are under way in our laboratory to study the important function of Lon in the pathogenesis of *P. aeruginosa*, as well as the complex gene regulation that is triggered in this pathogen by sensing aminoglycosides in the environment.

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