Cooperation and virulence of clinical *Pseudomonas aeruginosa* populations

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Bacteria communicate and cooperate to perform a wide range of social behaviors including production of extracellular products (public goods) that are crucial for growth and virulence. Their expression may be switched on by the detection of threshold densities of diffusible signals [Quorum-Sensing (QS)]. Studies using the opportunistic pathogen *Pseudomonas aeruginosa* suggest that QS "cheats"—individuals that don't respond to the QS signal, but are still able to use public goods produced by others—have a selective advantage in the presence of QS cooperators. It is, however, unclear whether this type of social exploitation is relevant in clinical contexts. Here, we report the evolutionary dynamics and virulence of *P. aeruginosa* populations during lung colonization of mechanically ventilated patients in the absence of antimicrobial treatments. We observed a large diversity of QS phenotypes among initial colonizing isolates. This diversity decreased over a matter of days, concomitant with a gradual increase in the proportion of QS cheating mutants (*lasR* mutants), which were found in 80% of the patients after 9 days of colonization. These mutants often evolved from initial wild-type genotypes. The fitness advantage of the *lasR* mutants is almost certainly due to social exploitation, because this advantage was only apparent in the presence of QS wild-type cells. Crucially, ventilator-associated pneumonia occurred significantly earlier in patients predominantly colonized by QS wild-type populations, highlighting the importance of QS in this clinical situation. These results demonstrate that social interactions can shape the short-term evolution and virulence of bacterial pathogens in humans, providing novel opportunities for therapy.

infection | quorum sensing | social interactions

Pseudomonas aeruginosa is an opportunistic pathogen responsible for acute infections in immunocompromised hosts and for chronic diseases in cystic fibrosis (CF) patients. In this organism, Quorum Sensing (QS) regulates expression of approximately 5% of the total genome, including many factors associated with virulence (1–3). QS signaling is mediated essentially by 2 secreted acyl-homoserine lactones (HSL), *N*-(3-oxododecanoyl)-HSL (3-oxo-C12-HSL) and *N*-butyryl-HSL (C4-HSL) synthesized by the LasI and RhlI enzymes, respectively. In vitro, these signaling molecules accumulate in the medium and, on reaching a threshold concentration, bind to their cognate transcriptional activators LasR and RhlR respectively to induce expression of target genes (4).

The importance of QS for the virulence of *P. aeruginosa* has been clearly established in various animal models (5–7). In patients, the detection of QS signaling molecules (8, 9) suggests QS does occur; however, recent reports on QS deficient strains isolated from both acute infections (10–13) and from chronically colonized CF patients (14) have cast doubts on the role of QS in clinical contexts. The evolution of QS mutants after several years of chronic colonization (14) could be explained by the physiological cost of QS gene expression (15, 16) or increased immunogenicity (14), but neither explanation suggests a link between QS and virulence. However, recent in vitro data (17) suggest that QS is a social phenomenon which provides a benefit to a bacterial population because it allows extracellular "public goods," such as catabolic enzymes, to be produced when bacteria are at a density when these public goods will be most beneficial for bacterial growth and survival (1). QS deficient *lasR* mutants were found to have a selective advantage in the presence of QS wild-type bacteria in vitro (17, 18) because they benefit from the extracellular public goods produced by QS cooperators in the vicinity without paying the metabolic costs themselves. If this explanation for the evolution of QS mutants is correct in vivo, then QS should be associated with effective host exploitation, and hence increased virulence, in a clinical context. Whether QS cheaters exploit QS wild-type strains and affect virulence during acute clinical situations remains to be established.

Here, we analyze the evolution of QS and associated virulence in acute clinical situations. Sampling methods in previous acute clinical studies (10–13) have not been sufficient to determine whether or not QS mutants have a selective advantage; what might be responsible for any realized advantage of the mutants; and whether or not mutants affect virulence. We followed the QS phenotype and the population dynamics of *P. aeruginosa* during the initial colonization (up to 20 days) and progression toward infection of intubated patients. We found that QS deficient *lasR* mutants rapidly increased in frequency, often evolving from QS wild-type genotypes in a matter of days. This growth rate advantage of QS mutants was only apparent in the presence of cocolonizing QS cooperators, suggesting that social exploitation provides a fitness advantage to the mutants. Furthermore we observed that virulence, as measured by the onset of *Pseudomonas* ventilator-associated pneumonia (VAP), was greatest in patients harboring only QS wild-type isolates.

Results and Discussion

Phenotypic Diversity of QS Phenotypes Among Colonizing Lung Isolates. We followed prospectively 31 intubated patients (intubation times of 3–20 days), hospitalized in intensive care units of 13 different European hospitals and colonized by *P. aeruginosa*. Importantly, none of the patients received antipseudomonal drugs during the observation period. This observation allowed us to follow the adaptive behavior of *P. aeruginosa* in the absence of any external selective pressure. Every day we collected from each patient one tracheal aspirate, from which we obtained one *P. aeruginosa* isolate. To gain a global picture of the QS phenotypes, we determined the production of elastase and rhamnolipids in the 364 collected isolates, which are mainly under the control of the *lasR* and *rhlR* QS systems, respectively

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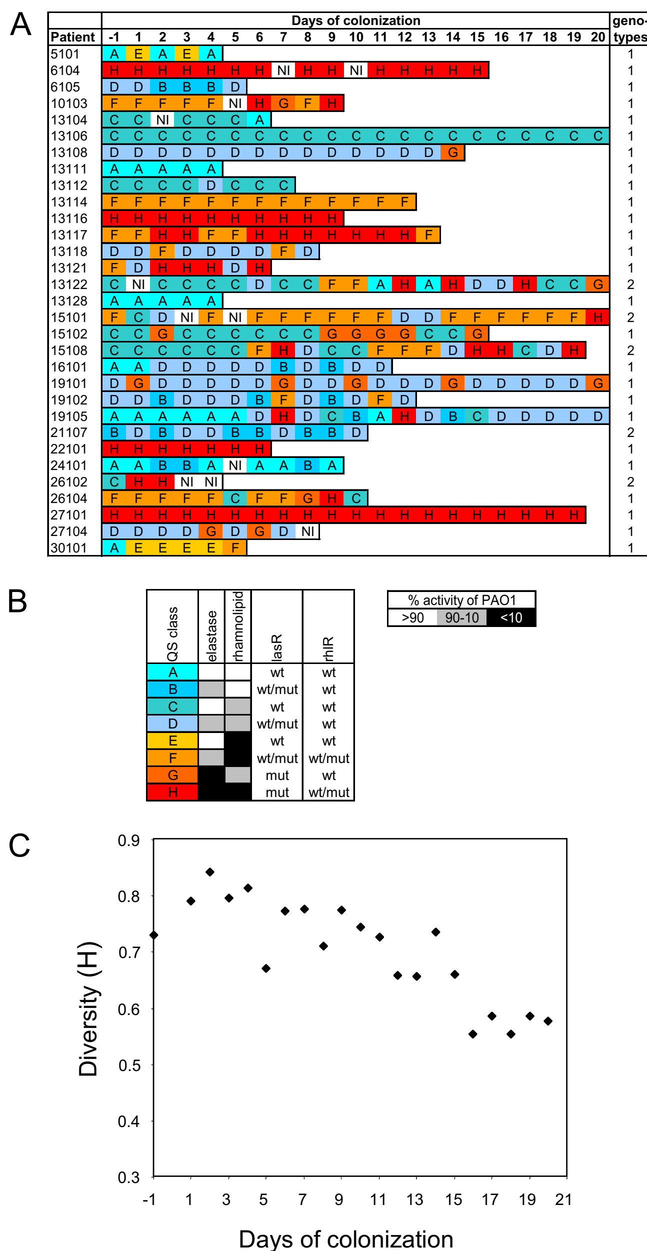

Fig. 1. QS phenotype diversity of isolates from intubated patients. (*A*) Elastase and rhamnolipid production of all 364 isolates was measured by ECR assay and halo diameter on modified SW-blue plates, respectively, and scored with respect to PAO1 activity (set to 100%), as shown in *B*. Genotypes of isolates from each individual patient were compared by RAPD. (*B*) The *lasR* and *rhlR* genes were sequenced in at least one isolate of each QS class from every patient. The presence of mutated *lasR* and *rhlR* alleles in the remaining isolates was assessed by high resolution melting (HRM) analysis of the corresponding PCR amplicons. Isolates of this class may carry either a wild-type (wt) or a mutated (mut) allele of the gene. (*C*) Diversity (H) of colonizing isolates across the 31 patients as calculated by the Shannon–Weaver index (19).

(Fig. 1). We observed a remarkable diversity in QS phenotypes, which allowed us to allocate the 364 isolates to 8 distinct classes (A through H) (Fig. 1 *A* and *B*). The majority of the patients (23 of 31) harbored at least 1 QS deficient isolate (classes E to H) at some point. We then calculated the diversity (H) of the QS phenotypes across the 31 patients through time by using the Shannon–Weaver index (19). Diversity among the initial colonizing isolates was high but decreased steadily during the colonization period (Fig. 1*C*). This loss in diversity was mainly due to a decrease in isolates of QS classes A and B, which were found in 30% of the patients on day −1, but had disappeared by day 15. The initial diversity probably reflects the different sources from which the isolates were acquired (endogenous, patient transmission, environment). Importantly this progressive loss of diversity was not due to selective pressure of antibiotics in our patient population and only resulted from the conditions that prevail in the lung environment.

Rapid Emergence of *lasR* Mutants Among Isolates from Intubated Patients. To understand how selection acts on QS during colonization of intubated patients, we sequenced both the *lasR* and *rhlR* genes in the first isolate obtained from each patient and then in subsequent isolates belonging to a different QS class (Fig. 1*B*, Table 1). As expected, QS proficient isolates (class A) all carried wild-type alleles of these genes, whereas QS deficient isolates (classes F to H) carried either a mutant *lasR* and/or *rhlR* allele(s). Surprisingly, isolates of classes C and E characterized by decreased rhamnolipid production all carried a wild-type *rhlR* gene, suggesting the presence of mutations in other regulatory loci. We identified 30 different genetic alterations in *lasR*, including 19 missense mutations, 7 base pair deletions and insertions, as well as 4 integrations of IS elements (Table 1). Most of the amino acid substitutions were predicted to be nontolerant by the SIFT algorithm (Table 1) (20) and were located either in the autoinducer binding-multimerization domain (amino acid 15–164) or in the DNA-binding domain (amino acid 174–231) of LasR (Table 1), suggesting loss of LasR function. We randomly chose 4 distinct *lasR* mutations (199IS, T115I, E196D, C79R) and complemented the corresponding isolates by inserting a Tn7::*lasR* cassette (10) at a neutral site on the chromosome. In all cases, wild-type elastase levels were restored, confirming that the *lasR* mutations were solely responsible for decreased elastase activity in these isolates.

To test whether the proportion of QS mutants fluctuates during colonization, we calculated the daily average number of *lasR* and *rhlR* mutant alleles present in the isolates from all 31 patients. Strikingly, we found a significant increase (logistic regression: $F_{1,\,19} = 13.95$, $P < 0.01$) in the proportion of patients harboring *lasR* mutants through time (filled circles in Fig. 2*A*), with peak frequency occurring on days 9 and 12. At this time, 80% of the patients were colonized by a *lasR* mutant, compared to 40% at the beginning of colonization. As expected, there was a highly significant relationship between the proportion of *lasR* mutants and mean elastase activity of the colonizing isolates (across all patients) through time ($F_{1,19} = 54.17$, $P < 0.001$) (Fig. 2*B*). In contrast, there was no significant change (logistic regression: $F_{1,19} = 3.57$, $P < 0.1$) in the proportion of patients harboring *rhlR* mutants through time (open circles in Fig. 2*A*). These data strongly suggest a short-term selective advantage of *lasR*, but not *rhlR*, mutants through time.

Why should there be a selective advantage of *lasR* but not *rhlR* mutants? The classical QS circuit assumes a hierarchy in which *lasR* partially controls *rhlR* expression (21), hence inhibition of genome-wide expression would be greater in *lasR* deficient mutants. The *lasR* deficient mutants are therefore likely to outcompete *rhlR* deficient mutants, and there would be little selection acting on *rhlR* in the presence of a *lasR* deficient mutation. It is also possible that some products which are under specific *rhlR*, but not *lasR*, control may in fact confer a direct fitness advantage.

We next addressed whether changes in the frequency of *lasR* mutants resulted only from selection acting on existing genetic variation or whether *lasR* mutants also evolved from wild-type genotypes during the course of colonization. We determined the genetic background of all isolates by RAPD [supporting information (SI) Fig. S1 and Fig. S2] and determined the clone type

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Table 1. Genotypes and LasR mutations of *P. aeruginosa* isolates

Patient	SNP-type*	LasR, D −1	LasR
05101	6C22	wt	
06104	AF9A	Y47-IS5	
06105	D421	L236P†	
10103	D421	V199-IS30	
13104	0C1A	wt	
13106	4F8A	wt	
13108	1BAE	wt	E196D (14)
13111	85AA	wt	
13112	C40A	wt	
13114	F469	L148P	
13116	F469	T222I	
13117	F469	L148P	L148P + D156G (2), L148P + R224H† (8)
13118	239A	wt	G191C†
13121	239A	wt	V184A (2), C79R (3)
13122	F661	wt	S204F† (6), T115I† (12)
	E429	NA	A231V† (20)
13128	062A	wt	
15101	0C2E	wt	R180G† (2)
	239A	NA	P85 + 1bp (2), T75 + 1bp (10), Q94End (20)
15102	E429	wt	D43Δ81bp (2), Y93Δ408bp (9), C203R† (15)
15108	E429	wt	
	239A	NA	L17Δ1bp (6), A134Δ6bp (15), I197-IS4 (16)
16101	0C2E	wt	Δ (3)
19101	F469	Δ	
19102	6D92	A231V†	
19105	7C2E	wt	F210L† (8)
21107	6D92	A231V†	
	F469	NA	wt (10)
22101	F469	P74L†	
24101	2C25	wt	T193I† (2)
26102	F469	wt	D156Δ1bp (1)
	6D92	NA	A231V† (2)
26104	F469	wt	D156Δ1bp (9)
27101	EC4A	IS	
27104	0812	L110Q†	
30101	D421	wt	T115I† (5)

Numbers in parentheses indicate day when mutation first occurred; Δ indicates undefined deletion; NA, isolate not available. Amino acids 15–164, autoinducer binding, multimerization domain; amino acids 174–231, DNA binding domain. IS sequences were identified with IS-Finder (http://www-is.biotoul.fr/is.html).

*SNP type was established using the Clondiag array as described in ref. 22.

†Amino acid substitutions predicted by the SIFT algorithm to be nontolerated.

of 1 representative isolate of each phenotype by using the Clondiag *P. aeruginosa* microarray (22). We identified 19 different clones colonizing the 31 patients (Table 1). Crucially, the majority of the patients (26/31) were colonized by a single genotype, and the remaining 5 patients harbored isolates with 2 genotypes (Table 1). In 7 patients colonized by a single genotype with an initially wild-type *lasR* allele, *lasR* mutants were subsequently detected and elastase production showed a significant decrease (paired $t = 3.17$, $P = 0.025$), relative to when the patients were initially colonized, at this time. Given the high diversity of SNP types across the whole patient cohort, it is highly unlikely that 2 colonizations by genotypes with the same SNP type would occur. Furthermore, we observed *lasR* mutants following wild-type *lasR* alleles, but not vice versa: If multiple colonizations were occurring, we would expect to see both. These findings strongly suggest that *lasR* mutants evolved from the colonizing genotypes during the course of the study.

As a cooperative behavior, QS is amenable to exploitation by noncooperators (cheats), which do not contribute to the production of public goods (17). Indeed, noncooperating *lasR* mutants were shown to emerge in vitro under conditions requiring QS for growth (18). To determine whether the fitness advantage of *lasR* mutants in the colonizing populations is due to social exploitation of QS wild-type, rather than other direct benefits, we compared bacterial population densities between patients in which only wild-type *lasR* alleles ($n = 10$) or only *lasR* mutants ($n = 11$) were present. There was no difference in bacterial load between these 2 groups (Fig. 2*C*) [repeated measures analysis using residual maximum likelihood (REML): $P > 0.2$], demonstrating that the fitness advantage of QS noncooperators is only apparent in the presence of QS wild-type isolates. Consistent with theory (23–26) and in vitro work (17), these data suggest that social exploitation of exoproducts might play a crucial role in determining fitness of QS nonresponders. More surprising is the lack of growth advantage of QS responders over QS nonresponders when in isolation from each other, given the clear and consistent benefit of QS for colonization and infection in animal models (27). It is possible that QS has both costs and benefits during initial colonization of intubated patients and that some of the beneficial exoproducts can be exploited by QS nonresponders. The benefits of QS may however be more apparent once infections are established.

Within-Host Population Dynamics. To further explore the selective advantage of *lasR* mutants, we followed the within-host dynamics

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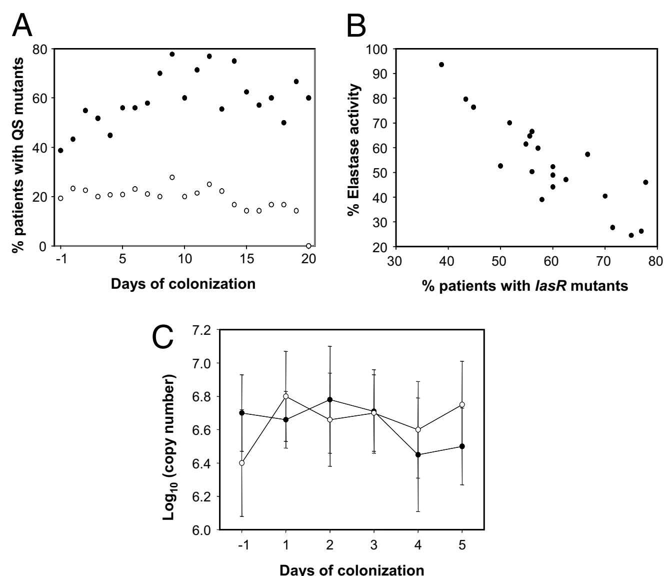

Fig. 2. Emergence of QS mutants and population densities. (*A*) The proportion of patients harboring isolates with mutations in *lasR* (●) and *rhlR* (○) genes through time. Day −1 denotes the first day of detectable *P. aeruginosa* colonization. Note that sample sizes decreased from 31 patients to 5 patients by day 20. (*B*) The relationship between proportion of patients harboring isolates with *lasR* mutations and mean elastase production of the given isolates. Each data point represents a single day. (*C*) Comparison of bacterial loads between patients. Data shows mean (SEM) genomic copy number in patients harboring isolates with only wild-type (●) or mutant (○) *lasR* alleles. *P. aeruginosa* genomic copy numbers were determined on total DNA extractions from daily tracheal aspirates and quantified by qRT-PCR. Data only shows the initial periods of colonization because of small sample sizes in the wild-type group after day 5.

of *P. aeruginosa* populations. We extracted total genomic DNA from the daily tracheal aspirates of 4 patients, 3 of whom were colonized by 2 genotypes (patients 21107, 15108, and 15101). The proportion of each genotype was determined by qRT-PCR by using genotype specific primer pairs as detailed in the legend of Fig. 3. In 3 cases (patients 15108, 15101, and 16101), *lasR* mutants evolved from wild type, which rapidly decreased in frequency (Fig. 3 *A*, *C*, *D*). In the 2 mixed genotype colonizations, the competing clones, both of which had wild-type *lasR* alleles, decreased in frequency with the increase in the *lasR* mutant (Fig. 3 *C* and *D*).

In the fourth patient (21107) (Fig. 3*B*) one initially colonizing clone was already a *lasR* mutant (Fig. S3). Despite a rapid increase during the first 5 days of colonization, this clone rapidly decreased in frequency and the cocolonizing wild-type *lasR* genotype increased in frequency (Fig. S3). It is not clear what caused this sudden reversion in frequencies, but it suggests that the outcome of short-term competition between wild-type and *lasR* mutants becomes much less predictable when the bacteria have different genetic backgrounds.

Another striking feature of the within-host dynamics is the apparent fluctuations in genotype frequencies of the wild-type and mutant *lasR* genotypes (Fig. 3 *C* and *D*, Fig. S1 and Fig. S2). The *lasR* mutants rapidly increased in frequency but generally failed to completely eliminate the wild-type. In the one patient where such fluctuations did not occur (16101) (Fig. 3*A*), the sampling period was terminated before potential fluctuations were observed. This result is consistent with our observation in the whole patient cohort, which showed a steady increase in the proportion of *lasR* mutants over the first 11 days, followed by a plateau or even a decrease (Fig. 2*A*). Such fluctuations are consistent with interactions between public good producers and exploiters in spatially structured environments (28) but not with a fitness advantage of *lasR* mutants that is independent of social exploitation. The occurrence of spatially structured microcolonies in the current study is further strongly supported by the observation that patients colonized by a single genotype sometimes contained multiple *lasR* alleles (patients 13117, 13121, 15102) (Table 1).

Virulence of QS Populations. Finally, we considered how QS affects virulence. Of the 31 patients, 6 developed *P. aeruginosa* (VAP). Two of them (13111, 13128) were colonized exclusively by *lasR*/*rhlR* wild-type isolates; 3 patients (16101, 21107, 24101) harbored both *lasR* wild-type and mutant isolates, and one patient (13116) carried only *lasR*/*rhlR* double mutants (Fig. 1*A*). The proportion of patients who developed VAP did not differ between patients harboring exclusively QS wild-type or mutant isolates (Fishers Exact Test, $P > 0.2$). However, VAP occurred significantly earlier (day 4–5) in the 2 patients exclusively colonized by QS wild-type isolates, but later (day 9–11) in patients also harboring *lasR* mutant isolates (*t* test; $t = 11.4$, $P = 0.001$) (Fig. 4). The bacterial factors that result in the progression from colonization to acute infection are unclear, but a positive relationship has been observed between bacterial density and the probability of an infection developing (29). This relationship suggests that the cooperative production of public goods through QS might in fact result in more efficient longer term exploitation of hosts, and hence increased virulence. A decrease in virulence with increasing within-host diversity (and hence competition) where extracellular public goods are important for within-host growth is consistent with recent theory (30, 31). However, in circumstances where extracellular products are less important for growth, within-host competition may favor genotypes that exploit the host most rapidly, resulting in the evolution of increased virulence (32). The relationship between within-host competition and virulence is further complicated where anti-competitor toxins are produced (33).

Conclusions

In summary, we have shown that *P. aeruginosa* isolates colonizing intubated patients present a remarkable diversity of QS phenotypes, most of which result from the rapid emergence of *lasR* mutants during the first 10 days of colonization. The most convincing explanation for this rapid adaptation is the massive fitness advantage of the *lasR* mutants gained by exploiting the cooperating wild-type population without contributing to the public good. This result suggests that QS plays an important role in bacterial social interactions in clinical contexts, as has been frequently suggested, and argues against the recent hypothesis that the production of QS molecules primarily provide a direct benefit to the producing bacteria through detecting when exoproducts are likely to diffuse away (diffusion sensing) (34, 35). It is therefore possible that genetic or ecological manipulation of the social environment (by inhibiting, for instance, cooperative systems such as QS) may impact on the severity of *P. aeruginosa* infections.

Materials and Methods

Patients and Clinical Collection. The patients included in this study were part of the placebo control group of a multicenter European trial (ClinicalTrials.gov ID# NCT00610623) that compared azithromycin to placebo for the prevention of pneumonia in intubated patients colonized by *P. aeruginosa*. We obtained approval for this study from the local ethical committees of the 13 participating hospitals and written consent from all patients or their legal representatives. We screened mechanically ventilated patients for respiratory tract colonization by *P. aeruginosa* every other day. Patients with ongoing *P. aeruginosa* infection, or having received therapies against the bacteria during the last 14 days, were not included. Starting the first day of proven colonization (D-1), we collected tracheal aspirates (usually 0.3 to 5 ml) and one *P. aeruginosa* isolate (collection period: 3–20 days) every day for a maximum of

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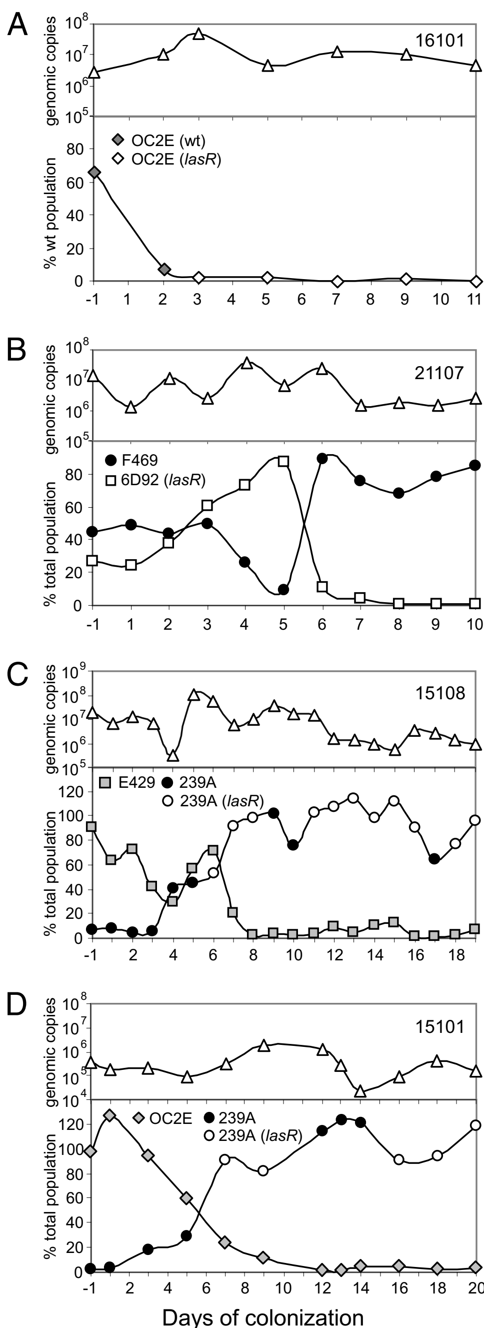

Fig. 3. *P. aeruginosa* population dynamics in intubated patients. Total *P. aeruginosa* genomic copy numbers were determined by qRT-PCR on total genomic DNA preparations from tracheal aspirates by using the *rpsL* primer pair and are expressed as genomic copies/g aspirate (*A–D*, *Upper*). (*A*) QS population dynamics in patient 16101 colonized by a single genotype. To discriminate in the genomic DNA preparations between *lasR* wild-type and mutant populations, a *lasR* primer pair was designed which amplifies a 200 bp DNA fragment that is absent in the *lasR* deletion mutants (isolates from days 3 to 11). The amount of total *P. aeruginosa* copies was determined in the same DNA samples by using the *rpsL* primer pair. (*Lower*) The graph shows the percentage of *lasR* wild-type copies among the total *P. aeruginosa* population. (*B*, *C*, *D*) QS population dynamics in patients colonized by 2 genotypes. In patient 21107, populations of clone F469 (*lasR* wild-type, *exoS*+, *exoU*−) and clone 6D92 (*lasR* mutant, *exoS*−, *exoU*+) were quantified by using primer pairs specific for *exoS* and *exoU*, respectively. The relative proportions of the 2 populations are expressed as a percentage of the total number of *P. aeruginosa* genomic copies determined in the same DNA preparations by using the *rpsL* primer pair (*B Lower*). The proportions of clones E429 (PA0636+, PA0722−) and 239A (PA0636−, PA0722+) in patient 15108 were quantified by using primer pairs specific for the variable genes PA0636 and PA0722, respectively, and expressed as percentage of total *P. aeruginosa* genomic copies (*C Lower*). The proportions of clone OC2E (PA0636+, PA0728−) and clone 239A (PA0636−, PA0728+) in patient 15101 were quantified similarly by using primer pairs specific for variable genes PA0636 and PA0728, respectively (*D Lower*) (22). Because only 1 isolate was available per day, which usually corresponded to the most abundant clone, the *lasR* allele of the isolate from the minor population could not be assessed.

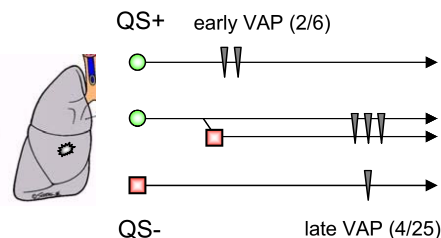

Fig. 4. QS and virulence of clinical *P. aeruginosa* populations. Intubated patients were colonized by either only QS wild-type isolates (QS+, 6 patients), only QS mutant isolates (QS, 5 patients) or mixed QS wild-type and mutant populations (20 patients). VAP (vertical arrows) occurred earlier (days 4–5) in patients harboring only QS wild-type populations and later (days 9–11) in patients cocolonized by QS mutant isolates.

20 days. Samples were frozen on site at −80 °C within 15 min. Reasons for early discontinuation included extubation, death, or proven *P. aeruginosa* VAP (as defined by strict clinical and laboratory data and confirmed by an external blind panel of experts).

Genotype and Mutation Analyses. For intrapatient comparison of isolates, we performed random amplification of polymorphic DNA (RAPD) (Fig. S1 and Fig. S2). Primer 207 was used for routine analysis. When distinct band patterns (>2 bands difference) were observed, RAPD was repeated with a second primer (primer 272) (36). The SNP type of one representative isolate from each RAPD type was determined by using the Clondiag array (22). Mutations in the *lasR* and *rhlR* genes identified initially by DNA sequencing, were confirmed in the other isolates from the same patients by high resolution melting (HRM) analysis by using the SensiMix Kit and Evagreen as the intercalating agent according to the manufacturer's instructions (Quantace Ltd.). Portions of the *lasR* and *rhlR* genes were amplified by using specifically designed primer pairs (all primer sequences are available on request). PCR amplifications and melt curves (0.1 °C increment) were performed in a RotorGene 6000 RealTime PCR machine (Corbett Research) and analyzed by using the integrated HRM software.

Exoproduct Assays. Elastase activity (Fig. S1 and Fig. S2) was determined in supernatants of cultures grown for 7 h in PB medium (37) at 37 °C, by using the Elastin Congo Red (ECR) assay (38). Rhamnolipid production was assessed on modified SW-Blue plates as described (39). PAO1 was used as the reference strain in each assay. Results of duplicate elastase determinations are expressed as the mean absorption ratios OD495/OD600.

Extraction of DNA from Tracheal Aspirates. Tracheal aspirates and one *P. aeruginosa* isolate from 31 intubated colonized patients were collected prospectively. Genomic DNA was extracted by using the DNAzol solution (Invitrogen). The final DNA pellet was dissolved in 0.2 ml 8 mM NaOH by heating at 56 °C for 10 min. Genomic DNA could be detected (>5 × 10^4 genomic copies/g aspirate) in 98% of the aspirates. All extractions were done in duplicate.

Determination of Bacterial Load in the Lung. The number of *P. aeruginosa* in tracheal aspirates was determined by qRT-PCR of genomic DNA preparations. The *rpsL* F/R primer pair (40) was tested for specificity against genomic DNA preparations from clinical isolates of *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii*. An amplification signal was obtained only with genomic DNA isolated from *P. aeruginosa*. Aliquots of genomic DNA preparations were diluted 10 fold into H_2O and 3 μl of this dilution were added to the PCR mix containing 1× Quantitect Sybr Green Master Mix (Qiagen) and 600 nM primers in a total volume of 15 μl. PCR conditions were as recommended by the manufacturer. A standard curve was obtained by addition of 10-fold dilutions of a *P. aeruginosa* culture to an

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aspirate collected from a patient not colonized by this organism. Genomic DNA was then isolated as described above and quantified by qRT-PCR. Under these conditions, we detected 10^4 genomic copies/g aspirate. Standard curves yielded reproducible values during the 3 month analysis period. *P. aeruginosa* was found in 301 of the 308 aspirates analyzed at levels varying from 5×10^4 to 1.8×10^8 genomic copies/g aspirate. The linearity and specificity of our assay was tested by adding serial dilutions of a *P. aeruginosa* laboratory strain culture to a tracheal aspirate that contained Gram-positive and Gram-negative bacteria but no *P. aeruginosa*. A linear correlation over at least 3 orders of magnitude was observed between the amount of bacteria determined on the genomic DNA extracts and the number of cfu added.

Statistical Analyses. Logistic regression was used to analyze the change in the proportion of *lasR* and *rhlR* mutants across patients through time; the data were slightly overdispersed and were therefore corrected by scaling factor to equalize the error deviance and degrees of freedom. To analyze the genomic copy data, we used REML to fit a linear mixed model with patient (random factor), time and *lasR* mutant/wild-type (both fixed factors). All analyses were carried out by using GenStat 10.

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