Quorum-sensing-deficient (*lasR*) mutants emerge at high frequency from a *Pseudomonas aeruginosa* *mutS* strain

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In *Pseudomonas aeruginosa*, quorum sensing constitutes a highly complex cell-to-cell communication system that, along with the cognate acylhomoserine lactone signals and regulators LasR and RhlR, modulates the production of virulence factors and a wide range of metabolic functions. In a previous paper, the authors reported that mismatch repair disruption in *P. aeruginosa* results in the spontaneous and reproducible emergence of defined morphological colony variants after a relatively short period of cultivation in an aerated rich medium, in contrast to the non-mutator parental strain, which does not display any kind of diversification under identical incubation conditions. One of the morphotypical variants, mS2, emerges at a high frequency and displays differences in virulence traits that could be regulated by major quorum-sensing regulators. The present study shows that *mutS* mS2 variants had defective LasR function due to simple but different point mutations along the *lasR* gene sequence, indicating that LasR inactivation is the main cause of mS2 phenotypic diversification. Moreover, it was determined that a non-functional LasR would confer a selective advantage in the late stationary phase, since viability was notably higher for mS2. Interestingly, in all mS2 variants analysed, no sequence alterations were found in the *gacA* and *rhlR* genes, suggesting that the selective pressures for GacA/RhlR and LasR were not the same and differed from those in other *Pseudomonas* species, which, when incubated in nutrient-rich liquid stationary-phase cultures, show specific high instability in the *gacA*–*gacS* genes.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that has an extraordinarily versatile metabolic potential allowing it to survive in a number of natural and nosocomial environments. It is also an opportunistic human pathogen that infects injured, burned and immunodeficient patients and causes severe and often fatal chronic infection in cystic fibrosis (CF) patients (Lyczak *et al.*, 2002). In addition, *P. aeruginosa* has remarkable versatility in its mechanisms of virulence, expressing and secreting a multiplicity of factors such as proteases, toxins, pigments and haemolysins that contribute to its survival and pathogenicity. The expression of these exoproducts is regulated, at least in part, by a cell-to-cell signalling system known as quorum sensing (QS) that directs the activation of specific genes at high cell densities in response to chemical signals released by *P. aeruginosa* itself (Whiteley *et al.*, 1999; Smith & Iglewski, 2003a). *P.*

aeruginosa QS involves the transcriptional regulators LasR and RhlR and their respective autoinducer synthases, LasI and RhlI, which direct the synthesis of their cognate signal molecules 3-oxo-C12 and C4 homoserine lactones (HSL) (Smith & Iglewski, 2003a). The two *P. aeruginosa* QS systems are linked, and operate in a hierarchical cascade, with the *las* system dominating over the *rhl* system at the transcriptional and posttranscriptional level (Pesci *et al.*, 1997). A third signal molecule recently found to be involved in *P. aeruginosa* QS is the 2-heptyl-3-hydroxy-4-quinolone *Pseudomonas* quinolone signal (PQS), which provides a further link between the *las* and *rhl* systems since both of them control the synthesis of PQS, which in turn controls the expression of RhlR and RhlI (Pesci *et al.*, 1999; McKnight *et al.*, 2000). Likewise, the *P. aeruginosa* QS cascade is regulated by a variety of other regulatory systems with a high potential to integrate and respond to multiple environmental signals, thereby generating a very complex signalling network that constitutes one of the bases for its exceptional adaptability (Schuster & Greenberg, 2006). One of these factors is GacA, a highly conserved two-component

Abbreviations: AHL, *N*-acylhomoserine lactone; CF, cystic fibrosis; HSL, homoserine lactone; MMRS, mismatch repair system; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing.

response regulator, which is generally responsible for the control of virulence factors, antibiotic production, biofilm formation and stress response in fluorescent pseudomonads (Parkins *et al.*, 2001; Heeb & Haas, 2001). In the *P. aeruginosa* regulatory hierarchy, GacA acts above LasR and RhlR, positively regulating the production of C4-HSL, pyocyanin, cyanide and lipase (Reimann *et al.*, 1997). In addition, the complex QS signalling circuitry includes, among others, the sigma factors RpoS and RpoN, the LuxR homologues QscR and VqsR, and global transcriptional regulators such as AlgQ, MvaT, DksA and Vfr (Schuster & Greenberg, 2006).

As in other bacterial species that develop in constantly changing environments, the adaptability of *P. aeruginosa* has been linked to the high frequency of clones with high mutation rates (mutators) (for a review see Denamour & Matic, 2006). Mutator phenotypes generally result from alterations in genes encoding DNA repair enzymes and for proteins that assure the accuracy of DNA replication. One of the main DNA repair systems in bacteria is the mismatch repair system (MMRS), which monitors the fidelity of DNA replication by repairing DNA polymerase errors and blocks homologous recombination events (Kunkel & Erie, 2005). As a consequence, MMRS-defective bacteria show both a mutator and a hyperrecombinogenic phenotype. Interestingly, it has been observed that *P. aeruginosa* mutator clones are extremely frequent in chronic CF lung infections but not in acute infections (Oliver *et al.*, 2000), suggesting that they play a crucial role in the adaptation required for long-term establishment in the heterogeneous and changing CF lung environment. It is important to mention that most of these mutator strains were found to be deficient in the MMRS with mutations in one of the main components of the system, the *mutS* gene (Oliver *et al.*, 2002). *P. aeruginosa* isolated from CF patients also differ from those obtained from acute infections, in that they show significant phenotypic variation, displaying a wide spectrum of morphotypic colony variants (Martin *et al.*, 1995; Oliver *et al.*, 2000). In a previous paper (Smania *et al.*, 2004), we described a mutator *P. aeruginosa* Hex1T *mutS* mutant strain which, if grown to late stationary phase, spontaneously gives rise to diverse colony morphologies, in contrast to the parental non-mutator (wild-type) strain, which, when incubated under the same conditions, does not display any diversification. The *mutS* variant, mS2, is easily distinguished by its particular colony appearance: translucent and hyperpigmented, with a flat periphery spreading out around the colony. The mS2 variant notably shows several altered virulence traits (decreased elastase activity, increased pigment production and swarming motility, and decreased cytotoxicity to eukaryotic cells) and it is reproducibly originated from a distinct *mutS* variant precursor, mS1, which is phenotypically more similar to the wild-type strain (Smania *et al.*, 2004). In the present study, we determined that the mS2 morphotypical diversification was based on distinct missense and nonsense point mutations in the *lasR* gene, while the *gacA* and *rhlR* genes,

positioned above and below in the QS regulatory cascade respectively, were not altered in the mS2 variants. We also determined that inactivation of LasR in the mS2 morphotype would confer selective advantages by increasing cell viability in the late stationary phase. It should be noted that the emergence of *P. aeruginosa lasR* mutants *in vitro* (Heurlier *et al.*, 2005) as well as in clinical isolates including CF has been described previously (Hamood *et al.*, 1996; Cabrol *et al.*, 2003; Schaber *et al.*, 2004; Dénervaud *et al.*, 2004; Salunkhe *et al.*, 2005). Thus, inactivation of LasR would constitute an important tool in adapting to particular environmental challenges, and the higher frequency of mS2 variants obtained from the mutator strain suggests a role for *mutS* hypermutability in acquiring these adaptive advantages.

METHODS

Bacterial strains, plasmids and media. The strains and plasmids used in this study are listed in Table 1. The mS1 and mS2 variants were obtained from a stock of a *P. aeruginosa* Hex1T mutator *mutS* strain which was frozen immediately after the interruption of the *mutS* gene (Smania *et al.*, 2004). For this purpose, the strain was incubated in LBS broth (10 g NaCl l⁻¹, 10 g soy-peptone l⁻¹, and 5 g yeast extract l⁻¹) at 30 °C for 200 h with constant aeration (220 r.p.m.). After that, mS1 (smooth and opaque) and mS2 (translucent and hyperpigmented) colonies were identified and several mS1 clones were isolated and frozen at -70 °C in the presence of 15 % (v/v) glycerol. These frozen mS1 cells were the starting stocks used for the rest of the experiments. To prepare inocula, bacteria were routinely subcultured on LBS broth from the frozen stocks and cultured overnight at 30 °C shaken at 220 r.p.m. Other growth media used were skim milk agar for protease activity assays (Sokol *et al.*, 1979), King's A medium for pigment quantification (King *et al.*, 1954), and Yep and ABm for *Agrobacterium tumefaciens* N-acylhomoserine lactone (AHL) detection assays (Chilton *et al.*, 1974). Media used for *Caenorhabditis elegans* bacterial virulence assays were brain heart infusion (BHI) (Britania) and nematode growth (NG) medium (2.5 g soy-peptone l⁻¹, 3 g NaCl l⁻¹, 5 mg cholesterol l⁻¹, 1 mM MgSO₄, 25 mM potassium phosphate, pH 6, 3.4 %, w/v, agar).

When required, antibiotics at the following concentrations were used: for *P. aeruginosa* 150 µg gentamicin ml⁻¹, 60 µg tetracycline ml⁻¹; for *A. tumefaciens* 30 µg gentamicin ml⁻¹. Standard molecular biology protocols were used throughout as described by Sambrook *et al.* (1989). The *C. elegans* N2 strain was maintained under standard culture conditions on NG agar and fed with *Escherichia coli* OP50 cells.

Phenotypic characterization assays

(i) Elastase activity determination. Supernatants from *P. aeruginosa* stationary-phase LBS cultures were tested for elastolytic activity (LasB protease) by the elastin-Congo red hydrolysis assay as described by Aendekerk *et al.* (2002).

(ii) Exoprotease activity determination. *P. aeruginosa* secreted protease (AprA) was detected on plates containing milk medium agar (Sokol *et al.*, 1979) consisting of peptone from casein 5 g l⁻¹ (Merck), skim milk 10 g l⁻¹, yeast extract 2.5 g l⁻¹, and 1 % (w/v) agar (Britania) supplemented with glucose (1 g l⁻¹). Cells were point-inoculated with a sterile toothpick. After 24 h incubation at 37 °C, the diameters of the clearing zones around the inocula were measured.

Table 1. Strains, plasmids and primers

Strain, plasmid or primers	Relevant genotype and phenotype*	Source or reference
Bacterial strains		
<i>Pseudomonas aeruginosa</i>		
Hex1T	Wild-type, isolated from hydrocarbon-contaminated soil	Pezza <i>et al.</i> (2002)
mS1	<i>mutS::kan</i> derivative of Hex1T; mutator strain, opaque colonies equivalent to the parent strain following diversification experiment; Km ^r	Smania <i>et al.</i> (2004)
mS2	<i>mutS::kan</i> derivative of Hex1T; mutator strain, transparent colonies emergent from mS1 following diversification experiment; Km ^r	This study
MPAO1	Wild-type	Jacobs <i>et al.</i> (2003)
MPAO1 <i>gacA</i>	<i>gacA::ISphoA-hah</i> (ID 37742) derivative of MPAO1; Tc ^r	Jacobs <i>et al.</i> (2003)
MPAO1 <i>lasR</i>	<i>lasR::ISlacZ-hah</i> (ID 17281) derivative of MPAO1; Tc ^r	Jacobs <i>et al.</i> (2003)
<i>Agrobacterium tumefaciens</i>		
NT1	Ti plasmid-cured derivative of strain C58; AHL non-producing strain	Watson <i>et al.</i> (1975)
NtL4(pTiC58ΔaccR)	NT1 carrying pTiC58ΔaccR; AHL constitutive expressing strain	Shaw <i>et al.</i> (1997)
NtL4(pZLR4)	NT1 carrying pZLR4, which expresses <i>traR</i> from its native promoter and carries a TraR-dependent <i>traG::lacZ</i> reporter fusion; exogenous long-chain AHL detecting strain; Gm ^r	Luo <i>et al.</i> (2003)
<i>Chromobacterium violaceum</i>		
CV026	<i>cviI::Tn5</i> derivative of ATCC 31532; exogenous short-chain AHL detecting strain; Km ^r	McClellan <i>et al.</i> (1997)
Nematodes		
<i>Caenorhabditis elegans</i>	Wild-type N2 strain	<i>Caenorhabditis</i> Genetics Center
Plasmids		
pME3827	pME6001 carrying a functional PAO1 <i>lasR</i> gene; Gm ^r	Pessi & Hass (2000)
pME3853	pME6010 with a 174 bp <i>lasI</i> upstream fragment and translational <i>lasI::lacZ</i> fusion containing the first 13 <i>lasI</i> codons	Pessi <i>et al.</i> (2001)
pME3846	pME6010 with a 666 bp <i>rhlI</i> upstream fragment and a translational <i>rhlI::lacZ</i> fusion containing the first 14 <i>rhlI</i> codons	Pessi <i>et al.</i> (2001)
Primers		
Sequence		
GA-for	5'-TGCGCCAAGCTAGGTCATGGG-3' upstream <i>gacA</i>	
GA-rev	5'-CCACGTAGAGAAGCTTGGCCT-3' downstream <i>gacA</i>	
LR-for	5'-CACGGGCGCATGCGCCTC-3' upstream <i>lasR</i>	
LR-rev	5'-AAGCTTCTATATAGAAGGGCAA-3' downstream <i>lasR</i>	
RR-for	5'-GCCATGATTTTGCCGTATCGG-3' upstream <i>rhlR</i>	
RR-rev	5'-CTAAAGGAGGATGAACGGCAG-3' downstream <i>rhlR</i>	

*Tc^r, tetracycline resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

(iii) **Pyocyanin quantification.** Pyocyanin was assayed by measuring the absorbance at 695 nm of cell-free supernatants after cell growth for 72 h at 30 °C in King's A broth.

(iv) **Swarming motility assay.** Swarming motility was assessed on swarm plates containing 8 g Bacto-peptone l⁻¹ (BD), 0.5 % w/v agar (Britania) supplemented with 5 g glucose l⁻¹. Cells were point-inoculated with a sterile toothpick. After incubation at 30 °C for 72 h, the diameter of the area covered by the swarm was measured.

(v) **Nematode slow killing assay.** The *C. elegans* slow killing assay was conducted as described by Tan *et al.* (1999a) with minor modifications. Briefly, 10 µl aliquots of 1/10 dilutions of overnight bacterial cultures grown in King's B broth (King *et al.*, 1954) were spread on NG medium in 5.5 cm Petri plates and incubated at 30 °C for 24 h. After incubation for 8–24 h at room temperature (23–25 °C), each plate was seeded with four L4-stage hermaphrodite wild-type

Bristol *C. elegans* strain N2 nematodes. Plates were incubated at 25 °C and examined for live worms after 5 days. Worms were considered dead when they failed to respond to a touch. Worms that died as a result of getting stuck to the walls of the plate were excluded from the analysis. The non-virulent *E. coli* strain OP50 was used as a control. Each independent assay consisted of three or four replicates.

(vi) **Nematode paralytic killing assay.** All paralytic killing assays were carried out according to Gallagher & Manoil (2001) with minor modifications. Briefly, 10 µl aliquots of 1/10 dilutions of BHI broth overnight cultures were spread onto 5.5 cm diameter BHI agar plates containing 12 ml BHI broth solidified with agar at 1.2 % (w/v). After incubation for 24 h at 30 °C, *C. elegans* N2 nematodes from stock plates were collected in M9 buffer (3 g KH₂PO₄ l⁻¹, 6 g Na₂HPO₄ l⁻¹, 5 g NaCl l⁻¹, 1 mM MgSO₄), and a 50 µl aliquot (containing approximately 40 adult worms) was spotted onto the *P.*

aeruginosa lawn. The plates were then incubated for 4 h at room temperature with the lid on, and the number of live nematodes was scored. Worms were considered paralysed when they did not move spontaneously and did not respond to mechanical stimulation. *E. coli* strain OP50 was used as non-virulent control and each independent assay consisted of three replicates.

(vii) Detection of AHLs. To detect the autoinducer 3-oxo-C12-HSL, a Petri dish bioassay based on the use of the *A. tumefaciens* NTL4 strain was carried out as previously described (Cha *et al.*, 1998). This reporter strain contained a *traG::lacZ* reporter fusion in the plasmid pZLR4, which allowed the detection of a range of exogenous long-chain homoserine lactones including 3-oxo-C12-HSL. Briefly, the *P. aeruginosa* variants were point-inoculated with sterile toothpicks. Following 24 h incubation at 28 °C, the diameters of the diffusing blue zones around the inocula were measured. *A. tumefaciens* NTL4pTiC58 Δ accR and *A. tumefaciens* NT1 were used as positive and negative assay controls respectively. The results were expressed in relation to the *P. aeruginosa* Hex1T parental strain, whose values were considered as the maximum.

To detect the autoinducer C4-HSL, a reporter plate bioassay that employed a *Chromobacterium violaceum* CV026 strain, whose endogenous AHL synthase gene (*cvil*) was disrupted allowing the detection of exogenous short-chain AHLs such as C4-HSL, was performed as previously described (McClean *et al.*, 1997). The analysis was carried out with 50 μ l culture supernatant obtained from each *P. aeruginosa* variant, grown overnight in AB minimal glucose medium (Chilton *et al.*, 1974) at 30 °C with aeration (220 r.p.m.).

LasR function reporter assay. *P. aeruginosa* Hex1T, mS1 and mS2-A to J variants, previously transformed with pME3853 and pME3846 plasmids containing translational *lasI::lacZ* and *rhlI::lacZ* fusions respectively (Table 1), were grown in LBS with aeration at 30 °C. β -Galactosidase measurements were performed by the Miller method (Sambrook *et al.*, 1989).

PCR assays. To amplify the coding regions of the *gacA*, *rhlR* and *lasR* genes (PA2586, PA3477 and PA1430 respectively) specific primers (Table 1) were designed on the basis of the PAO1 genome sequence (<http://www.pseudomonas.com>). PCR was performed using genomic DNA from different *P. aeruginosa* mS2-A to J variants as well as mS1 and Hex1T wild-type strains as templates under the following conditions: 3 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C, and a final extension of 10 min at 72 °C.

Sequencing and molecular analysis of the coding regions of the *gac*, *rhlR* and *lasR* genes. The PCR products were cleaned with a Gel Purification kit (QIAGEN) and directly sequenced by using the respective PCR primers. To identify mutations in *gacA*,

rhlR and *lasR*, the sequences obtained from the mS2 variants were compared with those obtained from the mS1 variant and the Hex1T wild-type strain by BLAST analysis.

Genetic complementation analysis of mS2 variants.

Complementation with the *lasR*⁺ gene was performed using the plasmid pME3827 (Table 1). The plasmid was electroporated into *P. aeruginosa* mS2-A to J variants following standard procedures (Ausubel *et al.*, 1992). Once electroporated, transformed clones were isolated and screened for alkaline protease activity and production of AHLs. *P. aeruginosa* MPAO1 *lasR* mutant (ID 17281) (Jacobs *et al.*, 2003) complemented with pME3827 was used as a control.

RESULTS

Phenotypic diversification upon *P. aeruginosa* MMRS disruption

In order to determine the factors that control mS2 emergence, we first performed a diversification experiment to confirm the reproducibility of this phenomenon and to obtain a frequency of mS2 emergence. For this purpose, 10 independent *P. aeruginosa* mS1 and 10 independent wild-type clones were incubated in LBS broth (10 ml in 100 ml flask) at 30 °C with constant shaking (220 r.p.m.) for 200 h. After that, appropriately diluted samples were plated on LBS agar (1 $\times$ 10³ colonies per culture) and mS2 variants were scored by visual inspection. We observed that mS2 reproducibly emerged from all mS1 cultures at an incidence of approximately 8–10 %, in contrast to the wild-type cultures, which maintained the same monomorphic colonies without any apparent morphological diversification (Fig. 1). In addition to their typical colony appearance, we determined that such mS2 variants had lower exoprotease activity than the wild-type strain (see below). These results confirmed that the mS2 variants were generated in a reproducible and consistent way. For further characterization, a total of 10 mS2 variants (named mS2-A to J) were isolated from three independent diversified cultures (culture 1, mS2-A; culture 2, mS2-B and C; culture 3, mS2-D to J).

Phenotypic characterization of mS2 variants

Considering that the phenomenon of mS2 emergence produced simultaneous alterations in several virulence

Fig. 1. Diversification in colony morphology of *P. aeruginosa* *mutS* strain. Equivalent inocula from single-colony overnight cultures were grown in 10 ml nutrient LBS broth at 30 °C with shaking (220 r.p.m.) for 200 h and appropriate dilutions were plated on LBS agar and grown for 48 h at 30 °C. (a) *P. aeruginosa* Hex1T wild-type strain. (b) *P. aeruginosa* Hex1T *mutS* strain. Arrows indicate mS2 among mS1 variants.

traits (Smania *et al.*, 2004) we investigated the possibility that the mS2 diversification was the result of mutations in major QS regulators that simultaneously control the expression of such virulence determinants. With this aim, we carried out a detailed phenotypic characterization including measurement of pyocyanin and elastase production and swarming motility, analyses of specific markers for the detection of *P. aeruginosa* QS deficiency such as alkaline protease activity, AHL production, and two *P. aeruginosa*–*C. elegans* killing assays, slow and paralytic killing.

The characterization was performed with the 10 mS2 clones previously isolated (mS2-A to J) and, as controls, the wild-type *P. aeruginosa* Hex1T strain, an mS1 clone, two engineered *P. aeruginosa* MPAO1 *lasR* and *gacA* mutants, and their MPAO1 parental strain (Jacobs *et al.*, 2003). The choice of LasR and GacA as candidates was based on previous (Smania *et al.*, 2004) and preliminary observations, mainly of avirulence and increased swarming motility, which suggested that mS2 phenotypic innovations may stem from mutations in the genes encoding any of these

regulators. In addition, analysis of GacA was of interest due to previous reports that the incubation of distinct strains of *Pseudomonas* in rich liquid cultures at late stationary phase produced spontaneous and highly frequent *gacA* mutants (Duffy & Defago, 2000; Bull *et al.*, 2001).

As shown in Table 2, the mS2-A to J clones all displayed, in comparison to the parental Hex1T wild-type and mS1 strains: 4–12-fold more pyocyanin production; half the exoprotease activity; barely detectable elastolytic activity; approximately 1.5-fold increased swarming motility; an avirulent phenotype in both *C. elegans* slow and paralytic assays; and scarcely detectable levels of C4-HSL and 3-oxo-C12-HSL, with the sole exception of clone mS2-B, which produced equivalent levels of long-chain AHLs to the wild-type and mS1 strains.

The *P. aeruginosa* MPAO1 *lasR* mutant showed alterations similar to the mS2 clones, except that pyocyanin was not hyperproduced. The *gacA* mutant exhibited increased swarming motility, diminished production of pyocyanin,

Table 2. Phenotypic characterization of *P. aeruginosa* mS2 variants

Strain	Pyocyanin*	Exoprotease†	Elastase‡	Swarming§	Slow-killing conditions	Paralytic conditions¶	Long-chain AHLs production#	Short-chain AHLs production**
mS2-A	1.16 ± 0.16	0.80 ± 0.24	0.01 ± 0.01	1.86 ± 0.45	–	–	–	–
mS2-B	1.25 ± 0.17	1.10 ± 0.28	0.04 ± 0.01	1.60 ± 0.02	–	–	++	–
mS2-C	1.16 ± 0.13	0.70 ± 0.12	0.04 ± 0.01	1.80 ± 0.40	–	–	–	–
mS2-D	1.07 ± 0.01	0.86 ± 0.08	0.02 ± 0.01	1.96 ± 0.50	–	–	–	–
mS2-E	1.15 ± 0.16	0.90 ± 0.14	0.04 ± 0.02	2.16 ± 0.50	–	–	–	–
mS2-F	1.13 ± 0.01	0.96 ± 0.08	0.02 ± 0.01	2.00 ± 0.28	–	–	–	–
mS2-G	1.07 ± 0.01	0.90 ± 0.14	0.04 ± 0.01	2.36 ± 0.22	–	–	–	–
mS2-H	1.18 ± 0.11	0.90 ± 0.16	<0.01	2.10 ± 0.70	–	–	–	–
mS2-I	1.14 ± 0.02	1.06 ± 0.22	<0.01	2.20 ± 0.28	–	–	–	–
mS2-J	1.07 ± 0.07	0.96 ± 0.08	0.03 ± 0.02	1.90 ± 0.50	–	–	–	–
Hex1T	0.23 ± 0.01	2.08 ± 0.40	0.23 ± 0.01	1.40 ± 0.02	++	++	++	++
mS1	0.10 ± 0.01	1.80 ± 0.32	0.15 ± 0.01	1.26 ± 0.25	++	++	++	+
MPAO1 <i>lasR</i>	0.12 ± 0.02	1.04 ± 0.02	0.01 ± 0.01	1.80 ± 0.26	–	–	–	–
MPAO1 <i>gacA</i>	0.04 ± 0.02	1.60 ± 0.10	0.14 ± 0.02	1.94 ± 0.40	–	++	++	ND
MPAO1	0.08 ± 0.01	1.68 ± 0.20	0.16 ± 0.04	ND	++	++	++	ND

ND, Not determined.

*A₆₉₅ (mean of three replicate cultures).

†Diameter of the clearing zones around the bacterial spots in cm (mean of three replicates).

‡A₄₉₅ (mean of three replicates).

§Diameter of motility area in cm. Assayed on 0.5 % agar plate as previously described.

||Rate of slow killing relative to wild-type Hex1T strain: ++, indistinguishable; +, moderately reduced; –, severely reduced (see Methods).

¶Rate of paralysis of worms relative to wild-type Hex1T strain: ++, indistinguishable; +, moderately reduced; –, severely reduced (see Methods).

#Assessed by *traG*::*lacZ* reporter. Size of diffusing blue colour in the overlay surrounding the bacterial spot relative to wild-type Hex1T strain: ++ indistinguishable (wide); +, moderately reduced (narrow); –, severely reduced (not detectable) (see Methods). Assays were performed twice, in duplicate.

**Assessed by *C. violaceum* CV026 Petri dish assay. Size of purple haloes around the bacterial spot relative to wild-type Hex1T strain: ++, indistinguishable (wide); +, moderately reduced (narrow); –, severely reduced (not detectable) (see Methods). Assays were performed twice, in duplicate.

and severely reduced virulence in the *C. elegans* slow killing assay, which was in accordance with previous reports (Reimann *et al.*, 1997; Tan *et al.*, 1999b). However, the levels of exoprotease and elastase activity and the behaviour in the *C. elegans* paralytic killing assays were equivalent to those obtained from the MPAO1, Hex1T and mS1 strains (Table 2). These data suggest that the pleiotropic phenotypes of the mS2 variants could be based on defective LasR function.

The emergence of mS2 variants is produced by spontaneous mutations in the *lasR* gene

In order to determine the molecular bases of *P. aeruginosa* mS2 emergence, the *gacA* and *lasR* coding regions of mS2-A to J variants and of mS1 and Hex1T wild-type strains were isolated by PCR amplification and sequenced. In this analysis the sequencing of the *rhlR* coding region gene was also included, with the intention of analysing the complete QS cascade. As shown in Table 3, every mS2 variant contained point mutations in the *lasR* coding region, whereas the *gacA* and *rhlR* sequences showed no change with respect to the Hex1T wild-type and mS1 sequences in all ten mS2 variants analysed. All the mutations found in the *lasR* sequences of the mS2 clones were single missense and nonsense base substitutions, mainly transitions resulting in amino acid changes or the introduction of a premature stop codon (Table 3). Some clones contained point mutations that produced changes in the amino acids of the N-terminal portion of LasR, which is known to be involved in binding with 3-oxo-C12-HSL and in protein multimerization (Kiratisin *et al.*, 2002) (Fig. 2). Moreover, most mutations altered highly conserved amino acids present in the LuxR-type transcription factors (Vannini *et al.*, 2002). Other clones contained point mutations generating substitutions in conserved amino acids of the C-terminal region (Table 3), which has been predicted to be the DNA binding domain of the protein (Kiratisin *et al.*, 2002) (Fig. 2).

Table 3. Mutations in the *lasR* gene of *P. aeruginosa* mS2 variants

mS2 variants	<i>lasR</i> mutation	Stop* or change†
mS2-A	G→A at 236	C79Y
mS2-B	C→T at 665	T222I
mS2-C	C→T at 692	A231V
mS2-D	C→T at 241	TAG at 241
mS2-E	C→T at 241	TAG at 241
mS2-F	T→C at 667	S223P
mS2-G	T→G at 452	L151R
mS2-H	T→C at 329	L110P
mS2-I	C→T at 244	T115I
mS2-J	C→T at 250	P117L

*Termination codon generated at the site of the alteration.
†Changes relative to the *lasR* sequence gene of *P. aeruginosa* Hex1T wild-type strain.

It is important to note that two clones (mS2-D and E) had the same point mutation in codon 241 (CAG→TAG), which would result in a truncated protein via the introduction of a premature stop codon (Table 3). These two clones were obtained from the same culture, suggesting that they were probably produced by the same mutation event. However, other clones (mS2-F, G, H, I and J) obtained from the same culture replica showed different kinds of sequence alterations, indicating that these variants had not arisen from mere selection of a single *lasR* mutation, but from independent mutational events. Equally, clones mS2-B and C, which arose from the same culture, showed distinct missense mutations, suggesting that mutational events in *lasR* have a notably high frequency.

Importantly, when plasmid pME3827, containing a functional *lasR*⁺ gene from strain PAO1 (Pessi & Haas, 2000), was introduced into every mS2 variant, the exoprotease activity, the production of 3-oxo-C12-HSL, and the opaque, smooth and convex colony morphology were restored to the phenotypes shown by Hex1T wild-type and mS1, clearly indicating that the mutations found in the *lasR* gene constituted the cause of the diversification into mS2. Furthermore, to determine the effects of each amino acid substitution on LasR function, we examined, in each of the mS2-A to J clones, the LasR-dependent expression of the *P. aeruginosa* genes encoding the AHL synthases, *lasI* and *rhlI*. By using the plasmids pME3853 and pME3846, carrying translational *lasI*::*lacZ* and *rhlI*::*lacZ* reporter fusions respectively, we observed that in wild-type and mS1 backgrounds, expression of *lasI* and *rhlI* was similar, reaching a maximum at the stationary phase (not shown). However, all mS2 variants showed drastically reduced expression levels of *lasI* and *rhlI* even in late stationary phase (OD₆₀₀ 3–5) (Fig. 3), indicating that independent of their nature and location, the *lasR* mutations would cause inactivation of LasR.

Emergence kinetics of *P. aeruginosa* mS2 in batch culture

Although the emergence of mS2 (*lasR*) mutants was clearly observed in the mutator *P. aeruginosa* Hex1T strain (Smania *et al.*, 2004), we examined whether it also occurs in the wild-type non-mutator strain. To investigate this possibility, we carried out a new diversification experiment, growing three independent cultures of the mS1 variant and of the wild-type *P. aeruginosa* Hex1T strain under the same conditions as described above, but for longer periods (300, 400 and 500 h). Again, the emergence of mS2-like morphotypes was scored by visual inspection and confirmed by exoprotease activity determination and production of AHLs.

As shown in Fig. 4(a), after 100 h incubation mS2 represented 0.5 % of the total cells in mS1 cultures, a percentage that exponentially increased with the incubation time, reaching almost 70 % at 500 h. In contrast, the wild-type cultures did not show any evidence of diversification until 400–500 h incubation, when a small proportion (4–5 %) of

Fig. 2. Mutations in the *lasR* genes of *P. aeruginosa* mS2 variants. The nucleotide sequence alterations in mS2-A to J were identified by alignment with the *P. aeruginosa* Hex1T *lasR* sequence. Nucleotide substitutions that occurred in each mS2 variant are indicated by encircled nucleotides. Bold letters indicate the amino acid substitutions, and asterisks indicate stop codons. Underlined triplets indicate the codon affected. The autoinducer-binding and DNA-binding domains are indicated by white and black bars, respectively. Letters in boxes show the amino acids involved in LasR multimerization according to Kiratisin *et al.* (2002).

translucent and hyperpigmented flat bordered mS2-like colonies emerged (Fig. 4a). Interestingly, by quantifying the total c.f.u. of each mS1 culture, it was observed that, at 50–200 h, the number of mS1 cells decreased. In contrast, the emergent mS2 showed a clear increase in cell numbers until 200 h when it also started to decline but with a flatter slope than mS1 (Fig. 4a). This growth of mS2 could be explained as a consequence of generation and/or selection events. In this context, it is important to remember that most of the mS2 clones isolated at 200 h (mS2-A to J variants), contained different kinds of *lasR* mutations, thus indicating the relevance of mutational generation events over selection events in the mS2 enrichment process. In addition, since at 500 h the number of mS2 cells overtook mS1, it may be suggested that mS2 would have a selective advantage in the late stationary phase.

To investigate if the translucent wild-type variants were based on *lasR* mutations, five mS2-like wild-type clones with low exoprotease activity were selected for nucleotide sequencing of the *lasR* gene. Interestingly, one of them showed an unaltered *lasR* gene sequence; another displayed a point mutation in codon 676 resulting in a V226I substitution; and the other three contained the same point mutation in codon 55 (TGG→TGA), producing a LasR truncation by the introduction of a premature stop codon.

From these experiments, we concluded that the frequency of emergence of *lasR* mutants from the mS1 mutator variant was significantly higher than that of the wild-type. Also, it seems evident that the tested incubation conditions are mainly acting as a selector for *lasR* mutants in the mutator, since all the mS2 analysed variants were based on mutations

Fig. 3. Expression of the AHL synthases encoded by the *lasI* and *rhII* genes. β -Galactosidase activities resulting from the translational fusions *lasI::lacZ* carried by plasmid pME3853 (a) and *rhII::lacZ* on plasmid pME3846 (b) were determined from stationary phase cultures of *P. aeruginosa* wild-type Hex1T, mS1 variant and mS2 variants A to J grown in LBS at 30 °C with aeration (220 r.p.m.). Each value is the mean $\pm$ SD of three cultures. Data for mS2-E are not included because it contains the same missense mutation as mS2-D.

in the *lasR* gene. On the other hand, although *lasR* mutants seemed to predominate in mS2-like wild-type populations, they could also originate from mutations in other genes that are at present unknown.

The inactivation of *lasR* increases viability of mS2 variants in late stationary phase

Finally, to evaluate the behaviour of mS2 (*lasR*) mutants, we determined the cell viability under the same conditions as in the previous diversification experiments. Equivalent inocula from single-colony overnight cultures of Hex1T wild-type, mS1, mS2-D and MPAO1 *lasR* mutants were grown in

Fig. 4. Emergence of mS2 variants from *P. aeruginosa* mS1 and Hex1T strains. (a) Single-colony overnight cultures of mS2, mS1 and wild-type strains were inoculated in LBS broth and grown in triplicate in three independent cultures of 10 ml LBS at 30 °C with shaking (220 r.p.m.) for 500 h. Appropriate dilutions of cultures were plated on LBS agar and mS2 colonies were distinguished by visual inspection (colonies with a flat transparent periphery) and decreased exoprotease activity. The percentages of mS2 with respect to the total number of colonies present in mS1 (●) and in Hex1T (○) cultures are indicated. Total viable cells of mS1 (□) and mS2 (■) are also indicated. (b) Growth of *P. aeruginosa* Hex1T, mS1, mS2-D and MPAO1 *lasR*. The strains were grown separately in 10 ml LBS at 30 °C with shaking (220 r.p.m.) for 500 h. Total viable cells were quantified by plating appropriate dilutions of the cultures of Hex1T (▼), mS1 (●), and mS2-D (○), and MPAO1 *lasR* (△) on LBS agar during the late stationary phase. Each point represents the mean of four parallel replicate cultures plated in duplicate.

triplicate in three independent cultures and appropriate dilutions were plated at different times on LBS agar in order to quantify the number of cells. As shown in Fig. 4(b), differences in numbers of c.f.u. were observed after 100–200 h incubation, when the mS1 and the wild-type showed a similar and notable decline in total cell numbers. Interestingly, the number of viable mS2-D variant was nearly constant until 200 h incubation, showing a gentle decrease over longer periods of time. Similar growth

behaviour was observed in the MPAO1 *lasR* mutant strain (Fig. 4b). It is important to note that the time at which the difference in viability was evident among mS2-D, mS1 and Hex1T wild-type strains (Fig. 4b) was coincident with the time at which mS2 began to emerge in the diversification culture (Fig. 4a). Another interesting observation was that after 200 h incubation, mS1 and wild-type cultures had a translucent appearance which was compatible with signals of cell lysis, whereas mS2-D and MPAO1 *lasR* maintained the characteristic dense appearance of saturated cultures. Recently, Heurlier *et al.* (2005) have shown the emergence of *lasR* mutants in cultures of *P. aeruginosa* PAO1 strain serially diluted in nutrient yeast broth. These authors determined that one of the potential causes of the emergence of *lasR* mutants is that they have a higher resistance to cell lysis produced by alkaline stress. In this context we determined that mS2, mS1 and Hex1T wild-type cultures quickly reached a pH of up to 9 after 24 h incubation.

DISCUSSION

The potential role of mutators in evolution has been an issue of major biological and medical interest. Since they would have the capacity to generate adaptive mutations more quickly than the wild-type population, it has been postulated that, particularly in changing and stressful environments, mutator cells hasten the evolution of bacterial populations. Most of the mutator bacteria isolated in nature have been shown to be defective in the MMRS (LeClerc *et al.*, 1996; Matic *et al.*, 1997; Oliver *et al.*, 2002), whose deficiency generates not only a mutator but also a hyperecombinogenic phenotype (Kunkel & Erie, 2005). Thus, MMRS-deficient cells constitute a source of genetic diversity; and as a consequence they would be selected and maintained in a bacterial population.

In a previous article, we noted that, upon inactivation of *P. aeruginosa* Hex1T MMRS by disruption of the *mutS* gene and incubation in rich aerated media, a spontaneous and reproducible morphotypical diversification is produced, in contrast to the non-mutator wild-type strain that, incubated under the same conditions, does not manifest any kind of diversification (Smania *et al.*, 2004). Based on these previous observations, we were particularly interested in the translucent and hyperpigmented mS2 morphotypical variant (Fig. 1), which reproducibly emerges from the smooth and opaque variant mS1 (Fig. 1) and displays differences in several phenotypic traits related to virulence and QS. In the present work, we isolated 10 mS2 variants from independent diversification experiments and performed a detailed phenotypic characterization and gene sequence analyses, which revealed that spontaneous mutations in *lasR* were responsible for the mS2 phenotype. In fact, all mS2 variants analysed showed increased pyocyanin production and swarming motility, diminished exoprotease and elastolytic activity, scarcely detectable production of AHLs, and a substantially reduced virulence in a *P. aeruginosa*–*C. elegans* pathogenesis model (Table 2). Examining the *lasR* gene by

PCR and sequencing, we observed that all *lasR* sequences of the mS2 variants harboured simple but different base substitutions, as compared to the unaltered Hex1T wild-type and mS1 *lasR* sequences. Moreover, since the expression of the AHL synthase genes *lasI* and *rhII* was reduced in all mS2 variants (Fig. 3) and the introduction of plasmid pME3827 containing a *lasR*⁺ allele restored an mS1-like phenotype, we conclude that such mutations, although different in nature and location, severely affect LasR function and constitute the basis for the change from mS1 to mS2.

Importantly, most *lasR* mutations found in mS2 variants consisted of transitions (90%), mainly C to T substitutions (Table 3). This observation is in accordance with previous reports on *E. coli* MMRS-deficient cells, which demonstrated that the spectrum of base substitutions was dominated by C to T changes (Schaaper & Dunn, 1987; Levy & Cebula, 2001). Actually, all the point mutations observed in our analysis originated from mismatches that are normally recognized by a functional MutS (Modrich & Lahue, 1996), indicating that an inefficient repair of such replication-related mismatches in the *mutS* mutant would explain the higher level of emergence of *lasR* mutants with respect to the non-mutator wild-type (Fig. 4).

It has been determined that the LasR N-terminal region is involved in the binding of 3-oxo-C12-HSL and protein multimerization, whereas the C-terminal region has been predicted to be the DNA-binding domain (Kiratisin *et al.*, 2002). Furthermore, by analysing single substitutions of conserved amino acids of LasR, Kiratisin *et al.* (2002) have observed that P117F severely affects the LasR multimerization and function whereas C79S and L110I have no effect on the ability of LasR to multimerize or function. Interestingly, we observed that the amino acids C79, L110 and P117 were substituted in the mS2-A, H and J clones respectively (Table 3). The substitution of P117L in clone mS2-J would have an equivalent effect to the P117F mutation reported previously. However, contrary to Kiratisin's observations, the spontaneous substitutions C79Y and L110P (mS2-A and H clones) altered LasR function (Table 2, Fig. 3). This discrepancy is not surprising since the amino acids involved in the substitutions carried out in Kiratisin's work (cysteine by serine and leucine by isoleucine) are structurally very similar whereas tyrosine, a large aromatic residue, and proline, a residue with highly restricted conformations, would generate substitutions that more drastically affect the protein structure. Thus, our results indicate that not only which residue is affected but also the nature of the substituted amino acid is important for LasR function. Furthermore, it seems that most of the LasR amino acid sequence is important for protein function since different kinds of simple amino acid substitutions throughout the protein affect its activity, suggesting that they have essential roles either in DNA and autoinducer binding or multimerization capacity (Figs 2 and 3).

Considering clone mS2-B, we observed that a single point mutation at *lasR* codon 665 (ACC→ATC) produced the substitution T222I (Table 3). As mentioned, unlike the other mS2 clones, the level of long-chain AHLs for mS2-B was equivalent to that of the wild-type and mS1 strains (Table 2). Nevertheless, as in the rest of the mS2 variants, the expression of both *lasI* and *rhlI* AHL synthases was reduced in mS2-B (Fig. 3) and the exoprotease activity and the opaque and smooth colony aspects were restored by complementation with a functional *lasR*⁺ gene. Additionally, it had no sequence alterations in *gacA* or in *rhlR*. One possible explanation could be a change in the promoter region of *lasI* that would allow the expression of the lactone synthase in spite of the fact that LasR activity was affected. Nevertheless, it would be based on a second suppressor mutation whose origin and nature remains to be elucidated.

The fact that *lasR*-negative mutants emerged as a consequence of several different point mutations indicates that the selection pressure acts on the *lasR* loss of function rather than on a particular mutational hot spot within the gene. Growth experiments showed that, under the conditions examined, the viability of *lasR*-defective mutants was increased, indicating that there would be a direct environmental selection in favour of them (Fig. 4). It is important to note that none of the mS2 variants analysed here contained any sequence alteration in the *gacA* and *rhlR* genes, suggesting that, in such an environment, the selective pressures for GacA/RhlR and LasR were different. We found this observation interesting since other *Pseudomonas* species show a high instability in *gacS*–*gacA* genes in similar nutrient-rich liquid stationary phase cultures (Duffy & Defago, 2000; Bull *et al.*, 2001) and some QS-negative *P. aeruginosa* clinical isolates were affected in both the *lasR* and *rhlR* systems (Schaber *et al.*, 2004; Dénervaud *et al.*, 2004).

The extent of QS-regulated genes of *P. aeruginosa* is still not completely known and may involve much complex cross-regulation between *las* and *rhl* systems, as well as other global regulators, thus exceeding the mere control of virulence. In this context, there is increasing evidence suggesting that the QS system could regulate a number of central metabolic functions such as glucose and adenosine catabolism (Schuster *et al.*, 2003; Heurlier *et al.*, 2005; Heurlier *et al.*, 2006) and denitrification (Wagner *et al.*, 2003). Recently, Heurlier *et al.* (2005) have proposed that alkaline lysis and death, induced by an increase in pH when *P. aeruginosa* grows in nutrient rich broth, may be the critical factor providing a selective survival advantage for *lasR* mutants. We also observed the alkalization of the media after 24 h growth and a lower level of cell lysis for the mS2 (*lasR*) variants compared to *P. aeruginosa* Hex1T and mS1. In this context, it is important to mention the observations obtained by Yates and collaborators (2002) which indicate that the concomitant increase in the pH of the LB medium during the growth of *P. aeruginosa* to the stationary phase generates the spontaneous and reversible

nonenzymic pH-dependent hydrolysis of the AHL ring lactone. Thus, it is highly suggestive that media alkalization not only would induce/select for *lasR*-deficient clones but also would 'turn off' the secreted AHLs, both as confluent phenomena leading to QS defectiveness.

It has been previously reported that under casein starvation conditions, *P. aeruginosa* can mutate and restore elastase and rhamnolipid production despite a non-functional *las* system (van Delden *et al.*, 1998). Such restoration requires an intact *rhl* system, since it is produced by a suppressor mutation that affects the transcription of the *rhlI* synthase gene (van Delden *et al.*, 1998). On the other hand, recent transcriptome analyses have allowed further insights into the particular role of each QS system, interestingly indicating that most of the *P. aeruginosa* QS-controlled virulence genes are primarily controlled by the RhlR-C4-HSL system (Schuster *et al.*, 2003; Schuster & Greenberg, 2006). Therefore there would be an intriguing QS redundancy, with overlapping regulation between the *las* and *rhl* systems as well as other global regulators that, acting in combination with the specific roles of each QS system, may constitute one of the bases of the exceptional plasticity of *P. aeruginosa* in responding to diverse environmental conditions. This trend and the high *las* system instability described here and by others (Heurlier *et al.*, 2005; Salunkhe *et al.*, 2005; Schaber *et al.*, 2004; Cabrol *et al.*, 2003; Hamood *et al.*, 1996), should be taken into account in the light of new therapies designed to specifically block the *las* QS (Smith & Iglewski, 2003b). In addition, it is important to consider that if QS plasticity based on mutational mechanisms, such as the acquisition of suppressor mutations (van Delden *et al.*, 1998), exists, a mutator phenotype would also be crucial to accelerate the generation of such suppressor mutants. However, we consider that, even in a mutator background, an ever-existing fine tuning between generation and selection forces would be present to ensure the emergence of adaptive variants avoiding the accumulation of non-beneficial or lethal mutations. In this context, it is important to mention that besides the observed mS2 diversification, which should be attributed to mutational events, the analysis of chromosomal DNA by pulsed-field gel electrophoresis revealed no obvious genetic rearrangements (unpublished data). This, together with transcriptome analyses of an *E. coli mutS* strain that shows that it does not display major differences from the wild-type at the transcriptional level (Robbins-Manke *et al.*, 2005), would be important to mitigate concern about the possible influence of additional mutations generated in a *mutS* background.

As mentioned above, accumulation of mutations in *lasR* after *in vitro* incubation in aerated rich liquid media has been reported previously for *P. aeruginosa* PAO1 (Heurlier *et al.*, 2005). Also, defects in the *lasR* gene have been detected in several clinical and environmental *P. aeruginosa* strains (Hamood *et al.*, 1996; Cabrol *et al.*, 2003; Schaber *et al.*, 2004; Dénervaud *et al.*, 2004). Furthermore, a recent report has described the isolation of *lasR*-defective mutants of the

virulent and highly transmissible *P. aeruginosa* Liverpool epidemic strains obtained from CF patients (Salunkhe *et al.*, 2005). These observations suggest that, in certain circumstances, the inactivation of the LasR-dependent regulatory system would be advantageous and would play an important role in *P. aeruginosa* pathogenesis. Our results additionally include a novel observation, that the acquisition of selective advantages through inactivation of LasR is greatly increased in *mutS*-deficient strains. Several other studies have supported the postulate that the acquisition of stable mutator phenotypes may confer selective advantages for bacteria mainly in stressful and fluctuating environments such as the infection and establishment processes of a pathogen (LeClerc *et al.*, 1996; Taddei *et al.*, 1997; Giraud *et al.*, 2001). Particularly in *P. aeruginosa*, almost 20% of the isolates obtained from chronically infected CF patients are stable mutators which are mainly produced by alteration of the *mutS* gene (Oliver *et al.*, 2000, 2002). Interestingly, the development of antibiotic resistance has been clearly established as a CF chronic infection phenotype that, driven by hypermutation, constitutes the main force contributing to fixation of mutators (Oliver *et al.*, 2000; Chopra *et al.*, 2003; Maciá *et al.*, 2005). Strikingly, by performing an analysis of mS2 grown in biofilms, we obtained a new spectrum of morphotypes, such as mucoid and small colony variants (unpublished data). This illustrates the increased versatility of *P. aeruginosa* MMRS-deficient cells and suggests that mutant phenotypes other than antibiotic resistance could act, probably by second-order selection processes, as alternative forces fixing mutator alleles in natural populations such as the CF lung. In this context, the findings reported here show the relevance of hypermutability via *mutS* deficiency favouring the acquisition of adaptive advantages through inactivation of LasR and provide interesting insights about the linkage between MMRS deficiency and the regulation of the QS system, a fundamental issue in *P. aeruginosa* adaptive processes.

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