Antibiotic Resistance Evolution Is Contingent on the Quorum-Sensing Response in *Pseudomonas aeruginosa*

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Abstract

Different works have explored independently the evolution toward antibiotic resistance and the role of eco-adaptive mutations in the adaptation to a new habitat (as the infected host) of bacterial pathogens. However, knowledge about the connection between both processes is still limited. We address this issue by comparing the evolutionary trajectories toward antibiotic resistance of a *Pseudomonas aeruginosa lasR* defective mutant and its parental wild-type strain, when growing in presence of two ribosome-targeting antibiotics. Quorum-sensing *lasR* defective mutants are selected in *P. aeruginosa* populations causing chronic infections. Further, we observed they are also selected in *vitro* as a first adaptation for growing in culture medium. By using experimental evolution and whole-genome sequencing, we found that the evolutionary trajectories of *P. aeruginosa* in presence of these antibiotics are different in *lasR* defective and in wild-type backgrounds, both at the phenotypic and the genotypic levels. Recreation of a set of mutants in both genomic backgrounds (either wild type or *lasR* defective) allowed us to determine the existence of negative epistatic interactions between *lasR* and antibiotic resistance determinants. These epistatic interactions could lead to mutual contingency in the evolution of antibiotic resistance when *P. aeruginosa* colonizes a new habitat in presence of antibiotics. If *lasR* mutants are selected first, this would constraint antibiotic resistance evolution. Conversely, when resistance mutations (at least those studied in the present work) are selected, *lasR* mutants may not be selected in presence of antibiotics. These results underlie the importance of contingency and epistatic interactions in modulating antibiotic resistance evolution.

Key words: **antibiotic resistance, *Pseudomonas aeruginosa*, quorum sensing, epistasis, *lasR*.**

Article

Introduction

Conventional wisdom establishes that evolution is sensitive to many stochastic influences so that it should be, mainly, unpredictable (Gould 1994). Nevertheless, many examples of convergent adaptation to similar conditions have been reported (Blount et al. 2018). Further, different studies based on experimental evolution have shown that the number of different evolutionary trajectories that bacterial populations submitted to the same selective pressure can follow is limited, hence presenting some degree of determinism (Barrick et al. 2009; Toprak et al. 2011; Lazar et al. 2013; Ibacache-Quiroga et al. 2018; Sanz-Garcia et al. 2018b). By combining experimental data and mathematical modeling (Huseby et al. 2017), as well as by blending information from molecular epidemiology studies with genetic reconstructions (Shcherbakov et al. 2010), different studies have reported also that mutation-driven bacterial evolution may present (at least in occasions) a certain degree of predictability.

In the case of antibiotic resistance (AR), the main factors that seem to constraint evolution are the mutation rate, the level of resistance and the impact of each mutation in bacterial fitness, and the strength of selection pressure (Hughes and Andersson 2017). Other elements that also restrict evolution are population bottlenecks, clonal interference, cross-selection, compensatory evolution, collateral sensitivity, and epistasis (Weinreich 2005; de Visser and Krug 2014; Szamecz et al. 2014; Maddamsetti et al. 2015; Gifford et al. 2016; Imamovic et al. 2018; Nichol et al. 2019; Rosenkilde et al. 2019).

Fitness costs associated with AR, when acquired by mutation, depend on the effect that resistance mutations have on bacterial physiology (Sander et al. 2002; Linares et al. 2005; Baquero et al. 2009; Fajardo et al. 2009; Andersson and Hughes 2010; Skurnik et al. 2013; Hernando-Amado et al. 2017). Conversely, the basal bacterial physiology, which can vary in different strains, may differentially affect fitness costs. This means that the type of resistant mutants present in a given genetic background might be restricted because of epistatic interactions (Baquero 2013; Agnello et al. 2016; Fuzi 2016), which may affect their level of resistance, their relative fitness, or both (Vogwill et al. 2016; Knopp and Andersson 2018). This situation is particularly evident in the case of combinations of AR mutations. The cumulative selection of AR mutations at different *loci* introduces historical constraints that reduce the variety of pathways leading to AR (Weinreich 2005; Weinreich et al. 2006; Schenk and de Visser 2013; Jochumsen et al. 2016). Further, the order in which mutations are selected is also important for the final outcome (Novais et al. 2010), which indicates the relevance of contingency in the evolution of AR.

Experimental evolution assays have been used to determine the importance of epistasis underlying evolutionary trajectories (Salverda et al. 2011; Tenaillon et al. 2012; Kryazhimskiy et al. 2014). Most studies in the field have analyzed epistasic interactions among different AR mutations (Trindade et al. 2009; Ward et al. 2009; Schenk et al. 2013; Wong 2017; Knopp and Andersson 2018). However, the effect of eco-adaptive mutations in shaping the evolution of AR through epistatic interactions has not been studied in such detail. Nevertheless, the pleiotropic effects caused by adaptive mutations, selected in the absence of selective pressure, but able to confer AR, have been explored (Rodriguez-Verdugo et al. 2013; Knoppel et al. 2017), indicating that AR can be interlinked with general aspects of the bacterial physiology (Martinez and Rojo 2011; Baquero and Martinez 2017).

It is worth mentioning that understanding the effect of eco-adaptive mutations in AR is of relevance for human health. Indeed, the study of *Pseudomonas aeruginosa* isolates producing chronic infections in cystic fibrosis (CF) or in chronic obstructive disease patients shows they present common patterns of evolution (Martinez-Solano et al. 2008; Huse et al. 2010; Yang et al. 2011). To what extent eco-adaptive evolution of bacteria, when facing a new environment, may influence the evolutionary trajectories toward AR of these host-adapted strains is a topic that remains to be analyzed in depth.

In a former study, we have applied adaptive laboratory evolution (ALE) and whole-genome sequencing (WGS) approaches to decipher the evolutionary trajectories of *P. aeruginosa* submitted to selective pressure by the ribosome-targeting antibiotics tobramycin or tigecycline (Sanz-Garcia et al. 2018b). We ascertained that the evolutionary trajectories of culture replicates presented common genetic elements, indicating that evolution toward AR has some degree of determinism. In addition, we found that mutants presenting two base pairs deletion (GA) in *lasR* (hereafter dubbed as *lasR**) were selected in all the control populations evolved in the absence of antibiotics. Opposite to this situation and despite the *lasR** mutation is likely an eco-adaptive mutation that improves the capability of *P. aeruginosa* for growing in the culture medium, *lasR** mutants were not selected in the populations challenged with antibiotics. This feature suggests the existence of epistatic effects between *lasR** and AR mutaions.

LasR is one of the regulators that, together with RhlR and PqsR, controls the three different, but interconnected, regulatory networks which hierarchically regulate the *P. aeruginosa* quorum-sensing (QS) response (Kiratisin et al. 2002). Although this cell-to-cell communication system constitutes a cooperative genetic program that enhances the colonizing ability of microbial population in the infected host (Williams et al. 2007), *lasR* cheaters are selected in vitro and are frequently found in clinical isolates of *P. aeruginosa* from patients with CF (Sandoz et al. 2007). In addition, this genotype enhances the fitness of the entire population during in vitro culture and in a mouse lung infection model (Smith et al. 2006; Sandoz et al. 2007; Hoffman et al. 2009; Zhao et al. 2016). Our results show the existence of negative epistasis between *lasR** and AR mutations. Further, they suggest that *lasR* mutations, besides their potential role for improving *P. aeruginosa* fitness during chronic infections, may also be relevant in shaping the evolution toward AR of *P. aeruginosa*.

Results and Discussion

lasR Deficient Mutants Are Early Selected during *P. aeruginosa* ALE

The evolutionary trajectories of *P. aeruginosa* in the presence of either tobramycin or tigecycline have been previously determined (Sanz-Garcia et al. 2018b). In this study, we found that *P. aeruginosa* mutants presenting two base pairs deletion in *lasR*, which produces a 223 frameshift in LasR, were selected in all control populations grown in absence of antibiotic selection. No mutation in this gene was found in any of the populations evolved under antibiotics selective pressure. To analyze the kinetics of emergence of the *lasR** mutation, this gene was Sanger-sequenced from the evolved populations at days 5, 10, 15, 20, 25, and 30 of evolution. All control populations presented the aforementioned genetic deletion at all evolutionary stages, whereas this mutation was not detected in any of the evolution steps of the populations challenged with either tobramycin or tigecycline. These results indicate that *lasR** mutants were early selected, likely as a mechanism of adaption for growing in the laboratory growth medium (see below), but this mutation may not provide a fitness gain when *P. aeruginosa* grows in presence of either tobramycin or tigecycline.

Characterization of the *lasR** Mutant

The aforementioned *lasR** mutation was detected at the population level. To further study the effect of this mutation in the behavior of *P. aeruginosa*, a clone from an evolved control population, at day 5 of evolution and containing the *lasR** mutation, was selected and sequenced. The clone contained the two base pairs deletion leading to the 223-frameshift mutation in LasR previously detected in the whole population. No further mutation was found in the genome of the *lasR** mutant. To ascertain the effect of this mutation on the regulatory activity of LasR, the expression of a set of QS-regulated genes was measured in *lasR** and compared with that of the wild-type parental strain PA14 during stationary phase, using the oligonucleotides described in supplementary table S1, Supplementary Material online. As shown in supplementary figure S1, Supplementary Material online, expression of QS-regulated genes was impaired in the *lasR** mutant. In addition, QS-related phenotypes were affected in this mutant: a reduced swarming motility and impaired biofilm formation (supplementary fig. S2, Supplementary Material online). Altogether these results indicate that the *lasR** mutation impairs *P. aeruginosa* QS response. It has been stated that triggering the QS response consumes an important amount of *P. aeruginosa* metabolic resources (Ruparell et al. 2016), in which case the *lasR** mutant should be fitter than its wild-type parental, at least at stationary growth phase, when the QS response is triggered. In agreement with this hypothesis, the *lasR** mutant reaches higher optical density at stationary

phase (maximum optical density of 0.84 and 0.71 in *lasR** and PA14, respectively; $P < 0.05$), while no significant differences in exponential growth rate were observed (supplementary fig. S3, Supplementary Material online). This result supports the notion that *P. aeruginosa lasR** mutants are selected in the laboratory growth medium because they reach higher cell densities (in this respect they are fitter) than the wild-type parental strain in these growing conditions, at least when antibiotics are absent. In addition, this emphasizes the metabolic burden imposed by the QS response and the growth advantage of deactivating this cellular response which, while not leading to faster growth, allows to reach higher cell densities.

A trivial explanation to the absence of *lasR** mutants when bacteria evolve in presence of antibiotics might be that this mutation increases the susceptibility of *P. aeruginosa* to the antibiotics used for selection, tobramycin and tigecycline. To ascertain this possibility, the susceptibility of both strains, *lasR** mutant and PA14 parental, to a set of antibiotics, was determined. As shown in table 1, the MICs of tigecycline and of tobramycin were exactly the same for both strains. This result indicates that the absence of *lasR** mutants in the antibiotics evolved populations is not merely a consequence of a higher susceptibility of these mutants to the selective agents that bacteria were confronted with along experimental evolution.

Evolutionary Landscapes of *lasR** *P. aeruginosa* toward AR

Given the above described results, it is possible that the presence of the *lasR** mutation modifies the evolutionary landscapes toward AR of *P. aeruginosa*. This may imply AR evolution to be contingent on the *P. aeruginosa* QS response. To analyze this possibility, 12 biological replicates of the *lasR** mutant were submitted to ALE (fig. 1), under the same conditions previously used for the wild-type PA14 strain (Sanz-Garcia et al. 2018b). These comprise four control populations (populations 1–4), four challenged with tigecycline (populations 5–8), and four challenged with tobramycin (populations 9–12). The MICs for each population of the antibiotic used for selection were determined every 5 days during the 30 days of the experiment (fig. 1). As shown in figure 2, a progressive increase in the level of resistance was observed for both antibiotics and in both genetic backgrounds, *lasR** and the previously analyzed PA14 strain (Sanz-Garcia et al. 2018b), suggesting either accumulation of consecutive mutations after each increase in antibiotic concentration or the displacement of low-level resistant mutants by higher-level resistant ones. The pattern of *lasR** phenotypic evolution was compared with that previously determined for the wild-type strain PA14 (Sanz-Garcia et al. 2018b). As shown in figure 2, all four *lasR** populations submitted to tobramycin selective pressure reached higher levels of resistance than the wild-type populations. The opposite was found in the case of tigecycline: most of the *lasR** evolved populations presented a lower MIC than the PA14 evolved populations. This indicates that the *lasR** mutation modifies the phenotypic evolutionary trajectories of *P. aeruginosa* when submitted to antibiotic selective pressure (fig. 3). To discard the possibility that the phenotypic differences observed might be due to a transient phenotypic adaptation to the presence of an antibiotic rather than to mutations (Levin and Rozen 2006; Martinez et al. 2009; Martinez and Rojo 2011), the evolved populations were sequentially subcultured in the absence of selection pressure (three sequential passages in medium without antibiotics) and the MICs again determined. No changes in MICs were detected, indicating that the observed phenotypes were due to inherited changes.

Table 1. Susceptibility to Antibiotics of the Studied Strains.

Strains	MIC (μg/ml)							
	Tgc	Tob	S	Ak	Cip	Atm	Caz	F
PA14	4	1	16	3	0.094	1	0.75	48
*lasR**	4	1	16	3	0.094	1.5	1	48
PA14-2[a]	64	24	192	256	0.38	3	2	3
PA14-2 *lasR**	96	16	256	96	0.38	3	2	3
PA14-5[b]	≥256	6	48	24	0.25	4	2	6
PA14-5 *lasR**	≥256	3	32	16	0.38	3	3	32

Note.—Tgc, tigecycline; Tob, tobramycin; S, streptomycin; Ak, amikacin; Cip, ciprofloxacin; Atm, aztreonam; Caz, ceftazidime; F, fosfomycin.
[a]Clone presenting mutations in *orfN*, *fusA*, *ptsP*, *fleQ*, and *pmrB*.
[b]Clone presenting mutations in *nfxB*, *orfN*, *mexD*, *rpsJ*, *parR*, *PA14_00180*, and *secA*.

Cross-Resistance and Collateral Susceptibility of the Evolved Populations

We have previously found that *P. aeruginosa* populations submitted to tobramycin or tigecycline present common changes in the susceptibility to other antibiotics besides those used along selection (Sanz-Garcia et al. 2018b). We wondered if the different phenotypic trajectories followed by *lasR** and PA14 populations could also result in differences in collateral susceptibility, reinforcing that the genetic background, either wild type or *lasR**, may constrain the evolutionary trajectories of *P. aeruginosa* in presence of antibiotics. To analyze this issue, the MICs of a set of antibiotics were determined for the 30 days populations that evolved from either PA14 or *lasR**.

In agreement with previous data (Sanz-Garcia et al. 2018a, 2018b), every population evolving in presence of antibiotics presented cross-resistance against antibiotics belonging to different structural families and were hyper-susceptible to fosfomycin (fig. 4 and supplementary table S6, Supplementary Material online). Fosfomycin hypersusceptibility has been associated with mutations in the genes that encode the *P. aeruginosa* peptidoglycan recycling pathway (Borisova et al. 2014; Hamou-Segarra et al. 2017), as well as with the inactivation of the fosfomycin resistance protein FosA (De Groote et al. 2011). Mutations in these genes were not found in the evolved populations. Nevertheless, it might be possible that their expression level could be altered in these populations, an issue that remains to be explored. Albeit, it is worth mentioning that quantitative differences in the strength of the phenotypic changes were observed between the two genetic backgrounds (fig. 4 and supplementary table S6, Supplementary Material online). *lasR**

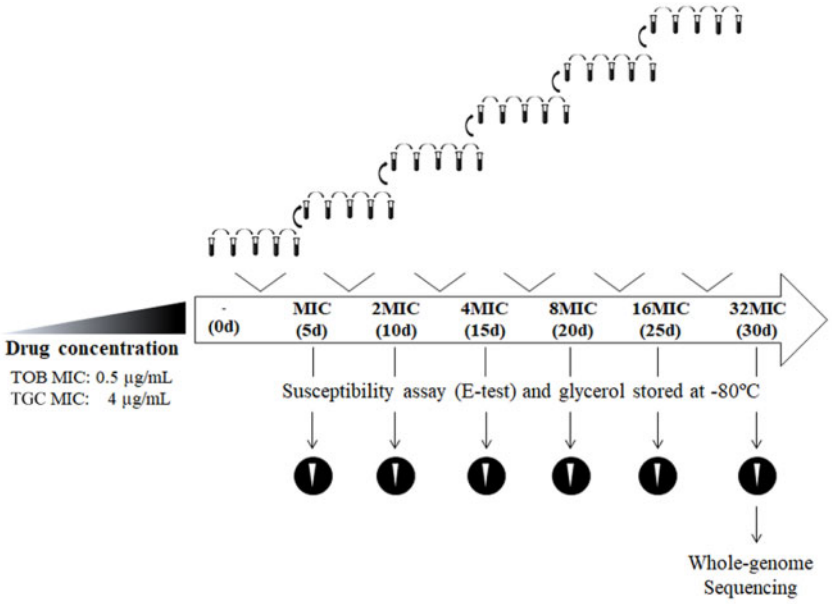

FIG. 1. Experimental evolution setup. Twelve independent bacterial populations of the *Pseudomonas aeruginosa lasR** mutant were grown in parallel in MH Broth (MHB) for 30 days. Four populations were challenged with tigecycline, four with tobramycin, and four were grown without any antibiotic. MICs of tigecycline and tobramycin were used to start the evolution experiment (0.5 µg/ml tobramycin; 4 µg/ml tigecycline). Every day cultures were diluted (1/250) in fresh MHB with the antibiotic concentration required. The concentration of the selection antibiotic was doubled every 5 days, increasing over the evolution experiment from the MIC up to 32MIC, and every replicate population from each step was preserved at −80 °C. MIC to the selection antibiotic for each replicate population was determined using *E*-test strips every 5 days. After 30 days of evolution, the genomic DNA of the 12 evolved populations was extracted and sequenced.

populations that had evolved in presence of tigecycline had higher ciprofloxacin and lower aminoglycosides (streptomycin and amikacin) MICs than PA14 populations evolved in presence of the same antibiotic. The tigecycline and tobramycin PA14 evolved populations showed a higher hypersusceptibility to fosfomycin than *lasR** populations that had evolved under the same conditions. Altogether, these results support the existence of epistatic interactions, which modulate the strength of the global phenotypes of susceptibility to antibiotics, between *lasR** and AR mutations. In a recent study of *P. aeruginosa* ALE, important differences in collateral susceptibility have been observed between parallel populations of the same genetic background adapted to the same antibiotic (Barbosa et al. 2017). These results and our findings suggest that historical contingency may be on the basis of the evolution of *P. aeruginosa* toward AR, including collateral susceptibility and cross-resistance phenotypic outcomes.

Analysis of Mutations Selected during *lasR** Experimental Evolution

To uncover the genetic modifications involved in the AR phenotype acquired by the populations of *lasR**, their genomes, as well as the genomes of the original *lasR** strain and of the control replicates, were sequenced at the end of the experiment (30 days), when the antibiotic concentration reached 32MIC. The mutations selected in the control populations were discarded for being considered to be involved in the adaptation to the growth medium. The presence of the mutations detected by WGS (supplementary table S2, Supplementary Material online) and the order of appearance of each mutation (supplementary table S3, Supplementary Material online) was confirmed by Sanger-sequencing of the corresponding genomic regions.

Population 5 presented, since the beginning of evolution, a mutation in *mutL*, a gene coding for a component of the mismatch repair system (Oliver et al. 2002), which inactivation renders a hypermutator phenotype characterized by an increased mutation rate and a particular mutational spectrum (mainly due to transitions) (Lee et al. 2012; Long et al. 2018). In agreement with this information, this population contains a larger number of mutations, most of them transitions, than the other ones (supplementary table S5, Supplementary Material online), not all of them necessarily involved in the acquisition of AR. Therefore, this population was not included in further comparative analysis.

The majority of the genetic changes found in the evolved populations resulted in amino acid alterations, frameshifts, or stop codons. Mutations in 20 different genes were candidates to be implicated in the acquisition of resistance to ribosome-targeting antibiotics in *P. aeruginosa lasR**. In agreement with results obtained when evolution was performed in PA14, these results indicate that increased resistance to tigecycline and tobramycin, two antibiotics targeting the same cellular machinery, comes about via distinguishable evolutionary trajectories, which have just two genes in common, *pmrB* and *orfN* (supplementary table S2, Supplementary Material online, and fig. 5). *pmrB* encodes the sensor of the two-component system PmrAB, involved in polymyxin and colistin resistance (Moskowitz et al. 2012; Jochumsen et al. 2016), whereas *orfN* encodes a putative glycosyl transferase needed for the

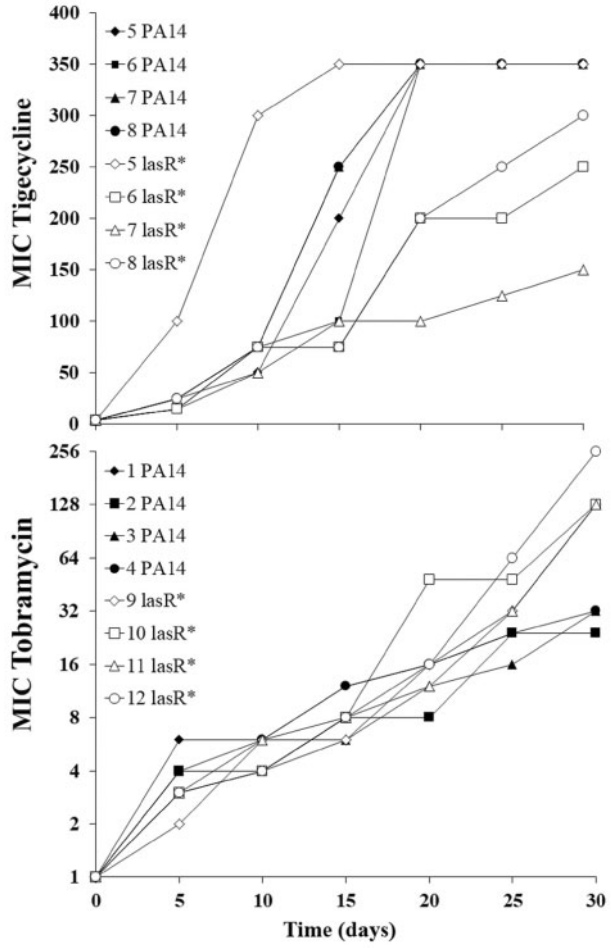

FIG. 2. Phenotypic evolutionary trajectories of the wild-type PA14 and *lasR** *Pseudomonas aeruginosa* population replicates evolving under antibiotic selective pressure. The figure shows the MIC values (μg/ml) for four PA14 and *lasR** replicates evolved in the presence of the indicated antibiotics. MIC values were determined using *E*-test strips in the case of tobramycin and a broth dilution method in the case of tigecycline, for which the detection limit of the *E*-test strip (256 μg/ml) was not high enough to determine the MICs.

glycosylation of type A flagellins (Schirm et al. 2004). Both genes have been described to mediate *P. aeruginosa* virulence and AR (Arora et al. 2005; Wong et al. 2012; Sanz-Garcia et al. 2018b). Interestingly, the populations lacking mutants in *orfN* contained mutations in *orfK* (9, 10 replicates) or in *orfH* (11 replicate), both genes belonging to the same genomic island implicated in flagellin glycosylation (Arora et al. 2004). In addition, it should be noted that these mutations are usually selected in early steps of the tigecycline evolution, suggesting that these genes may be important not only for bacterial virulence but also during the first stages of *P. aeruginosa* evolution toward resistance to the studied antibiotics.

The specific pathways toward resistance to each ribosome-targeting antibiotic were sought. In the case of tobramycin, mutations in *fusA* and *ptsP* were found in the first steps of all the replicates that evolved in presence of tobramycin (supplementary table S3, Supplementary Material online). *fusA* codes for an elongation factor whose mutations appear to be essential in the appearance of aminoglycosides resistance (Wang et al. 2015; Feng et al. 2016), whereas *ptsP* mutants have been selected under tobramycin pressure in a previous *P. aeruginosa* ALE study (Sanz-Garcia et al. 2018b).

Mutations in *nfxB*, encoding the transcriptional repressor of the multidrug efflux pump MexCD-OprJ (Purssell and Poole 2013), likely leading to *mexCD-OprJ* overexpression, were selected in all populations that had evolved in presence of tigecycline. An additional mutation in *mexD*, which codes for the transmembrane protein of the MexCD efflux pump, was detected in replicate 5 (supplementary table S5, Supplementary Material online). This mutation is located in one of the two large periplasmic loops involved in substrate specificity (Elkins and Nikaido 2002) and might improve MexD tigecycline specificity, a feature already described in the case of mutations in *acrB*, the *Enterobacteriaceae* orthologue of *mexD* (Blair et al. 2015). In addition of developing resistance to tigecycline, all the tigecycline evolved replicates showed increased resistance to antibiotics belonging to other structural categories such as quinolones, some beta-lactams and chloramphenicol (fig. 4 and supplementary table S6, Supplementary Material online), a set of antimicrobials that are MexCD substrates (De Kievit et al. 2001).

Comparison of Genetic Modifications Selected along *lasR** and PA14 Tobramycin or Tigecycline ALEs

As above described, the phenotypic evolutions of *lasR** and PA14 toward AR were different (fig. 3), which should be due to different genotypic trajectories followed by *P. aeruginosa* when submitted to antibiotic challenge in the two genomic backgrounds. Although a common set of mutated genes was selected in both cases, new mutations were selected in *lasR** challenged with either of the antibiotics (fig. 5). Moreover, some mutations selected in the wild-type strain PA14 were not selected in *lasR**. These results show that, besides affecting phenotypic evolutionary trajectories, the genetic background (either wild type or *lasR**) impacts as well the genotypic evolutionary trajectories of *P. aeruginosa* when submitted to selective pressure by either tobramycin or tigecycline.

In presence of tigecycline, mutations in *parRS*, *secAG*, and *PA14_00180*, which were selected during PA14 evolution (Sanz-Garcia et al. 2018b), were not selected in the *lasR** background, whereas new mutations were selected, in such *lasR** background, in *phoQ*, *rpoA*, and *mutL* (fig. 5). Population 5, which presents a *mutL* mutation, harbors also a larger number of mutations (supplementary table S2, Supplementary Material online). However, since this is a hypermutator population, these mutants were not included in further analysis. PhoP-PhoQ is a two-component regulatory system implicated in resistance to the polymyxin B and to aminoglycosides (Macfarlane et al. 2000). In addition, this two-component regulatory system regulates the expression of an ABC transporter system (PA4456-4452) which effluxes tetracycline (Chen and Duan 2016). *rpoA* encodes the RNA polymerase alpha subunit. It has been described that exposure to biocides selects *Salmonella enterica* serovar Typhimurium *rpoA* mutants presenting decreased

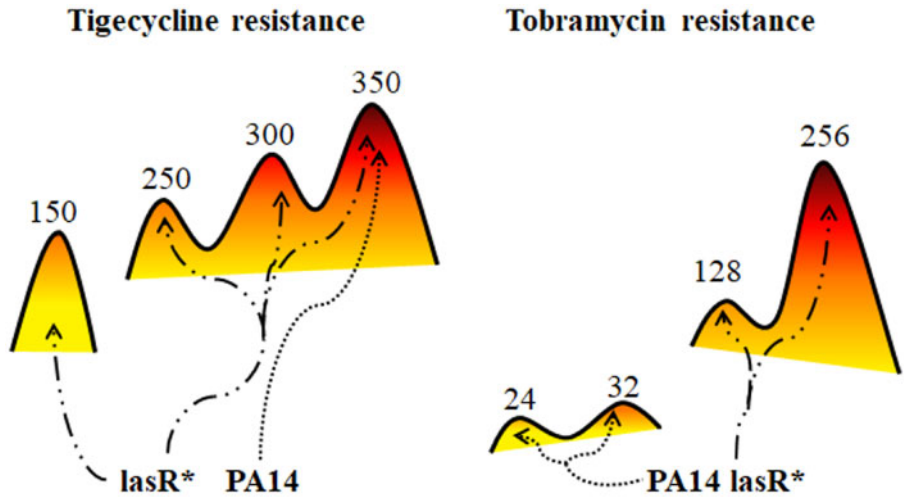

FIG. 3. *lasR** genotype constrains the evolutionary pathways of *Pseudomonas aeruginosa* toward AR. The evolutionary landscapes have been modeled as mountains with genotypes starting at the *X* axis and MIC values at the *Y* axis. High and low levels of resistance are represented as mountains with different altitude and levels of resistance inside each mountain are represented as mountain summits. The potential evolutionary trajectories followed by each genotype are represented.

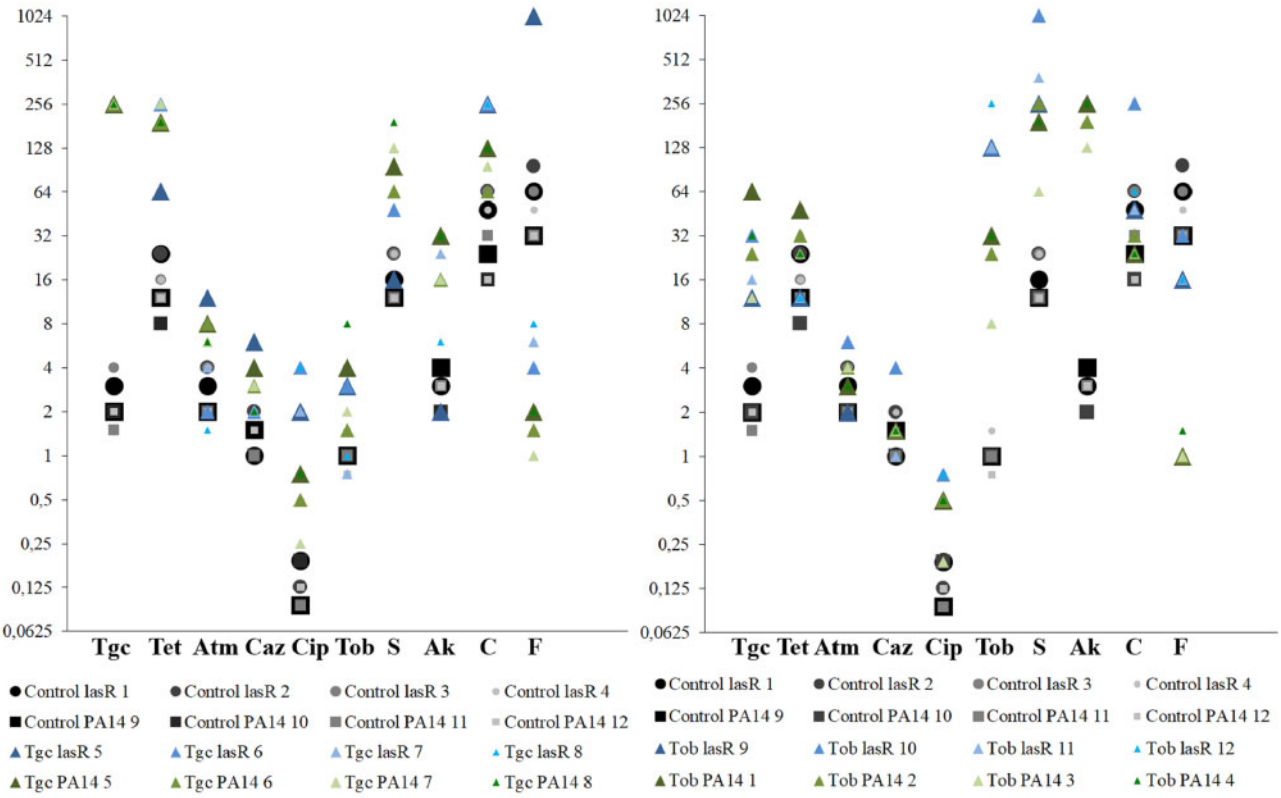

FIG. 4. Evolution of collateral sensitivity and cross-resistance. The collateral profiles of all experimentally evolved populations in tigecycline (left) and tobramycin (right) are represented. The antibiotics against which these populations were challenged are indicated in the *X* axis and the level of resistance for each individual replicate is indicated by a MIC value (μg/ml) in the *Y* axis. Squares and circles represent control replicates of *lasR** and PA14, respectively. Triangles represent each antibiotic treated replicate (different size for each replicate), being blue *lasR** and green PA14. Tgc, tigecycline; Tet, tetracycline; Atm, aztreonam; Caz, ceftazidime; Cip, ciprofloxacin; Tob, tobramycin; S, streptomycin; Ak, amikacin; C, chloramphenicol; and F, fosfomycin.

susceptibility to quinolones (Webber et al. 2015). Additionally, it has been reported that mutations in this gene are selected to compensate fitness defects of rifampicin resistant *Mycobacterium tuberculosis* isolates carrying *rpoB* mutations (Li et al. 2016; Nusrath Unissa and Hanna 2017). Whether *rpoA* mutations contribute to AR or they were selected as a compensation mechanism in our experiments remains to be established. As stated above, the mutation of

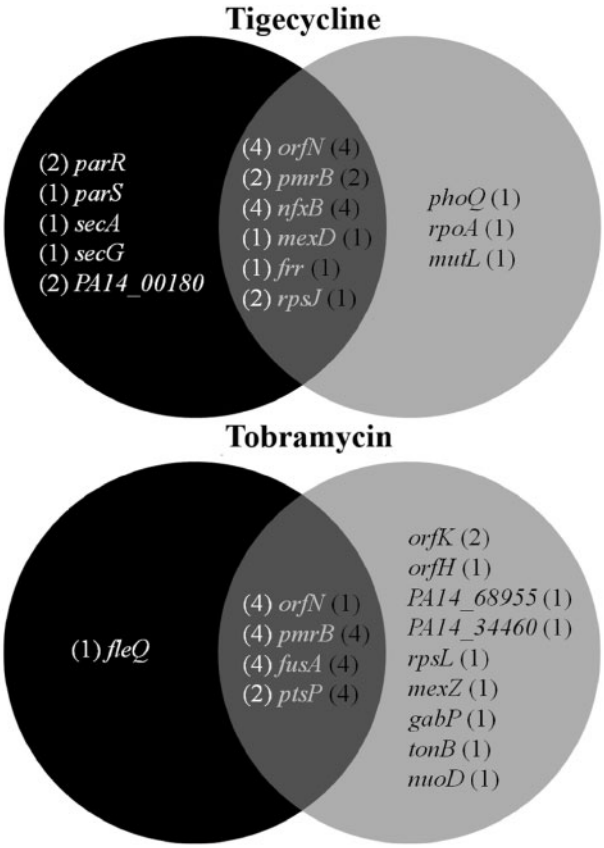

FIG. 5. Common and different genetic changes identified in *lasR** and PA14 populations of *Pseudomonas aeruginosa* evolved for 30 days in tigecycline and tobramycin. The graph shows the genes in which mutations were selected after evolution in 32MIC of antibiotics in *lasR** (gray) and wild-type PA14 (black) *P. aeruginosa* genomic backgrounds (Sanz-Garcia et al. 2018b). In parentheses, the number of replicates containing mutations in each gene is given.

mutL produces a hypermutator phenotype. This phenotype may allow a faster adaptation to antibiotic stress as described in the case of *P. aeruginosa* isolates causing chronic infections in CF patients (Oliver et al. 2002). It has been suggested that the inactivation of the DNA repair system may favor the selection of *lasR* mutations along *P. aeruginosa* chronic infections (Smania et al. 2004). However, other authors have described that mutations in *lasR* arise before mutations in the DNA repair system, showing that hyper-mutability is not a requisite for the acquisition of loss-of-function QS mutants (Ciofu et al. 2010). The results obtained in the current work are in agreement with this second possibility.

In the case of evolution in presence of tobramycin, loss-of-function of *lasR* gave rise to the selection of a set of new mutations, not previously selected in a wild-type PA14 background (Sanz-Garcia et al. 2018b), in *orfH*, *orfK*, *gabP*, *rpsL*, *tonB*, *nuoD*, *PA14_68955*, *PA14_34460*, and *mexZ*. The role played by *gabP*, *PA14_68955*, and *PA14_34460* in AR has not been previously described. *PA14_68955* and *PA14_34460* code for a 2-octaprenyl-3-methyl-6-methoxy-1, 4-benzoquinol hydroxylase and a hypothetical protein, respectively. *gabP* encodes a γ-aminobutyric acid permease. Its orthologous contributes to the growth and virulence of *P. syringae* in tomato plants (McCraw et al. 2016), but a role in AR of this gene has not been reported. The implication of *rpsL* mutations in resistance to ribosome-targeting antibiotics as streptomycin has been previously described in *Escherichia coli* (Pelchovich, Schreiber, et al. 2013) and *Mycobacterium tuberculosis* (Pelchovich, Zhuravlev, et al. 2013). *tonB* codes for an energy-transducing protein that couples the energy of the cytoplasmic membrane to a variety of outer membrane receptors required for the import of siderophores (Poole et al. 1996; Zhao and Poole 2002; Shirley and Lamont 2009). In addition, some *tonB* mutants are hyper-susceptible to antibiotics because of the involvement of TonB in the activity of some efflux pumps, such as MexAB (Zhao and Poole 2002). However, the nucleotide change selected in *tonB* in *lasR** replicate 10 did not produce any amino acid change, and this population was not susceptible to substrates typically extruded by MexAB, such as beta-lactams (Morita et al. 2001). *nuoD* is an already described tobramycin resistance determinant (Schurek et al. 2008). It encodes a proton-translocating NADH ubiquinone oxidoreductase that catalyzes the oxidation of NADH by ubiquinone. Mutations in *nuoD* lead to resistance to benzimidazole derivatives in *Helicobacter pylori* (Mills et al. 2004). It is possible that the Gln184* *nuoD* mutation causes a disruption of the electron transport chain (and the proton motive force), blocking

tobramycin uptake and increasing resistance. The Val43Gly substitution in MexZ, a transcriptional repressor of the *mexXY* multidrug efflux pump operon (Matsuo et al. 2004), should increase the expression of MexXY. Consistent with this situation, the Val43Gly *mexZ* mutation is associated with a reduced susceptibility to ciprofloxacin and tobramycin, two well-known MexXY substrates (Morita et al. 2001).

Genetic Analysis of Epistatic Interactions between *lasR** and Endpoint AR Mutations

Despite the mutations selected in both genetic backgrounds (wild type and *lasR**) present some degree of overlapping (fig. 5), the evolutionary trajectories were not the same, neither at the phenotypic (fig. 3) nor at the genotypic (fig. 5) levels. Further, although the *lasR** mutation was selected at early stages in the evolution of all the control PA14 populations, this mutation was not observed in any of the populations evolved in presence of antibiotic, neither at the first stages (5 days) nor at the end of the evolution (30 days). As shown in table 1, this is not the consequence of a reduced MIC on the *lasR** mutant to the antibiotic selective agents. A suitable hypothesis for explaining the observed counterselection of *lasR** mutants in the presence of antibiotics would be that the *lasR** allele reduces the fitness under selection (measured as growth in the presence of antibiotics) of the selected AR mutants. In other words, counterselection of *lasR** mutation in presence of antibiotics may be due to the existence of a negative epistasic interaction between *lasR** and some AR mutations (fig. 6).

To test this hypothesis, we isolated two resistant clones, containing a representative set of endpoint mutations, obtained at the end of PA14 evolution in presence of either tobramycin or tigecycline. For this purpose, ten independent clones were selected from each endpoint evolved population, the mutations present in the populations were confirmed by Sanger-sequencing. Representative clones containing a large number of mutations were chosen for further studies and their genomes were sequenced to ascertain that no other mutation was present. The clone obtained from the population PA14-2, which had evolved in presence of tobramycin, contained the following mutations: OrfN (Val50fs), FusA (Ala595Pro), PtsP (Glu677ST), FleQ (Thr241Pro), and PmrB (Ser8Ala). The clone obtained from the population PA14-5, which had evolved in presence of tigecycline, contained the mutations NfxB (Leu151Pro), OrfN (Val50fs), MexD (Phe608Cys), RpsJ (Val57Leu), ParR (Glu87Lys), PA14_00180 (Arg49Leu), and SecA (Ala492Val).

The *lasR** allele was introduced in both clones, and the genomes of the resulting strains were sequenced to ascertain that no other mutation was present. The effect of this mutation in the susceptibility to antibiotics was analyzed. As shown in table 1, the introduction of the *lasR** allele in the tobramycin resistant clone PA14-2 led to an increased susceptibility to this antibiotic, which was the one used for *P. aeruginosa* experimental evolution. This result supports the hypothesis that *lasR** mutants are counterselected in presence of this antibiotic as the consequence of negative epistasis with some AR mutations (fig. 6). Additionally, the introduction of the *lasR** allele led to changes in the cross-resistance phenotypes of clone PA14-2. In the *lasR** background, the mutant presents a higher susceptibility to amikacin and a decreased susceptibility to tigecycline and streptomycin (table 1) when compared with the wild-type PA14 background. These results further support the existence of epistasis between certain AR mutations, present in clone PA14-2, and *lasR**. In the case of the resistant clone PA14-5, isolated in a population that had evolved in presence of tigecycline, resistance to this antibiotic of both strains (PA14 or *lasR** background) was above the limit of detection of the test used for MIC determination. However, when bacteria grow in presence of this antibiotic, a clear impairment in growth rate and in the final optical density reached is found in the *lasR** genomic background, when compared with the original PA14-derived mutant (fig. 7). Besides, the introduction of the *lasR** allele modified the susceptibility to other antibiotics. In comparison with the situation in PA14, a *lasR** background increased the susceptibility to tobramycin, streptomycin and amikacin, and decreased the susceptibility to ciprofloxacin and fosfomycin of the tigecycline resistant clone PA14-5 (table 1). Again, these data support the existence of negative epistasis between *lasR** and certain AR mutations present in the tigecycline resistant clone PA14-5.

Epistatic Interactions between *lasR** and AR Mutations Occur at Early Stages of Evolution in Presence of Antibiotics

Our work indicates that the genomic background, either wild-type PA14 or QS-defective *lasR** mutant, modifies the strength of resistance as well as the collateral sensitivity and cross-resistance phenotypes of endpoint mutants selected in presence of either tobramycin or tygecycline. However, these results do not provide evidences on early stages of evolution, which would have been contingent for the establishment of the observed differential evolutionary trajectories. To address this issue, we focused in a mutation present in *orfN* (OrfNVal50fs), hereafter dubbed as *orfN**. This mutation was selected, at early stages of evolution (day 5), in all the *P. aeruginosa* PA14 populations evolved in the presence of either tigecycline or tobramycin (Sanz-Garcia et al. 2018b), so it may be contingent on PA14 evolution in presence of both antibiotics. As in the case of the wild-type PA14 background, *orfN** mutants were selected early in all *lasR** populations evolving in presence of tigecycline and in one of the populations evolving under tobramycin selection. These results indicate that, even in a *lasR** background, *orfN** mutations are frequently selected. *lasR** mutants are enriched in the first steps of evolution in absence of antibiotics, likely because they present an increased fitness on rich medium (supplementary fig. S3, Supplementary Material online). Therefore, it would be expected that, when fitter *lasR** mutants emerge and are mixed with the less fit PA14 population, *orfN** mutants should be more frequently selected in the *lasR** background than in the wild-type PA14 one. However, as above stated, the *lasR*orfN** mutant was not selected during evolution in presence of antibiotics, suggesting the existence of negative

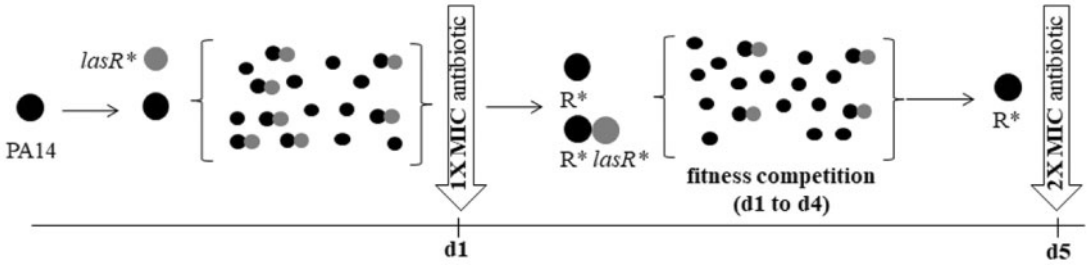

FIG. 6. Negative epistasis hypothesis between *lasR** and AR mutations. During growth in medium without antibiotic, the starting drug susceptible genotype (black circle) may present a mutation in *lasR* gene (gray circle) before the antibiotic treatment at day 1, this mutation is selected in all the control populations evolved in the absence of antibiotic. When antibiotic is applied, an AR mutation (R*) may be selected in both, PA14 and *lasR**. During maintenance of antibiotic concentration (day 1 to day 4) fitness competition may lead to a reduction of *lasR** mutants from day 1 to 4, being absent at day 5 and at the end of the evolutionary assay (see text for details).

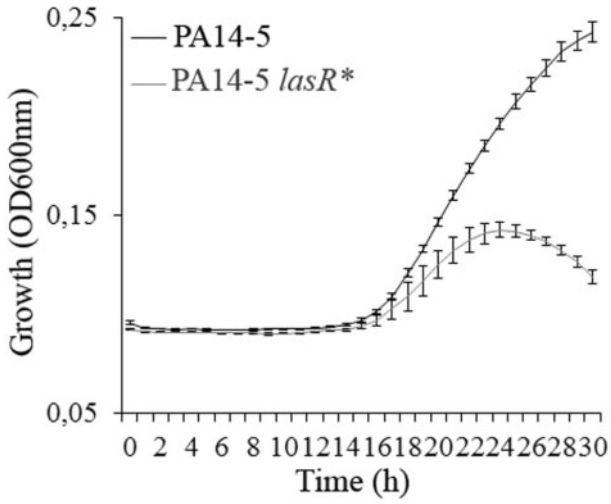

FIG. 7. Introduction of *lasR** in antibiotic resistant PA14 endpoint evolved clone reduces *Pseudomonas aeruginosa* fitness in presence of tigecycline. Growth curves on MHB containing tigecycline (50 μg/ml) of *P. aeruginosa* resistant mutant PA14-5 (*nfxB*, *orfN*, *mexD*, *rpsJ*, *parR*, *PA14_00180*, and *secA*) which contains (gray line) or not (black line) a *lasR** loss-of-function mutation. Mean ± SEM values of three replicates are represented.

epistatic interactions between *lasR** and *orfN** mutations. To address this possibility, single-step tigecycline resistant mutants were selected in plates containing tigecycline 32 μg/ml. *orfN* was Sanger-sequenced in 100 of the selected mutants. One *orfN** mutant was chosen for further studies and its genome was whole sequenced. The mutant presents the *orfN** mutation (OrfNVal50fs), without any further genetic modification. Since flagellum is relevant in *P. aeruginosa* motility (Overhage et al. 2007), it is possible that this mutation may alter such motility. Indeed, as shown in supplementary figure S4, Supplementary Material online, the *orfN** mutant presents a strongly reduced swarming motility (supplementary fig. S4, Supplementary Material online).

To analyze the effect of the genomic background in the level of resistance achieved by the *orfN** mutation, the *lasR** mutation was introduced into the *orfN** mutant. The genome of the resulting double mutant was whole sequenced, not presenting any further mutation. Growth curves of *orfN** and *lasR*orfN** mutants, in the presence of tobramycin or tigecycline, at the concentrations used in the previous evolution assay in PA14, were compared. As shown in figure 8, the introduction of the *lasR** mutation into the *orfN** mutant negatively affects the growth of *P. aeruginosa* in presence of either tobramycin or tigecycline, by reducting the final optical density reached and by increasing the lag phase, respectively. These results indicate that, when the *orfN** and *orfN*lasR** mutants coexist, the first should prevail over the second. In other words, the absence of *lasR** mutants in *P. aeruginosa* PA14 populations evolved in presence of either tobramycin or tigecycline was the consequence of negative epistasis between *lasR** and AR mutants, as *orfN**, selected early along evolution.

Conclusions

Although evolution is considered as largely unpredictable (Gould 1994), important efforts have been made to predict the evolution of microorganisms, particularly in the case of health relevant processes as the acquisition of AR (Martinez et al. 2011; Palmer and Kishony 2013; Hughes and Andersson 2017). It has been invoked that selective pressure, fitness costs, mutational load, and epistatic interactions modulate all of them the evolutionary trajectories of bacterial pathogens toward AR (Hughes and Andersson 2017). In addition, the order of acquisition of AR mutations—the historical contingency—is also in the basis of the evolutionary trajectories toward AR (Salverda et al. 2011). Herein, we analyze another aspect that may modulate the pathways toward AR, as is the existence of mutations in *loci* unrelated to the acquisition of resistance, in particular, those improving the fitness of the microorganisms when confronted with a new habitat (as the infected host). For this purpose, we studied the effect on *P. aeruginosa* evolution toward AR of a *lasR** loss-of-function mutation that improves bacterial fitness in absence of antibiotics, while impairing the QS response.

lasR mutations are frequently selected in vivo in *P. aeruginosa* populations causing chronic infections. It has been suggested that these mutations modify bacterial virulence, hence favoring chronic infections (Hoffman et al. 2009; Wilder et al. 2009; LaFayette et al. 2015). However, their selection in laboratory growing conditions suggests that optimization in the

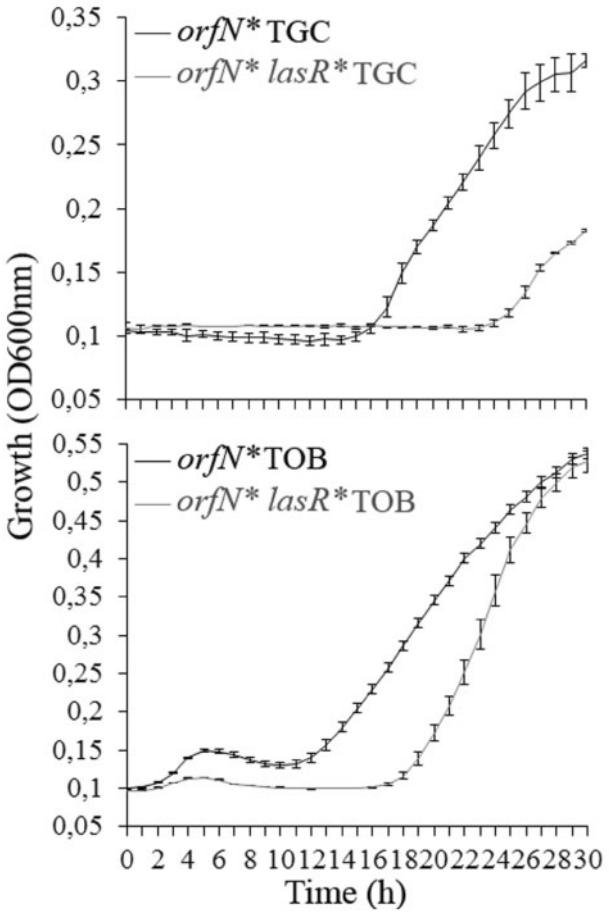

FIG. 8. Validation of the negative epistasis hypothesis between *lasR** loss-of-function and *orfN** mutations. Growth curves on MHB containing either tobramycin (0.5 μg/ml) or tigecycline (4 μg/ml) of *Pseudomonas aeruginosa orfN** mutant containing (gray line) or not (black line) a *lasR** mutation. Mean ± SEM values of three replicates are represented.

use of nutrients resources, more than fine tuning of bacterial/host interactions, is on the basis of the selection of these mutants. The current work indicates that negative epistasis and historical contingency modulate *P. aeruginosa* evolution toward resistance.

When *lasR** mutations are selected and predate the bacterial population early during evolution, before selection pressure is applied, AR mutants are selected in this genetic background similarly as it happens in a wild-type background. Nevertheless, *lasR** constrains the type and AR level of resistant mutants and therefore, competition between antibiotic resistant mutants, presenting either QS defective or QS wild-type backgrounds, is expected to happen. In turn, this indicates that the cross-talk between AR and virulence (Martinez and Baquero 2002) may depend on epistatic interactions among resistance and virulence determinants and that the trajectories leading to AR can be contingent on previously selected eco-adaptive mutations. Conversely, since QS-defective *lasR* mutants are not selected when AR mutants arise, QS-defective mutants are also contingent on previous acquisition of AR, at least in our experimental setting.

Materials and Methods

Experimental Evolution and Determination of Susceptibility to Antibiotics

Twelve independent *lasR** bacterial populations (four populations grown in presence of tigecycline, four populations challenged with tobramycin, and four control populations grown without antibiotic) were subjected to experimental evolution under the same conditions described in Sanz-Garcia et al. (2018b).

Cultures were grown at 37 °C and 250 rpm. Every day, along 30 days, the cultures were diluted (1/250) in fresh Mueller Hinton (MH) Broth (Pronadisa). Every 5 days the concentration of the selecting antibiotic was doubled, increasing over the evolution experiment from the MIC up to 32MIC. Every replicate population from each concentration step was preserved at −80 °C. In addition, every 5 days, the MIC of the antibiotic used for selection in population was determined at 37 °C in MH agar using *E*-test strips (MIC Test Strip, Liofilchem). A broth dilution method was performed in the case of tigecycline, for which the detection limit of the *E*-test strip (256 μg/ml) was not enough to evaluate the resistance level acquired by the evolved populations.

At the end of the experiment, the level of resistance acquired by the evolved populations and the susceptibility of these populations to a wide range of antibiotics—tetracycline, aztreonam, ceftazidime, ciprofloxacin, streptomycin, amikacin, chloramphenicol, and fosfomycin—was examined at 37 °C in MH agar using *E*-test strips.

WGS and Genomic Analysis

The genomic DNA of the 12 populations evolved during 30 days and of the mutants constructed in this work (*lasR**, *orfN**, *orfN*lasR**, *PA14-2*, *PA14-2 lasR**, *PA14-5*, and *PA14-5 lasR**) was extracted using the Gnome DNA kit (MP Biomedicals). WGS was performed by Sistemas Genómicos S.L. The quality analysis was done using a 4200 Tapestation High Sensitivity assay and the DNA concentration was determined by real-time polymerase chain reaction (PCR) using a LightCycler 480 device (Roche). Libraries constructed were pair-end (2× 100) and sequenced in an Illumina 2500 sequencer. The average number of reads per sample represents a coverage >200×. A similar approach was followed for sequencing the mutants used in the study.

WGS data were analyzed using the CLC Genomics Workbench 9.0 (QIAGEN) software. To that end, genomic information was trimmed and the reads were aligned against the *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1). Mutations detected in the DNA samples obtained from populations kept under selective pressure were filtered against those mutations present in control populations.

Validation of Single Nucleotide Polymorphisms

Presence and order of appearance of the mutations detected in the WGS analysis was determined by Sanger-sequencing. Twenty pairs of primers, which amplified around 200 base pair regions containing each genetic modification, were used

(supplementary table S4, Supplementary Material online) to recover de DNA region by PCR. The DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN) and Sanger-sequenced at GATC Biotech.

RNA Preparation and Real-Time Reverse Transcription-PCR

An overnight culture of *P. aeruginosa* PA14 was used to inoculate Erlenmeyer flasks with 20 ml of Lysogeny Broth (LB) Lennox medium to a final $OD_{600\,nm}$ of 0.01. The flasks were incubated until stationary phase of growth ($OD_{600\,nm} = 2, 5$). Ten milliliters of each culture were spun down at 4 °C (7,000 rpm) and frozen in dry ice. Total RNA was extracted with the Rneasy Kit (Qiagen). DNA was removed by treatment with the DNA-Free Kit (Ambion). To check that no residual DNA was present in the RNA samples, PCRs were performed with primers *rplU_Fw* and *rplU_Rv* (supplementary table S1, Supplementary Material online). cDNAs were obtained using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as indicated by the manufacturer.

Real-time reverse transcription-PCR was performed using a Power SYBR Green Kit (Applied Biosystems) in an ABI Prism 7300 real-time system (Applied Biosystems). The expression level of QS-related genes was measured using the primers described in supplementary table S1, Supplementary Material online. Gene expression data were normalized to that of the *rpsL* gene. All the primers used were designed with Primer3 Input software; their specificity was tested by BLAST alignment against *P. aeruginosa* PA14 genome from Pseudomonas Genome Database (http://www.pseudomonas.com/) and their efficiency was analyzed by reverse transcription-PCR using serial dilutions of cDNA. The relative amount of mRNA was calculated following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). In all cases, the mean values of relative mRNA expression obtained in three independent triplicate experiments were considered.

Motility and Biofilm Assays

Swarming assays were carried out as described (Ha et al. 2014). The culture medium used contained 0.5% (w/v) Bacto Casaminoacids (BD), 3-mM $MgSO_4$, 3.3-mM K_2HPO_4, 0.5% (w/v) glucose, and 0.5% Bacto Agar (BD). Four microliters of bacterial overnight cultures diluted to an $OD_{600\,nm}$ of 1.0 were used to inoculate the plates.

Biofilm production was quantified using a microtiter plate assay (Merritt et al. 2005) with some minor modifications, using LB as culture medium, 0.1% (w/v) crystal violet in ethanol as staining solution and 0.25% Triton X-100 in ethanol as biofilm solvent solution. After 48 h of culture in static conditions at 37 °C, the $OD_{570\,nm}$ of stained, washed, and solubilized contents of each well, were measured using a Tecan Infinite 200 plate reader (TECAN).

Growth Curves

Growth curves were determined using a Tecan Infinite 200 plate reader (TECAN) by measuring the $OD_{600\,nm}$ of the bacterial cultures (inoculated at an $OD_{600\,nm}$ of 0.01) every 10 min during 30 h at 37 °C in MH media and MH media supplemented with different concentrations of tobramycin and tigecycline.

Mutant Construction

Strains containing the *lasR** selected mutation were constructed by insertion of the mutant allele by homologous recombination. *lasR** allele was obtained by PCR from the mutant strain obtained in the evolutionary assay, leaving ~500 bp upstream and downstream of the corresponding single nucleotide polymorphism using the oligonucleotides 5′-*AAGCTT*AGCGCCATCCTGCAGAAGAT-3′ and 5′-*AAGCTT*GCCGACCAATTTGTACGATC-3. The PCR product containing *Hin*dIII restriction sites was cloned into *Hin*dIII-digested and dephosphorylated pEX18Ap vector (Hoang et al. 1998), and then introduced by transformation into the conjugative *Escherichia coli* S17-1 strain. Subsequently, conjugation and mutant selection were performed as described elsewhere (Hoang et al. 1998) using 350 µg/ml carbenicillin and 10% sucrose.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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