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Codon-usage frequency mediated SNPs selection in *lasR* gene of cystic fibrosis *Pseudomonas aeruginosa* isolates

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen with high clinical relevance for hospital infections of patients. Accumulating DNA sequencing results of clinical *P. aeruginosa* isolates have revealed frequent mutations in *lasR* gene, which encodes the highest arches component of quorum-sensing system (QS). We analyzed the sequencing data of *lasR* gene from a large collection of cystic fibrosis (CF) *P. aeruginosa* isolates. Our systematical analyses revealed that single nucleotide polymorphisms (SNPs) selection in *lasR* gene were largely constrained by codon-usage frequency. As a whole, SNP-substituted codons encoding unconserved amino acid resulted in unfavored codons with relatively low codon-usage frequency, while those associating with conserved amino acid were not strictly regulated in such way. These SNPs substitutions gives rise to diverse functional LasR isoforms and contributes to the relative growth fitness of recombinant *lasR* variant strains. Our survey reveals a novel pattern of SNPs selections in *lasR* gene of CF isolates. Our findings could be served as a powerful resource for understanding adaptive mechanism of clinical isolates under environmental constrains and developing antibacteria drugs for CF patients.

1. Introduction

Despite substantial advances in cystic fibrosis (CF) treatment, resistance of *P. aeruginosa* to therapy remains a main challenge (Burns et al., 2001). *P. aeruginosa* strains isolated from CF patient lungs were frequently found to acquire a series of adaptive mutations in the genome (Smith et al., 2006; D'argenio et al., 2007). Mutations in *lasR* gene, coding for a transcriptional factor of quorum sensing system (QS), have long been detected and are common in clinical *P. aeruginosa* isolates, especially those isolated from chronically infected CF patients (D'argenio et al., 2007). The presence of *lasR* mutants were associated with worse disease progressions to airways of CF patients (Hoffman et al., 2009).

P. aeruginosa lasR mutants were described as exhibiting a few phenotypic characteristics, such as defects in the QS-regulated virulence (Köhler et al., 2009; Hoffman et al., 2010), resistance to the cell lysis in high-density cultures(Heurlier et al., 2005), enhanced resistance to the antibiotic treatment (Hoffman et al., 2010) and oxidative stress (D'argenio et al., 2007). LasR mutants have also been proposed acting as a social cheater in a mixed population containing *lasR* intact strains. LasR mutants can exploit the secreted LasR-regulated public goods from *lasR*-intact kins, and thus gain the relative growth advantage without suffering energetic costs for producing such goods (Sandoz et al., 2007; Dandekar et al., 2012).

The identification and characteristics of adaptive mutations in clinical *P. aeruginosa* isolates revealed a range of strong selective pressures in those isolates (Smith et al., 2006). One consequence brought by those constraints is the selective usage of different genetic codons (Hershberg and Petrov, 2008). For example, comparative sequencing analysis for some genes, such as *oriC*, *citS* and *ampC*, in different genotypes of *P. aeruginosa* isolates showed that codon biases of such genes were consistently high (Kiewitz and Tümmler, 2000). Such biased codon usage potentially affect general gene expression levels (Gingold et al., 2014), influence protein folding (Pechmann and Frydman, 2013) and mRNA stability (Presnyak et al., 2015a). Biased using codons is generally regarded as a strategy allowing clinical isolates to have better adaptation under harsh conditions.

Mutation patterns identified in *lasR* gene of clinical isolates were found to be diverse, including single nucleotide polymorphisms (SNPs), insertion and deletions (In/Del) (Cabrol et al., 2003; Köhler et al., 2009; Feltner et al., 2016). Those mutations brought either missense or nonsense amino acid substitutions to LasR (Cabrol et al., 2003; Köhler et al., 2009; Feltner et al., 2016). Interestingly, mutations in *lasR* gene does not always lead to loss-function of LasR in each clinical isolate

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studied (Feltner et al., 2016). While most *lasR* variant isolates lost LasR activity, some still bear functioning LasR (Feltner et al., 2016). Though mutational selections are thought to be associated with combined factors, those functioning LasR isoforms of clinical isolates are interesting, considering those isolates were under strong-selection conditions.

Prior studies isolated and sequenced a large collection of *P. aeruginosa lasR* variants from CF patients (Feltner et al., 2016). The majority of those isolates were LasR-null mutants, with either amino acid substitution or stop codon introduction. Interestingly, about one-sixth of those isolates observed still retain functioning LasR (Feltner et al., 2016). Here we reanalyzed this sequencing data, particularly focusing on SNPs selections in *lasR* gene. We found that those isolates generally select SNPs resulting in corresponding codons with relatively less usage frequency. On the other hand, some selected SNPs, which arised in or surrounding codons responsible for conserved amino acids, escaped such genetic constrains. Site-direct mutagenesis of those predicted conserved amino acids fully disrupted LasR activity. Further, we also demonstrated that the codon-usage frequency regulated SNP selection pays a role in relative fitness of *lasR* variant recombinant strains. Our studies here revealed a novel codon-usage frequency mediated SNPs selection in *lasR* gene, and would contribute to understanding the environmental adaptations for CF *P. aeruginosa* isolates.

2. Materials and methods

2.1. Bacterial strains and growth

The *P. aeruginosa* wildtype PAO1 strain and derivative Δ*lasR* mutant strain with *lasR* gene clean deletion (Wang et al., 2015), are generous gifts from E. Peter Greenberg (University of Washington, US).

2.2. Bacterial growth

The *P. aeruginosa* and derivative recombinant strains, and *E.coli* DH5a were grown in Luria-Bertani broth with aeration at 37 °C.

2.3. Expressing of SNPs-modified lasR copy in the background of P. aeruginosa ΔlasR;1; strain

The *lasR* gene fused with native promoter was cloned into pUC18T-mini-Tn7T-Gm vector (GenBank accession number: AY599232.2) (Choi and Schweizer, 2006), generating pUC18T-mini-Tn7T-Gm-lasR. Corresponding SNPs from CF clinial *lasR* variant isolates were introduced by site-directed mutagenesis into *lasR* gene, using pUC18T-mini-Tn7T-Gm-lasR as template and followed *Dpn*I digestion for amplification products. Constructs were verified by Sanger sequencing and transferred into *P. aeruginosa* Δ*lasR* mutant with a helper plasmid, pTNS2 (GenBank accession number: AY884833), by electroporation (Choi and Schweizer, 2006). Primers used for cloning and site-direct mutagenesis are listed in Table S5.

2.4. Proteolysis assay

LasR activity of a series of recombinant PAO1 strains was evaluated in proteolysis assay, in which those recombinant strains form a zone of clearing on skim milk agar plate (Sandoz et al., 2007; Dandekar et al., 2012). Individual colonies were spotted on skim milk agar plate (25% LB, 4% skim milk, 1.5% agar). The LasR-dependent protease-catalyzed zones were photographed after incubation at 37 °C for 18 h.

2.5. Relative fitness of recombinant strains

All *Pseudomonas* strains were grown in Luria-Bertani broth for overnight until reaching to the stationary phase. Such starter culture of each recombinant strain was then cocultured with WT PAO1 strain at the start ratio of 1:99 in PM medium (Dandekar et al., 2012), respectively. The mixed bacterial population was sub-cultured with the ratio of 1:150 by exchanging fresh PM medium daily. After 3 or 6 days of subculture, cell counts were determined by spreading bacteria onto LB agar plate with no antibiotics or Gm-resistant LB plate for counting total cell numbers or recombinant cell numbers. The relative fitness is calculated as $W_{ij} = D_i/D_j = m_i / m_j$, which is estimated as the ratio of the numbers of doublings of the two competitors according to previous definition (Lenski et al., 1991).

2.6. Bioinformatic analyses

The DNA sequencing data of clinical CF *P. aeruginosa* isolates was obtained from the previously published article (Feltner et al., 2016). The entire CDS of *P. aeruginosa* PAO1 strain (accession number: NC_002516.2) was download from NCBI and the codon-usage frequency of each genetic codon was computed using customized Perl scripts, which is available upon request. The protein sequence of LasR and the homologues, LasR from *Pseudomonas aeruginosa* (NP_250121.1), AhyR from *Aeromonas salmonicida* (YP_855090.1), CarR from *Erwinia carotovora* (Q46751.1), EsaR from *Pantoea stewartia* (P54293.1), LuxR from *Vibrio fischeri* (YP_206883.1), PhzR from *Pseudomonas fluorescens* (YP_206883.1), VanR from *Vibrio anguillarum* AAC45213.1) and YenR from *Yersinia enterocolitica* (AAC45213.1), were aligned using MEGA6 software and the following similarity plot was conducted using JEMBOSS (version 1.5) and R (version 3.2.3).

2.7. Statistical analyses

Statistical analyses were performed using Excel and R (http://www.R-project.org/).

3. Results

3.1. SNPs selection in lasR gene is mediated by codon-usage frequency

To investigate genetic mechanisms for SNPs selection when a bacterium is subjected to strong selective pressures, we analyzed the sequencing data from a large collection of clinical *P. aeruginosa* isolates in CF patients (Feltner et al., 2016). We particularly focused on *lasR* gene because mutations in that gene were frequently identified in clinical *P. aeruginosa* isolates (D'argenio et al., 2007). We started from a total of 205 such isolates, which have insertions, deletions (InDels) and single nucleotide polymorphisms (SNPs) in *lasR* gene. To eliminate factors potentially complicate the bioinformatic analysis, InDels as well as SNPs introducing early stop codons were excluded. In the end, total 92 *lasR* variants of those CF isolates were included for further analyses, each one containing one single SNP in *lasR* coding region (Table S1).

Since SNPs substitution always confers synonymous or non-synonymous alteration, we asked whether SNPs substitutions introduced alterations of codon-usage frequency in *lasR* gene of those isolates. We first computed the codon-usage frequency for codons affected by SNPs substitutions. The codon-usage frequency here was calculated as the observed frequency of a codon divided by the frequency of all codons used genome-wide in *P. aeruginosa* strain PAO1, a reference laboratory strain (Table S2). The poor or favored codons here are referred to those codons with relatively low or high genome-wide usage frequency, respectively. We next compared SNPs-introduced alterations of codon-usage frequency in *lasR* gene of each isolate with that of strain PAO1. The majority of isolates selected SNPs resulting in corresponding poor codons, with the exception of a small sample size of isolates (Fig. 1A and B, $p = 2 \times 10^{-4}$, *t* test, also seen in Table S3).

We noticed that among total 92 isolates analyzed, a subpopulation of isolates (49 isolates) identified from different places share common mutational hotspots in LasR, in which same amino acids were repeatedly mutated by SNP substitutions (Table S4). We were particularly interested in this special subpopulation for studying the possible

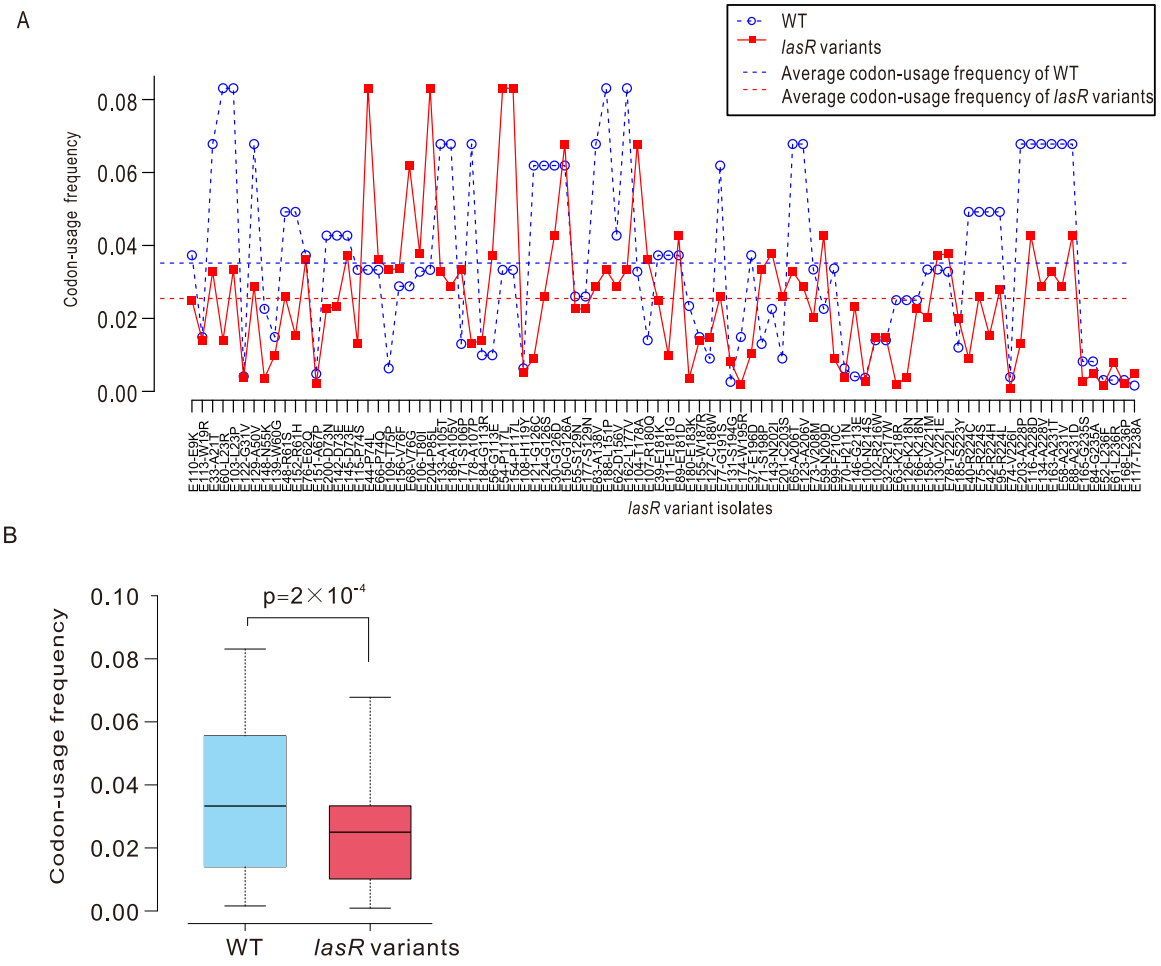

Fig. 1. Analysis of codon-usage frequency alteration in cystic fibrosis *lasR* variant isolates.
(A) Plot of codon-usage frequency alteration in cystic fibrosis *lasR* variant isolates. The *lasR* gene of laboratory strain PAO1 (NC_002516.2) serves as a reference. Values at the y-axis indicate codon-usage frequency of mutated or referential codons. The x-axis shows the *lasR* variant isolates with SNP substitutions, which are in the order of mutated codons positions in *lasR* gene. Each cystic fibrosis isolate contains one single SNP in *lasR* gene. The *lasR* variant isolates, N = 92; Blue hollow circle: WT PAO1, red solid square: cystic fibrosis *lasR* variant isolates.
(B) Boxplot of average codon-usage frequency of all mutated genetic codons in *lasR* gene of cystic fibrosis isolates. Bars represent the standard deviation of the average, $p = 2\times10^{-4}$, *t* test.

common SNPs selection mechanism. Reduction of codon-usage frequency for mutated codons was much more significant in this subpopulation than in the total 92 isolates. In detail, thirty-seven isolates (75.5%) in this subpopulation selected SNPs giving rise to infrequently used codons, while only 12 isolates (24.5%) did conversely (Table S4). These results suggested that codon-usage frequency mediated SNPs selection is even stronger in some sub-categorized clinical *P. aeruginosa* isolates.

Taken together, we reasoned that SNPs selection in CF *lasR* variant isolates we analyzed is largely mediated by codon-usage frequency, leading to rare codons with relative less codon-usage frequency.

3.2. Codons encoding conserved amino acids escape constrains of codon-usage frequency

While the majority of mutated coding codons in *lasR* gene selected SNPs leading to biased usage frequency, a few exceptions were also observed to escape such constrains (Table S3). For example, SNPs substitutions in codons encoding amino acid P74 resulted in either favored or poor codons, as examined in isolate E44, E66 and E155 (Table S4). This resulting flexible alteration in codon-usage frequency prompt us to look for particular features of P74 in LasR, such as whether P74 is functional importance for LasR. As expected, P74 is one of such amino acids essential for AHL encapsulation activity of LasR (Bottomley et al., 2007) (Kiratisin et al., 2002). Similar phenomena were also found for G126, as shown in isolate E30, E121, E124 and E150 (Table S4). Meanwhile, it should be stressed that, G126 is such an amino acid being conserved both in TraR of *Agrobacterium tumefaciens* and SdiA of *Escherichia coli* (Bottomley et al., 2007). We thus hypothesis that those amino acids associated may be evolutionarily conserved and essential for LasR activity.

To test above hypothesis, we chose LasR homologues from reference strains as previously reported (Nasser and Reverchon, 2007), and performed amino acid sequences alignment analyses. Indeed, all such amino acids examined, either positioned at or surrounded the conserved sites (Fig. 2). This analysis suggested that codons encoding evolutionarily conserved amino acids are not constrained by the codon-usage frequency for SNPs substitutions.

These predicted conserved amino acids were further mapped onto *lasR* coding region and they on the whole located in two known functional domains, AHL binding and multimerization domain (amino acids 15–164) and DNA binding domains (amino acid 174–231) (Fig. S1). Based on above analyses, we therefore assumed these codons encoding predicted conserved amino acids might be essential for LasR activity.

B

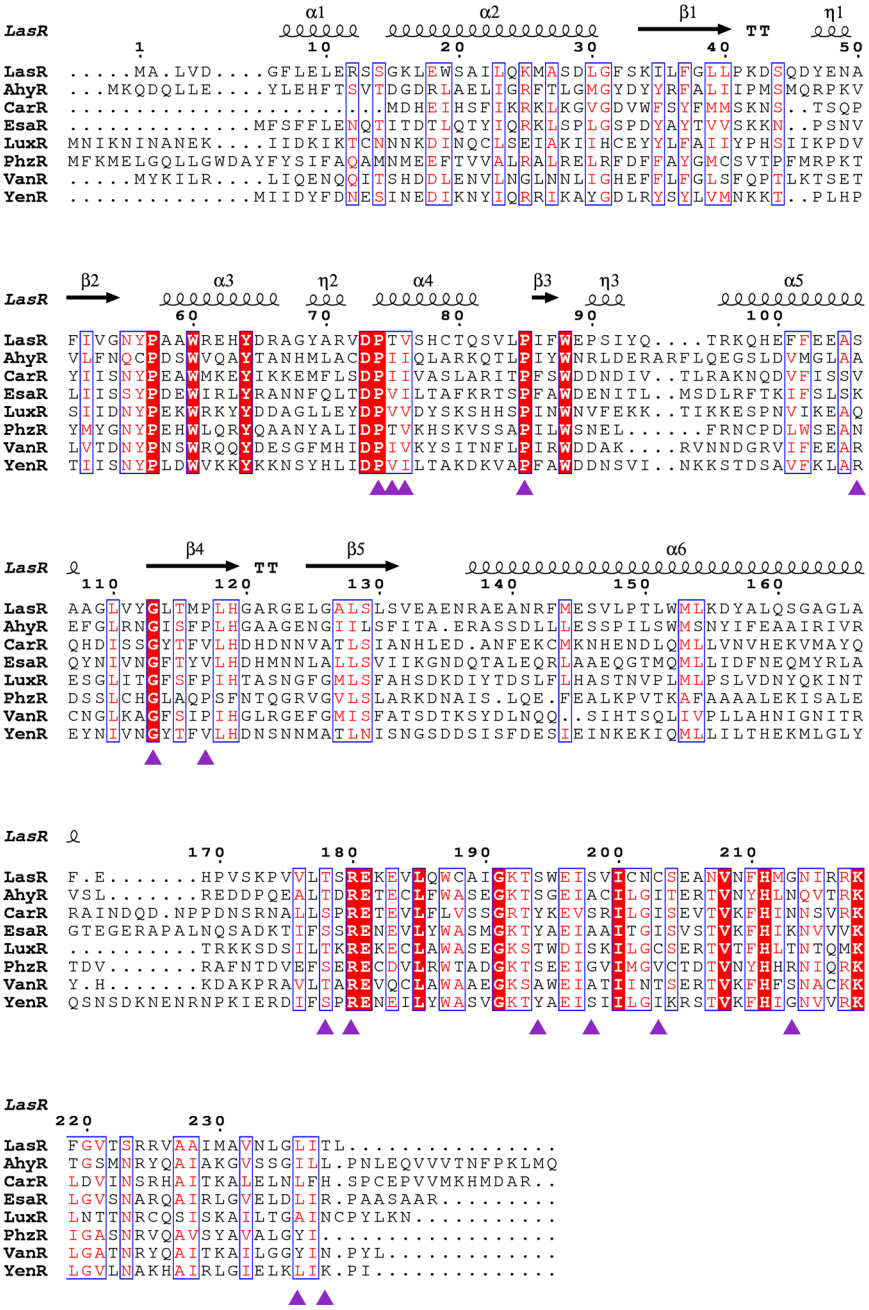

Fig. 2. LasR homologue sequences alignment. The protein sequences of LasR and homologues are aligned using MEGA6 software. The major secondary structure elements of LasR are shown above the alignment. The amino acids are framed and red highlighted according to the conserved degree. The amino acids encoded by SNP-mutated poor codons are highlighted as purple triangle, which are locating at or close to the conserved sites. LasR from *Pseudomonas aeruginosa* (NP_250121.1), AhyR from *Aeromonas salmonicida* (YP_855090.1), CarR from *Erwinia carotovora* (Q46751.1), EsaR from *Pantoea stewartia* (P54293.1), LuxR from *Vibrio fischeri* (YP_206883.1), PhzR from *Pseudomonas fluorescens* (YP_206883.1), VanR from *Vibrio anguillarum* (AAC45213.1) and YenR from *Yersinia enterocolitica* (AAC45213.1).

3.3. Predicted conserved amino acids are required for LasR proteolytic activity

We next sought to test whether those predicted conserved amino acids are functionally essential for LasR. We took advantage of a strategy by generating different recombinant *P. aeruginosa* strains bearing the SNP-modified *lasR* copies for test. These recombinant strains were all originated from uniform *lasR*-null genetic background and were chromosomally inserted with SNP-modified *lasR* copies, which were derived from corresponding CF isolates (Fig. 3A).

Since functional LasR regulates extracellular enzymes, such as LasB elastase and alkaline protease(Gambello and Iglewski, 1991; Gambello et al., 1993), activity of SNPs-modified *lasR* could be evaluated by forming zone of proteolysis on the skim milk agar plate (Sandoz et al., 2007; Dandekar et al., 2012). We tested representatives of those SNP-modified *lasR* copies (Table S3). The *lasR* recombinant variants, with substitutions in codons responsible for conserved amino acids (A21 T and A50 V), both substantially lost LasR proteolytic activity (Fig. 3B and C), similar to that of the negative control, LasR-null mutant. By comparison, SNPs substitutions in codons encoding nonconserved amino acids, (T75 P and G213E), brought little effect to LasR proteolytic activity (Fig. 3B and C). As such, these results suggested that codons encoding for conserved amino acids could escape constrains of codon-usage frequency on SNPs selection, and are at least required for LasR proteolytic activity.

3.4. Codon-usage frequency regulation contributes to relative fitness of lasR variant recombinant strains

We have showed that introduction SNPs into *lasR* gene results in

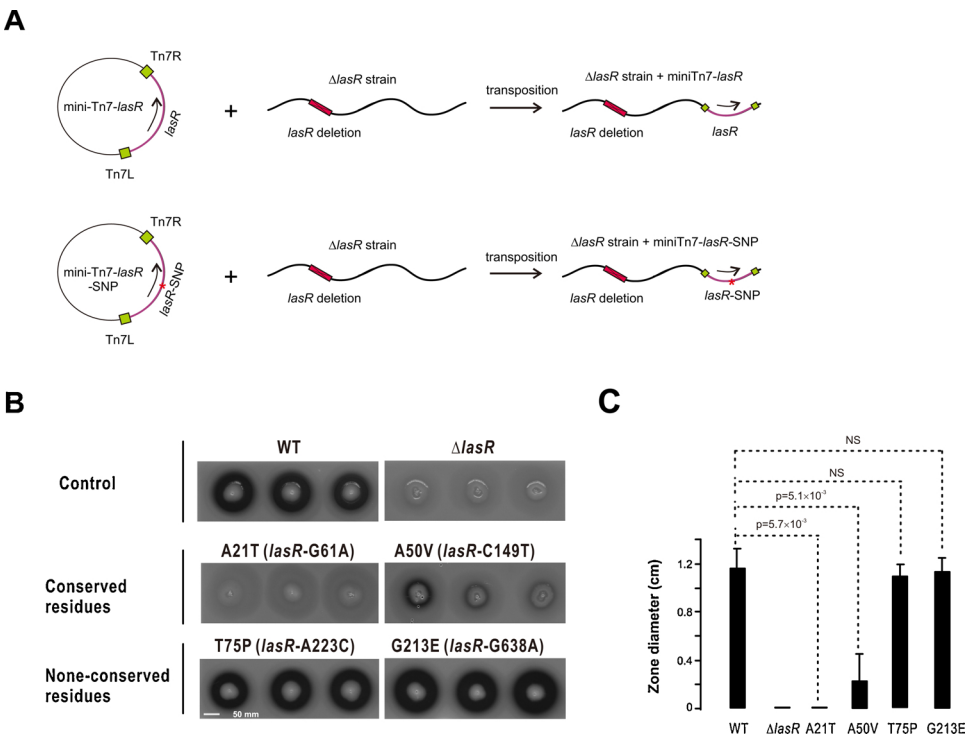

Fig. 3. Proteolytic activities of SNP-modified LasR.
Representative SNPs from CF isolates were tested for effects on the LasR-regulated protease activity.
(A) The schematic presentation for constructions of *lasR* copies bearing with or without SNPs. The miniTn7 vector (GenBank accession number: AY599232.2) containing SNP-modified *lasR* copies were transferred into Δ*lasR* mutant, creating a series of recombinant strains as indicated.
(B) These recombinant strains expressing different SNP-modified *lasR* copies were spotted onto skim milk agar and LasR-dependent protease-catalyzed zones were photographed after 18 h. WT: miniTn7-*lasR* wild type in Δ*lasR* strain; Δ*lasR*: miniTn7- empty vector in Δ*lasR* strain; A21T: miniTn7-lasR- G61A in Δ*lasR* strain. SNP(G61A) derived from E33 isolate, locating at the codon encoding non-conserved amino acid A21; A50V: miniTn7-lasR- C149 T in Δ*lasR* strain. SNP(C149 T) derived from E129 isolate, locating at the codon responsible for non-conserved amino acid A50; T75P: miniTn7-*lasR*-A223C in Δ*lasR* strain. SNP (A223C) derived from E109 isolate, locating at the codon coding for conserved amino acid T75; G213E: miniTn7-lasR- G638A in Δ*lasR* strain. SNP(G638A) derived from E146 isolate, locating at the codon associating with amino acid G213 around conserved sites. Scale bars, 50 mm.
(C) Quantification of the data in (B) showing that groups of SNP-modified LasR either retained LasR activity displaying clear proteolysis zone (corresponding T75 P and G213E), or inactivated LasR activity presenting little or nearly no proteolysis zone (corresponding A21 T and A50 V). Bars represent standard deviation of the average between 3 replicates.

non-proteolytic or proteolytic LasR (Fig. 3). The non-proteolytic *lasR* mutants have been previously reported to bear higher relative growth fitness against wild-type strain by exploiting exocellular public goods (Diggle et al., 2007; Sandoz et al., 2007). However, it is not clear about the relative growth fitness of *lasR* mutants remaining proteolytic ability. Thus, we here focused on SNPs leading to active proteolytic LasR. By using similar strategies as above, we generated two *lasR* versions, SE129-H (*lasR*-C149 G, mutated codons with corresponding relative high codon-usage frequency) and SE129-L (*lasR*-C149 T, mutated codons with corresponding relative low codon-usage frequency), by introducing SNPs into *lasR* gene genertating different codons at the same amino acid. The designed strategy of these two *lasR* versions are explained at Table S5. We chose this representative A50 because this amino acid is dispensable for LasR activity and not conserved across LasR homologues (Fig. 2). As expected, recombinant strains bearing both those *lasR* versions showed clear zones of proteolysis, similar to that of WT control (Fig. S2, $p = 0.07$ and $p = 0.26$, respectively, *t* test), and likewise no statistically significant difference between each other (Fig. S2, $p = 0.42$, *t* test). This proteolytic assay showed that LasR activity was not affected by the SNP substation at A50.

We next co-cultured WT strain with each recombinant strain respectively, in order to reveal whether relative growth fitness could potentially be affected by SNP-modifed *lasR* versions. Indeed, strain SE129-L showed higher relative fitness index than strain SE129-H at a later time point (6 day) but not earlier (3 day) (Fig. 4, $p = 0.03$, *t*-test). These results demonstrated that SNPs-introduced codon-usage frequency regulation contributed to the relative growth fitness of *lasR* variant recombinant strain, even when SNP substitutions arise at a dispensable amino acid, and this effect gradually accumulated upon the co-culture progress.

Taken together, these data suggested a functional role of SNPs-introduced codon-usage frequency regulation in administrating relative growth fitness of *lasR* variant recombinant strains. Since these two *lasR* variant versions has undistinguishable LasR activity, future experiments need to be performed to investigate mechanisms leading to this differential relative growth fitness with wild-type strain.

4. Discussion

Although SNPs substitutions in *lasR* gene are common phenomena in clinical *P. aeruginosa* isolates, relatively little is known about the relevance between those coding SNPs organizations and corresponding effects for LasR activities. Here, we systematically analyzed the sequencing data, specially focusing on *lasR* gen from a large collection of CF *P. aeruginosa* isolates (Feltner et al., 2016). We generated a reference list of SNPs from 92 *lasR* variants among the CF isolates and defined the profile of altered codon-usage frequency for *lasR* gene. The results presented here provide a comprehensive probe of codon-usage frequency mediated SNPs regulation in *lasR* variant isolates. Importantly, our studies also provide a template for conducting similar systematic studies on SNPs substitutions in other bacterial species for gaining valuable insights into their regulatory roles. The strategies used in our studies will likely be particularly helpful for the study other important human pathogens of interest.

Substitution frequency of non-synonymous mutations is often much lower than that of synonymous mutations (Li, 1997), a fact is thought to be due to higher selective pressures non-synonymous mutations have (Tomoko, 1995). In our studies, SNPs identified in all 92 CF isolates are all non-synonymous (Table S1). Since CF isolates are expected to encounter complexed environmental constrains, such as chemical and physical stresses, variable nutrients and limited irons (Palmer et al., 2005, 2007), these identified non-synonymous SNPs presumably suggested that CF isolates encountered relatively strong selective pressures. Meanwhile, given that bacterium usually pays cost of coding mutations for obtaining adaptive benefits (Giraud et al., 2001), biased SNPs selection discovered in our studies may probably suggest potentially important roles for these SNPs in *lasR* gene, probably in order to modulate LasR in CF isolates. In general, CF isolates with coding SNPs

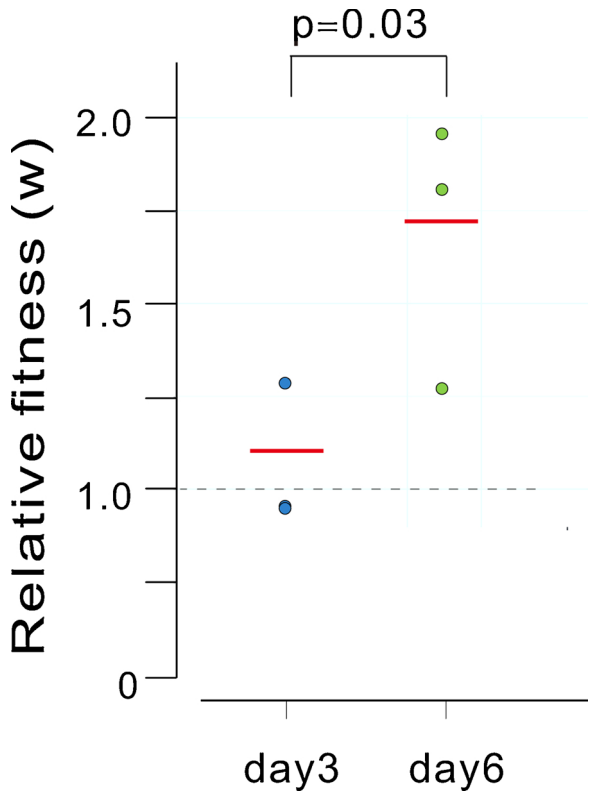

Fig. 4. Codon-usage frequency regulates relative growth fitness of recombinant *lasR* variant strains.
SNP-modified *lasR* copies were transferred into Δ*lasR* mutant, creating a series of recombinant strains as indicated, similar to the methods descripted in Fig. 3. We generated two *lasR* versions, SE129-H (*lasR*-C149 G, corresponding to have codons with relative high codon-usage frequency) and SE129-L (*lasR*-C149 T, corresponding to have codons with relative low codon-usage frequency), by introducing SNPs into *lasR* gene creating codons with different codon-usage frequency at the same amino acid (also seen at Table S5). The miniTn7 constructs bearing these two *lasR* copies transferred into Δ*lasR* mutant strain respectively. Each recombinant mutant strain was then co-cultured with WT PAO1 in PM medium at a start ratio of 1:99 and subjected to daily subculture (very 24 h). Relative fitness of SE129-L to SE129-H was computed as the ratio of Malthusian growth parameters (w) at the time point of 3 days or 6 days. Relative growth fitness of SE129-L to SE129-H is statistically significant at day 6 ($p = 0.03$, *t*-test).

in *lasR* gene were thought to be LasR-null mutants (Köhler et al., 2009). However, a large screening for LasR activities in CF isolates revealed some *lasR* variants still retain LasR activities (Feltner et al., 2016). Consistent with previous reports, our studies also verified active SNP-modified LasR in our in vitro recombinant analysis system. Yet the mechanism and role of coding SNPs leading to active LasR under strong selection pressures still remains an open question for future studies.

SNPs substitutions in coding region could affect protein expressions in several means, such as through modulating mRNA translation efficiencies, influencing mRNA folding structures and regulating mRNA decay level (Goodman et al., 2013) (Pechmann and Frydman, 2013) (Gu et al., 2010; Spencer et al., 2012; Presnyak et al., 2015b). Accordingly, SNPs substitution in *lasR* gene may expect to affect LasR expression and could display LasR-dependent phenotypes, especially given that those SNPs were selected in CF isolates. Two representatives of SNPs tested in our studies, giving rise to amino acid A21 T and A50 V respectively, almost completely abolish LasR proteolytic activity (Fig. 3). The inactive LasR brought by these two SNPs substitutions were reasonably expected, because they are conserved across LasR homologues in different bacterial specis and hence predicted as essential for LasR proteolytic activity. On the other side, other group of SNPs analyzed here showed comparable level of proteolysis with that of wild-type LasR (Fig. 3 and Fig. S2). These undistinguished LasR-dependent proteolytic phenotypes probably lies in that sensing a single amino acid substitution in LasR may be beyond our detection sensitivity, when those SNPs altered such codons responsible for unconserved amino acids. Another possible explanation is that such SNP-modified LasR expression might be easier to be detected when subjecting *lasR* variant recombinant strains to host-like conditions rather than artificial laboratory conditions we used here.

In general, LasR activities in clinical *P. aeruginosa lasR* variant isolates is evaluated in the way of measuring readout of LasR-responsive reporter transferred in those isolates. We are aware of that under certain circumstances, this approach has its limitations and might bring about inaccurate interpretations. One of the reasons is that *P. aeruginosa* owes complexed QS systems, consisting of four sub-systems partly overlapping with redundant QS functions (Lee and Zhang, 2015). As previously reported, under some circumstances in some clinical *P. aeruginosa* isolates, LasR functions can be compensated by activating RhlR simply through a mutated transcriptional regulator (MexT) (Oshri et al., 2018), or be partially taken over by another IQS system if under phosphate limited conditions (Lee et al., 2013). In those circumstances, direct measurement of SNP-modified LasR activity might be misled. To circumvent those limitations, we took use of a recombinant system to assess LasR activity (Fig. 3). This recombinant system has advantage in sequestering and evaluating SNP-modified LasR activity in a uniform *lasR*-null genetic background. This strategy could rule out potential influences from other non-*lasR* sites, given that genome-wide secondary mutations are rather common in clinical *P. aeruginosa* isolates (Oliver et al., 2000).

We here analyzed a limited proportion number of CF *P. aeruginosa lasR* variant isolates from a single source and observed a trend for Codon-usage frequency mediated SNPs selection in those isolates. To better consolidate our observations, for future studies, extending more isolate resources and including more isolates for test could be helpful for illustrating selection force on observed codon-usage frequency. For example, environmentally isolated *P. aeruginosa* strains could be a valuable option. Likewise, LasR mutants acting as cheaters derived from laboratory evolutions under limited nutritional conditions (Sandoz et al., 2007; Dandekar et al., 2012), would also be of interest. Rather, the strategies used in our studies could be applied in other functional genes as well as in other human important pathogens.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.05.002.

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