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Original article

Macrolide therapy in *Pseudomonas aeruginosa* infections causes uL4 ribosomal protein mutations leading to high-level resistance

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ABSTRACT

Objectives: *Pseudomonas aeruginosa* colonizes the cystic fibrosis (CF) airways causing chronic bacterial lung infections. CF patients are routinely treated with macrolides, however, *P. aeruginosa* is considered insusceptible as consequence of inadequate susceptibility testing leaving resistance mechanism completely overlooked. Here, we investigated a new mechanism of macrolide resistance caused by ribosomal protein mutations.
Methods: Investigating a longitudinal collection of 529 isolates from CF patients and analysing 5758 protein sequences from different sources, mutations in *P. aeruginosa*'s ribosomal proteins connected to macrolide resistance were identified. Using a modified susceptibility testing protocol, isolates harbouring a mutated uL4 ribosomal protein were tested for resistance against macrolide antibiotics and macrolide-induced quorum sensing modulation. Proteome and ribosome profiling were applied to assess the impact of the mutations on the bacterial physiology.
Results: Five uL4 mutations were identified in isolates from different CF patients. Most mapped to the conserved loop region of uL4 and resulted in increased macrolide tolerance (>10-fold relative to wt strains). Greater concentrations (>10-fold) of macrolide antibiotic were needed to inhibit the growth, reduce swimming motility, and induce redox sensitivity of the uL4 mutants. 16 proteins involved in ribosome adaptation displayed altered expression possibly to compensate for the uL4 mutations, which changed the ribosome stoichiometry without negatively affecting bacterial physiology.
Conclusions: Macrolide antibiotics should, therefore, be considered as active antimicrobial agents against *P. aeruginosa* and resistance development should be contemplated when patients are treated with prolonged courses of macrolides. Importantly, improved macrolide susceptibility testing is necessary for the detection of resistant bacteria. **Lise Goltermann, Clin Microbiol Infect 2022;28:1594**
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Introduction

Cystic fibrosis (CF) patients suffer from chronic bacterial lung infections, which can last for more than 30 years [1]. They are treated with a plethora of antibiotic, immunomodulatory and supportive treatments [2,3]. Courses of macrolide treatment are typically prescribed as anti-inflammatory supportive treatment of patients infected with *P. aeruginosa* [4–8]. However, according to the EUCAST guidelines [9], *P. aeruginosa* is not susceptible to macrolides due to its intrinsic resistance phenotype [15]. Recent reports have highlighted the shortcomings of standardized antimicrobial susceptibility testing (AST) in accurately predicting macrolide susceptibility in *P. aeruginosa.* The introduction of physiologically relevant media for AST allowed identification of 23S rRNA mutations causing macrolide resistance [10,11]. However, mechanisms which confer tolerance and/or resistance have not been systematically investigated.

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Alterations in specific regions of the uL4 and uL22 ribosomal proteins have been described to cause macrolide resistance through remodeling of the nascent peptide exit tunnel (NPET) of the ribosome in *Escherichia coli*, *Streptococcus pneumoniae*, *Legionella pneumophila* and *Neisseria gonorrhoeae* [12,13]. However, this has never been investigated in *P. aeruginosa*, most likely because of the assumption that macrolides do not exhibit a direct antibacterial effect on this pathogen [14].

In this study, we investigated collections of *P. aeruginosa* clinical isolates spanning different continents and infection scenarios [15,16], where we identified mutations in the uL4 (*rplD*) and uL22 (*rplV*) proteins. The investigated mutations reduce the bacteriostatic effects of macrolide antibiotics but also the macrolide-induced modulation of quorum sensing properties. Importantly, differences in macrolide susceptibility are only apparent using a modified AST broth microdilution method with 50% LB as the test medium and not through the standardized AST.

Methods

Detailed information is provided in supplementary text S1.

Bacterial strains and media

P. aeruginosa clinical isolates were isolated at the Copenhagen CF Center and Department of Clinical Microbiology, at Rigshospitalet, Copenhagen, Denmark [15,16]. The collection is comprised of 529 isolates collected from 55 patients. The *P. aeruginosa* laboratory strain PAO1 was used as laboratory reference [17]. Strains were routinely grown in LB, 50% LB, RPMI or cation adjusted Muller-Hinton broth (CA-MHB) at 37°C with shaking.

Antibiotic susceptibility testing

Minimum Inhibitory Concentration (MIC) was determined by broth microdilution [9] in 50% LB. The MIC was determined as the lowest concentration, which inhibited visible growth (OD630 < 0.05) in the wells. For post-MIC effect, cell cultures from the MIC assay were spotted onto LB-agar plates. For measurement of efflux pump activity, Phenylalanine-Arginine β-Naphthylamide was included during MIC determination. E-tests (bioMérieux, Marcy-l'Étoile, France) were performed according to manufacturer's guidelines but on 50% LB agar. For re-growth experiments, cultures were incubated with 4x MIC for 2h and then 100-fold diluted into fresh 50% LB without antibiotic to allow re-growth.

Complementation assay

For complementation assays, the *rplD* gene from strains PAO1, LJR04 (ΔKPW) and 102 (+G) was PCR amplified using primers *rplD*_forward and *rplD*_reverse and inserted through USER-cloning technology into the pHERD30T vector amplified with primers pHERD30T_forward and pHERD30T_reverse (table S1). Clinical strains were transformed as previously described [18].

Redox sensitivity assay

Strains were grown o. n. in 50% LB medium containing 0% or 2% MIC of azithromycin. Cultures were then mixed with molten 0.5% LB agar and poured onto pre-cast LB-agar plates. After solidification, a filter paper disc saturated with 30% H_2O_2 was placed on top. Clearing zones around the H_2O_2 discs were measured after 24h incubation at 37°C [8].

Swim assay

Plates containing 50% LB and 0.3% agar, were prepared with or without 7% MIC of azithromycin, point inoculated and incubated at 30°C for 24h for swimming diameter quantification.

Sucrose density gradient analysis of ribosome complexes

Sucrose gradients were performed according to [19] and fractions analysed using a BioLogic LP system.

Proteomic analysis

Proteomic analysis was performed according to [20].

Protein alignments

uL4 sequences from *P. aeruginosa*, *E. coli*, *B. cepacia* and *H. influenzae*, *S. aureus* and *S. pneumoniae* were aligned using CLUSTAL O (1.2.4). uL4 and uL22 protein sequences were obtained from NCBI and aligned using the MUSCLE algorithm using the software SnapGene 5.3.2. The AMAS conservation score was calculated using the software Jalview.

Results

uL4 and uL22 mutations in clinical P. aeruginosa isolates

Analysis of the sequences of 529 clinical *P. aeruginosa* isolates at the CF clinic at Rigshospitalet, Copenhagen, Denmark [15,16], revealed 14 isolates from different clone types (>10,000 Single Nucleotide Polymorphisms (SNPs) difference) and 4 different patients containing 5 different mutations in the ribosomal protein uL4 (Fig. 1a, b). The ΔKPW and ΔRA deletions and the +G and +T insertions are all localized in the extended loop region of uL4 that normally protrudes into the NPET (Fig. 1a, b). The +VT duplication is, instead, localized outside of the extended loop region and far from the NPET of the ribosome (Fig. 1a, b). In all cases, the uL4 mutations appeared after the patients had commenced macrolide treatment (Fig. 1b).

Only a single mutation (a SNP) was identified in the *rplV* gene encoding the uL22 protein (Fig. 1d). None of the mutant strains contained 23S rRNA mutations or any additional mutations in other ribosomal proteins [10,15,16].

The alignment of the uL4 protein in a panel of four Gram-negative and two Gram-positive species shows a 100% conservation at the specific residues altered in isolates ΔKPW, ΔRA, +G and +T indicating a conserved function of the uL4 extended loop (Fig. 1c).

By analyzing 2909 uL4 protein and 2849 uL22 protein sequences originating from previously published *P. aeruginosa* strains spanning different countries and infection scenarios, mutations at highly conserved residues both identical and distinct from the ones found in our collection were identified (Fig. 1d).

Mutations in the uL4 loop region provide macrolide resistance

RPMI medium has been suggested for macrolide susceptibility testing using the broth microdilution methodology [11,21], however, the analyzed isolates did not grow in this medium (Fig S1a) possibly because of auxotrophies arisen in the isolates during in-patient evolution. Therefore, AST was performed using the broth microdilution methodology in the conventional cation adjusted Muller-Hinton broth (CA-MHB) as well as in 50% dilute LB broth (50% LB). To compare isolates with mutant and wild type copy of

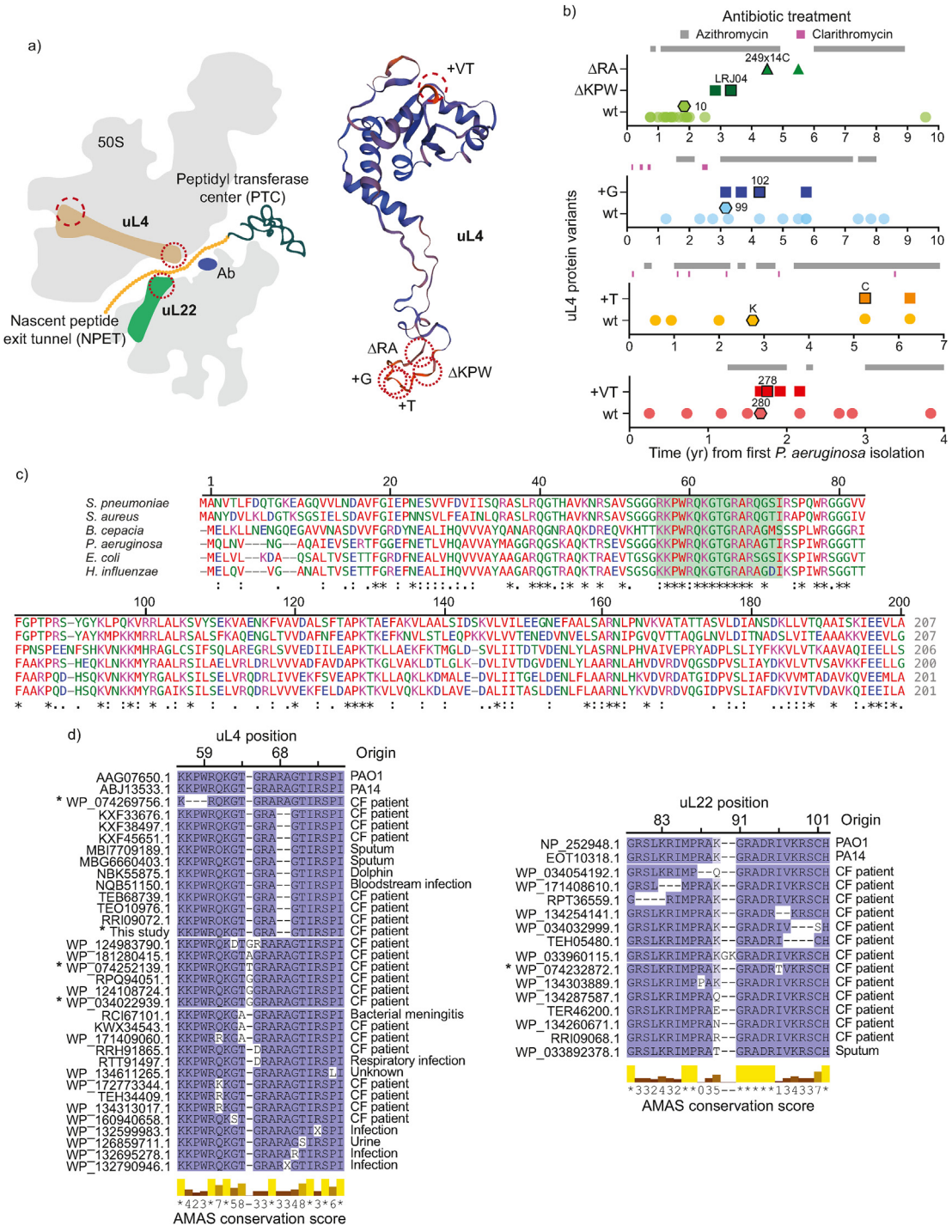

Fig. 1. uL4 and uL22 protein mutations in clinical strains of *Pseudomonas aeruginosa*. a) Localization and structure of the ribosomal uL4 protein in *P. aeruginosa* ribosomes (PAO1 6SPG). Circles indicate the position of mutations in the uL4 protein in isolates sampled from cystic fibrosis patients subsequent to antibiotic treatment. b) Emergence of uL4 mutants over time aligned with patient macrolide treatment. Whole genome sequences were analysed to determine if the isolate contained a wt uL4 (circles and hexagons) or a mutant uL4 (squares and triangles). Hexagons mark the strains containing a wt uL4 used as control for the respective uL4 mutants within the specific clone type. Outlined squares or triangles mark the uL4 mutant isolate used. Isolate LRJ04 (DK06 clone type) contains a 57-KPW-59 (ΔKPW) deletion, isolate 249x14C (DK06 clone type) contains a 68-RA-69 (ΔRA) deletion, isolate 102 (DK12 clone type) contains a glycine 65 (+G) insertion, isolate C (DK17 clone type) contains a threonine 64 (+T) insertion, and isolate 278 (DK36 clone type), contains a 189-VT-190 (+VT) duplication. Strains 10 (ΔKPW and ΔRA mutants), 99 (+G mutant), K (+T mutant) and 280 (+VT mutant), are ancestor isolates containing a wild type copy of the uL4 protein. c) Alignment of protein sequence of uL4 in 4 Gram-negative (*Pseudomonas aeruginosa, Escherichia coli, Burkholderia cepacia* and *Haemophilus influenzae*) and 2 Gram-positive species (*Staphylococcus aureus* and *Streptococcus pneumoniae*). The loop region (shaded green) is highly conserved across species. Numbering refers to *Pseudomonas aeruginosa*. d) Alignments of 2909 uL4 and 2849 uL22 protein sequences from *P. aeruginosa*. Only sequences with alterations compared to PAO1 in otherwise highly conserved regions of uL4 and uL22 are shown. The asterisk denotes strains in our collection of clinical strains of *P. aeruginosa*.

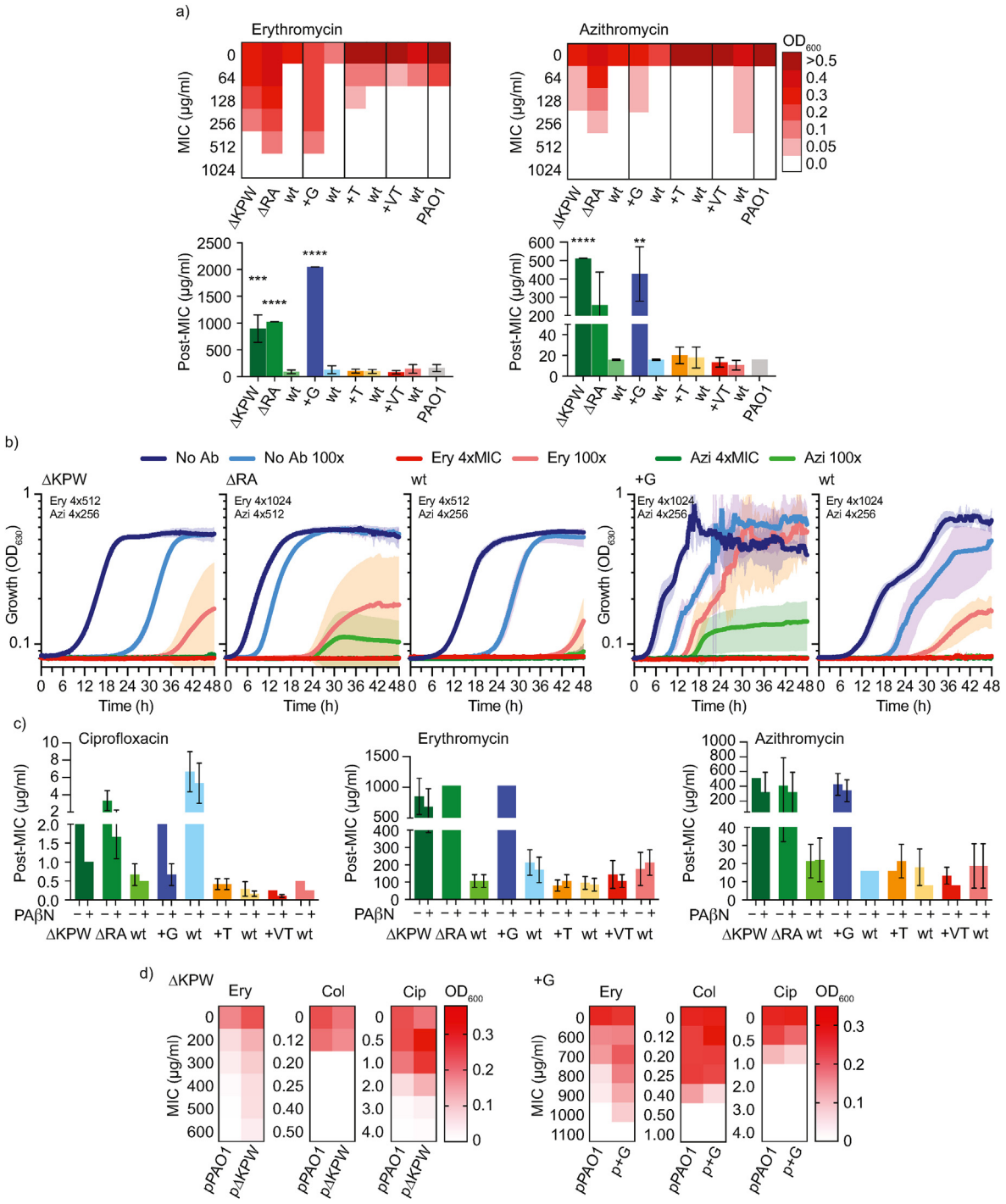

Fig. 2. Bacteriostatic effect of macrolide antibiotics on clinical strains of *Pseudomonas aeruginosa*. a) Erythromycin or Azithromycin Minimum Inhibition Concentration (MIC) measured by endpoint optical density (OD_{630}) after 24h incubation in a MIC assay in 50% LB supplemented with the indicated antibiotic concentrations and post-MIC effect determined as the minimum concentration needed to prevent re-growth when spotted onto LB-agar after 24h MIC incubation. The MIC value is the lowest concentration of antibiotic resulting in an endpoint OD of 0. The data represent the mean ± SD of 3-6 replicates. Differences relative to the wt strain antibiotic were computed by Student's t-test where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$. b) Bacteriostatic effect of erythromycin and azithromycin. Re-growth was monitored for 48h upon short treatment at 4xMIC followed by 100-fold dilution into non-antibiotic containing medium. Antibiotic concentrations used for each strain are given in each panel, (No Ab, no antibiotic; Ery, erythromycin; Azi, azithromycin; 100x, post-treatment upon 100-fold dilution into non-antibiotic containing medium). c) Post-MIC effect determined after 24h of incubation in a MIC setup in 50% LB with (+) or without (-) addition of the efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) at 2 µg/ml. d) Complementation of the uL4 mutations by over-expression of the wt copy of the uL4 protein from an inducible plasmid. Strain ΔKPW over-expressed either the wt uL4 from PAO1 (pPAO1) or its own copy (pΔKPW) while strain +G over-expressed either the wt uL4 from PAO1 (pPAO1) or its own copy (p+G). The graph represents the endpoint optical density (OD_{630}) after 24h incubation in a MIC assay in 50% LB supplemented with the indicated antibiotic concentrations.

the uL4 protein, we selected phylogenetically related ancestral strains previously isolated from the same patients to serve as controls with non-mutated uL4 (Fig. 1b).

Using 50% LB resulted in a better separation between mutants and ancestors, less variability between experiments and overall lower MICs compared with CA-MHB (Fig. 2 and S1b). All further analyses were, therefore, performed in 50% LB. Isolates ΔKPW, ΔRA and +G, compared with their respective ancestral wt isolates, showed a larger than 10-fold difference in MIC for erythromycin increasing from 64 μg/ml to 1024 μg/ml (Fig. 2a). Since aggregation and cell lysis could confound the MIC readings, we also determined the viability of bacteria after the MIC assay. For azithromycin, the MIC measurements were somewhat confounded by low level growth, which however, did not result in viable colonies when the post-MIC effect was evaluated after the end of the MIC assay (Fig. 2a). For that reason, the post-MIC effect was a much better predictor of azithromycin susceptibility. For ΔKPW, ΔRA and +G mutant strains, the difference in post-MIC effect relative to the ancestral strains was greater than 20-fold increasing from 16 μg/ml for the ancestral strains up to 512 μg/ml for the mutants (Fig. 2a). Both +T and +VT mutant strains, in contrast, did not have a detectible effect on susceptibility towards either macrolide relative to the respective ancestral wt isolates neither with respect to the MIC nor to the post-MIC effect (Fig. 2a). PAO1 laboratory strain mirrored in all cases the behavior of the ancestral wt clinical isolates (Fig. 2a). None of the mutant strains showed collateral resistance or sensitivity toward other classes of antibiotic indicating that the uL4 loop region mutations are specific for macrolide resistance (Fig. S3). Of note, macrolide resistance could not be accurately assessed on solid medium probably due to diffusion, growth rate or other factors which differ between growth on static solid plates or in aerated liquid culture.

Macrolides work through a bacteriostatic action, which was confirmed by the re-growth of most strains upon removal of antibiotic even after treatment at high antibiotic concentrations regardless of any uL4 mutations. As expected, strains ΔKPW, ΔRA and +G required shorter incubation times relative to the respective wt to show re-growth of the culture (Fig. 2b and S2).

None of the uL4 mutant isolates showed any significant reduction in the post-MIC value for erythromycin or azithromycin when assessed in combination with the efflux inhibitor Phenylalanine-Arginine β-Naphthylamide (PAβN), thus ruling out any increased efflux of macrolide antibiotics (Fig. 2c).

To further confirm that macrolide resistance was dependent on the uL4 mutations, plasmids encoding either the wt uL4 copy from PAO1 (pPAO1) or the mutant uL4 variant from ΔKPW (pΔKPW) or +G (p+G) isolates were expressed in strains ΔKPW or +G (Fig. 2d). As expected, a reduction in erythromycin susceptibility was measured only upon expression of the wt uL4 copy from PAO1 (Fig. 2d). The differences in resistance between the strains expressing wt or mutant variants of the uL4 protein are less pronounced than for the clinal isolates. This is most likely due to the competition of the wt and mutant proteins for the assembly of a functional ribosome and therefore not all ribosomes will contain a wt uL4. No changes in antibiotic susceptibility were instead shown for the non-ribosome targeting antibiotics colistin and ciprofloxacin (Fig. 2d).

Macrolide mediation of quorum sensing dependent phenotypes

Macrolides supposedly reduce *P. aeruginosa* virulence through modulation of the quorum sensing systems and motility [8]. All strains displayed an increase in redox sensitivity after azithromycin pre-treatment, however, isolates ΔKPW, ΔRA and +G required higher concentration of H_2O_2 to elicit a similar effect as in the wt strains (Fig. 3a). Likewise, strain +G (the only strain to retain swimming motility) required higher concentration of azithromycin to prompt a reduction in motility (Fig. 3b).

Molecular effects of the ribosome mutations

uL4 mutant strains were largely unaffected by addition of low concentrations of macrolide, while the generation time of the ancestral wt strains and the control PAO1 strain increased as the erythromycin concentration approached the MIC of the wt (Fig. 4a).

Whole-cell proteomics (Table S2) showed that, overall, each wt and uL4 mutant strain was characterized by a specific proteome which, in the case of strains ΔKPW, ΔRA and +G changed convergently as consequence of the uL4 mutations (Fig. S4). Ribosomal protein uS12 was the only ribosomal protein downregulated in all mutant isolates ΔKPW, ΔRA and +G (Fig. 4b). As expected, the enrichments based on the COG categories are strain specific (Fig. S4e), however, 16 proteins within the translation, ribosome structure and biogenesis category were all differentially expressed in the macrolide resistant mutant strains ΔKPW, ΔRA and +G (Fig. 4c). This suggests a common compensatory mechanism to counteract the uL4 mutations including ribosome remodeling and pseudouridylation factors, 16S rRNA processing, tRNA modifications, ribosome-binding factors and the hibernation promoting factors (Fig. 4c).

Of note, the proteomic data confirmed that the MexAB-OprM and MexXY-OprM systems are not significantly upregulated in

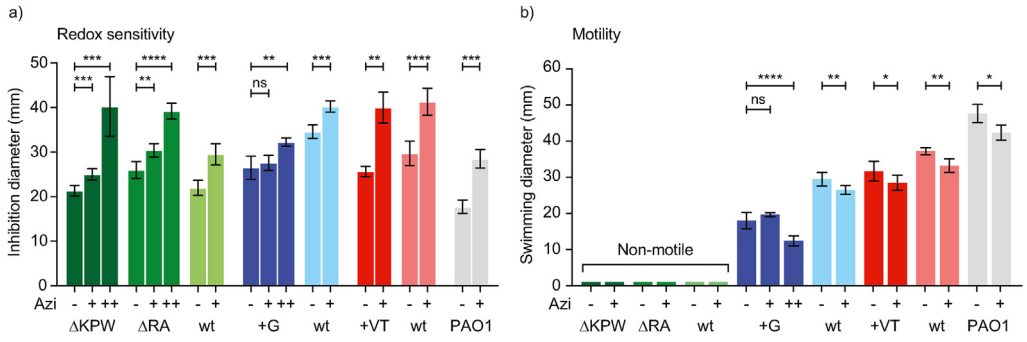

Fig. 3. Phenotypic traits modulated by macrolide antibiotics. a) Macrolide induced redox sensitivity measured as H_2O_2 induced clearing zones on soft overlay agar. Liquid cultures treated with 0% (-) or 2% MIC (of the ancestor +, or the mutant ++) of azithromycin for 24h were encased in a thin layer of soft LB agar, spread on solid LB agar with H_2O_2 saturated filter paper discs placed on top. Clearing diameter was measured after 24h. b) Swimming motility measured as the swim zone diameter on soft (0.3%) agar plates with or without (-) addition of azithromycin at a concentration of 7% of the MIC (of the ancestor +, or the mutant ++). The data represent the mean ± SD of 3-8 replicates. Differences relative to the strain without antibiotic were computed by Student's t-test where $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.001$.

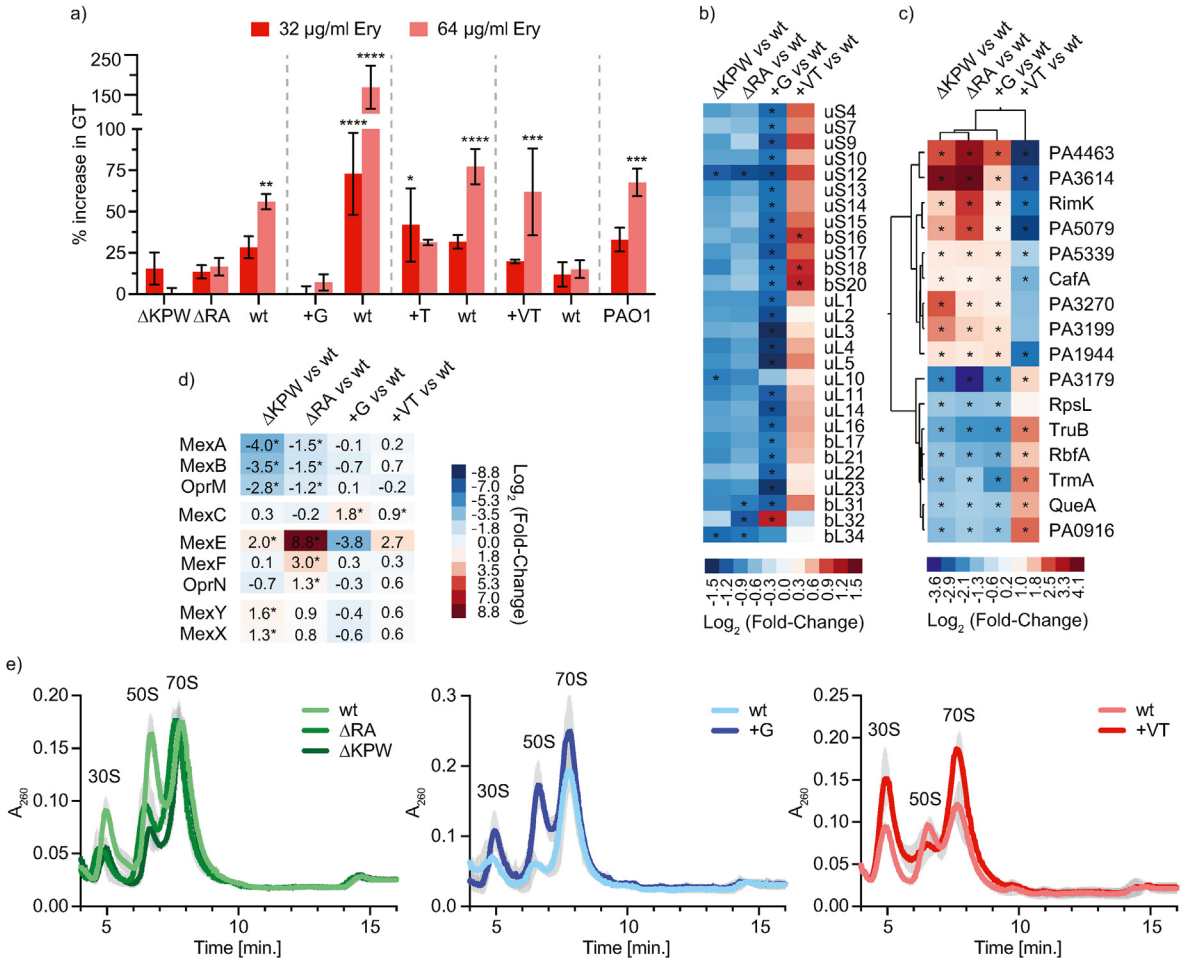

Fig. 4. Effect of the uL4 ribosomal protein mutations on the physiology of the cell. a) Increase in generation time in the presence of 32 µg/ml (dark red) or 64 µg/ml erythromycin (light red) expressed in percentage and relative to the absence of antibiotic. The data represent the mean ± SEM of 3-6 replicates. Differences in the generation time between strains and conditions were computed by Two-way ANOVA followed by Šídák's multiple comparisons test where *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$. b) Expression profile of the proteins belonging to the small and large subunit of the ribosome. Differentially expressed proteins (Log_2(Fold-Change) ≥ |0.6| and P value ≤ 0.05) in the mutant relative to the ancestor wild type strain are denoted by an asterisk. c) Hierarchical clustering analysis of the differentially expressed proteins in the mutant relative to the ancestor wild type strain within the translation, ribosomal structure and biogenesis COG category. Differentially expressed proteins (Log_2(Fold-Change) ≥ |0.6| and P value ≤ 0.05) are denoted by an asterisk. d) Expression profile of the proteins belonging to the multidrug efflux systems associated with macrolide efflux. Differentially expressed proteins (Log_2(Fold-Change) ≥ |0.6| and P value ≤ 0.05) in the mutant relative to the ancestor wild type strain are denoted by an asterisk. e) Polysome profiles of wt and mutant ribosomes. Sucrose gradients were used to compare ribosome assembly defects. The data represent the mean ± SD of 3 replicate experiments.

the uL4 mutant isolates consolidating the conclusion that macrolide resistance was not achieved through increased efflux (Fig. 4d and S5).

Ribosome assembly and subunit stoichiometry analyses showed consistent individual differences between mutant strains and their respective ancestral strains (Fig. 4e). The ΔKPW, ΔRA and +G mutations do not seem to cause any defects in ribosome biogenesis albeit the distribution of the 30S, 50S and 70S subunit distribution varies relative to each wt ribosome. This is in accordance with the previous literature [22–25].

Discussion

Macrolide antibiotics are heavily used to treat CF patients because of their anti-inflammatory properties. However, the effectiveness of these antibiotics in the patients is still unclear since conventional AST does not reflect the in-situ susceptibility. As also proposed elsewhere, this could explain why *P. aeruginosa* is deemed non-susceptible to macrolide antibiotics [11]. The optimization of the AST for macrolides has shed new light on the resistance profile of *P. aeruginosa*, making it possible to identify a new mechanism implicated in macrolide tolerance.

Surprisingly, several uL4 mutations were identified originating primarily from CF patient samples, but also from bacterial meningitis, bacteremia, urine and ventilator associated respiratory infections. This is well in line with the fact that the uL4 protein is under CF niche specific selection [26], which could possibly be a result of the extensive macrolide use in this environment [26]. It is not clear why so few uL22 mutants are observed in our strain collection but, it seems that overall they are found less often than the uL4 mutations and exclusively in isolates from CF patients.

As strains go through years of in-patient adaptation, the physiology of the cell is fine-tuned [1,27]. While some mutations provide an advantage under certain selection criteria, they may be lost or outcompeted once the selection pressure is lifted. Remarkably, uL4 mutations are prevalent across different clone types and patients, indicating that they can be easily accommodated by the bacteria, or that the maintenance of the selective

pressure (antibiotic treatment) specifically enriches for this phenotype. ΔRA-mutant strains, for example, were identified more frequently than any other uL4 mutant across the available *P. aeruginosa* collections. Similarly, the +G mutation was also prevalent among the analyzed collections and remained in this particular patient for at least three years suggesting that is it not readily outcompeted in the lung environment as long as azithromycin treatment persists.

The proteomic analysis combined with the characterization of ribosome assembly suggest that the ribosome undergoes adaptive changes in order to accommodate either of the uL4 mutations which are, however, well tolerated by the cell. It is worth noting that not all uL4 mutations cause macrolideresistance since the specific quaternary conformation of the ribosome might be key to both accommodate the antibiotic in its binding site and to allow the passing of the nascent peptide through the NPET. Further molecular characterization of the uL4 mutations can improve our understanding of the interaction between the ribosome and antibiotics and can aid in the rational design of new ribosome targeting antimicrobial compounds.

Importantly, the uL4 mutations not only reduce the bacteriostatic effects of the tested macrolide antibiotics, but also the virulence associated traits such as motility, and macrolide-induced redox sensitivity, which are attenuated in the uL4 mutants. While some studies have started questioning the benefit of continuous low dose macrolide treatment [28–30], others still recommend long-term azithromycin treatment [31]. The use of macrolide drugs extends beyond CF and include primary ciliary dyskinesia (PCD) and chronic obstructive pulmonary disease (COPD) as the Global initiative for chronic Obstructive Lung Disease (GOLD) recommendations now also suggest addition of macrolide compounds to reduce the risk of exacerbations [32,33]. However, due to the lack of efficient bacterial screening, the extensive use of macrolides is producing a reservoir of resistance completely overlooked and neglected. Therefore, when designing prolonged courses of macrolide treatment, AST and resistance development needs to be evaluated and carefully considered to avoid the insurgence of super resistant bacteria impossible to eradicate.

Transparency declaration

We declare no competing interests.

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Contribution

LG, RLR and SM designed the project. LG, KLA and RLR performed the experiments and verified the data. HKJ provided the strains and the clinical data. LG and RLR wrote the first draft of the paper, which after review by all authors, was revised according to the co-authors’ suggestions. All authors approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2022.08.003.

References

[1] Rossi E, La Rosa R, Bartell JA, Marvig RL, Haagensen JAJ, Sommer LM, et al. Pseudomonas aeruginosa adaptation and evolution in patients with cystic fibrosis. Nat Rev Microbiol 2021;19:331–42. https://doi.org/10.1038/s41579-020-00477-5.
[2] Flume PA, O'Sullivan BP, Robinson KA, Goss CH, Mogayzel PJ, Willey-Courand DB, et al. Cystic fibrosis pulmonary guidelines. Am J Resp Crit Care 2012;176:957–69. https://doi.org/10.1164/rccm.200705-664oc.
[3] Jr. PJM, Naureckas ET, Robinson KA, Mueller G, Hadjiliadis D, Hoag JB, et al. Cystic fibrosis pulmonary guidelines. Am J Resp Crit Care 2013;187:680–9. https://doi.org/10.1164/rccm.201207-1160oe.
[4] Nagai H, Shishido H, Yoneda R, Yamaguchi E, Tamura A, Kurashima A. Long-term low-dose administration of erythromycin to patients with diffuse panbronchiolitis. Respiration 1991;58:145–9. https://doi.org/10.1159/000195915.
[5] Jaffé A, Francis J, Rosenthal M, Bush A. Long-term azithromycin may improve lung function in children with cystic fibrosis. Lancet 1998;351:420. https://doi.org/10.1016/s0140-6736(05)78360-4.
[6] Hoiby N. Diffuse panbronchiolitis and cystic fibrosis: east meets West. Thorax 1994;49:531–2. https://doi.org/10.1136/thx.49.6.531.
[7] Hansen CR, Pressler T, Koch C, Høiby N. Long-term azitromycin treatment of cystic fibrosis patients with chronic Pseudomonas aeruginosa infection; an observational cohort study. J Cyst Fibros 2005;4:35–40. https://doi.org/10.1016/j.jcf.2004.09.001.
[8] Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J, Häussler S. Quorum-sensing antagonistic activities of Azithromycin in Pseudomonas aeruginosa PAO1: a global approach. Antimicrob Agents Ch 2006;50:1680–8. https://doi.org/10.1128/aac.50.5.1680-1688.2006.
[9] (ESCMID) EC for AST (EUCAST) of the ES of CM and ID. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infec 2003;9. https://doi.org/10.1046/j.1469-0691.2003.00790.x. ix–xv.
[10] Mustafa M-H, Khandekar S, Tunney MM, Elborn JS, Kahl BC, Denis O, et al. Acquired resistance to macrolides in Pseudomonas aeruginosa from cystic fibrosis patients. Eur Respir J 2017;49:1601847. https://doi.org/10.1183/13993003.01847-2016.
[11] Buyck JM, Plésiat P, Traore H, Vanderbist F, Tulkens PM, Bambeke FV. Increased susceptibility of Pseudomonas aeruginosa to macrolides and ketolides in eukaryotic cell culture media and biological fluids due to decreased expression of oprM and increased outer-membrane permeability. Clin Infect Dis 2012;55:534–42. https://doi.org/10.1093/cid/cis473.
[12] Wittmann HG, Stöffler G, Apirion D, Rosen L, Tanaka K, Tamaki M, et al. Biochemical and genetic studies on two different types of erythromycin resistant mutants of Escherichia coli with altered ribosomal proteins. Mol Gen Genet Mgg 1973;127:175–89. https://doi.org/10.1007/bf00333665.
[13] Gregory ST, Dahlberg AE. Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23 S ribosomal RNA. J Mol Biol 1999;289:827–34. https://doi.org/10.1006/jmbi.1999.2839.
[14] Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song Z, Johansen HK, et al. Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of Pseudomonas aeruginosa and attenuates chronic P. aeruginosa lung infection in cftr −/− mice. Antimicrob Agents Ch 2007;51:3677–87. https://doi.org/10.1128/aac.01011-06.
[15] Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome Analysis of a transmissible lineage of Pseudomonas aeruginosa reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. Plos Genet 2013;9:e1003741. https://doi.org/10.1371/journal.pgen.1003741.
[16] Marvig RL, Sommer LM, Molin S, Johansen HK. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. Nat Genet 2015;47:57–64. https://doi.org/10.1038/ng.3148.
[17] Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 2000;406:959–64. https://doi.org/10.1038/35023079.
[18] Choi K-H, Kumar A, Schweizer HP. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Meth 2006;64:391–7. https://doi.org/10.1016/j.mimet.2005.06.001.
[19] Halfon Y, Jimenez-Fernandez A, La Rosa R, Portero RE, Johansen HK, Matzov D, et al. Structure of Pseudomonas aeruginosa ribosomes from an aminoglycoside-resistant clinical isolate. Proc Natl Acad Sci 2019;116:22275–81. https://doi.org/10.1073/pnas.1909831116.
[20] Bongers M, Perez-Gil J, Hodson MP, Schrübbers L, Wulff T, Sommer MO, et al. Adaptation of hydroxymethylbutenyl diphosphate reductase enables volatile isoprenoid production. Elife 2020;9:e48685. https://doi.org/10.7554/elife.48685.

[21] Belanger CR, Hancock REW. Testing physiologically relevant conditions in minimal inhibitory concentration assays. Nat Protoc 2021;16:3761–74. https://doi.org/10.1038/s41596-021-00572-8.
[22] Chittum HS, Champney WS. Ribosomal protein gene sequence changes in erythromycin-resistant mutants of Escherichia coli. J Bacteriol 1994;176:6192–8. https://doi.org/10.1128/jb.176.20.6192-6198.1994.
[23] Zengel JM, Jerauld A, Walker A, Wahl MC, Lindahl L. The extended loops of ribosomal proteins L4 and L22 are not required for ribosome assembly or L4-mediated autogenous control. Rna 2003;9:1188–97. https://doi.org/10.1261/rna.5400703.
[24] Zaman S, Fitzpatrick M, Lindahl L, Zengel J. Novel mutations in ribosomal proteins L4 and L22 that confer erythromycin resistance in Escherichia coli. Mol Microbiol 2007;66:1039–50. https://doi.org/10.1111/j.1365-2958.2007.05975.x.
[25] Fulle S, Gohlke H. Statics of the ribosomal exit tunnel: implications for cotranslational peptide folding, elongation regulation, and Antibiotics binding. J Mol Biol 2009;387:502–17. https://doi.org/10.1016/j.jmb.2009.01.037.
[26] Dettman JR, Kassen R. Evolutionary genomics of niche-specific adaptation to the cystic fibrosis lung in Pseudomonas aeruginosa. Mol Biol Evol 2020;38. https://doi.org/10.1093/molbev/msaa226. msaa226-.
[27] La Rosa R, Rossi E, Feist AM, Johansen HK, Molin S. Compensatory evolution of Pseudomonas aeruginosa's slow growth phenotype suggests mechanisms of adaptation in cystic fibrosis. Nat Commun 2021;12:3186. https://doi.org/10.1038/s41467-021-23451-y.
[28] Samson C, Tamalet A, Thien HV, Taytard J, Perisson C, Nathan N, et al. Long-term effects of azithromycin in patients with cystic fibrosis. Resp Med 2016;117:1–6. https://doi.org/10.1016/j.rmed.2016.05.025.
[29] Bush A. Azithromycin is the answer in paediatric respiratory medicine, but what was the question? Paediatr Respir Rev 2020;34:67–74. https://doi.org/10.1016/j.prrv.2019.07.002.
[30] Nichols DP, Happoldt CL, Bratcher PE, Caceres SM, Chmiel JF, Malcolm KC, et al. Impact of azithromycin on the clinical and antimicrobial effectiveness of tobramycin in the treatment of cystic fibrosis. J Cyst Fibros 2017;16:358–66. https://doi.org/10.1016/j.jcf.2016.12.003.
[31] Nichols DP, Odem-Davis K, Cogen JD, Goss CH, Ren CL, Skalland M, et al. Pulmonary outcomes Associated with long-term Azithromycin therapy in cystic fibrosis. Am J Resp Crit Care 2019;201:430–7. https://doi.org/10.1164/rccm.201906-1206oc.
[32] Global Initiative for Chronic Obstructive Lung Disease (GOLD). Global strategy for the diagnosis, management and prevention of COPD, GOLD 2017. GOLD website. 2016. http://goldcopd.org/gold-2017-global-strategy-diagnosis-management-prevention-copd/.
[33] Kobbernagel HE, Buchvald FF, Haarman EG, Casaulta C, Collins SA, Hogg C, et al. Efficacy and safety of azithromycin maintenance therapy in primary ciliary dyskinesia (BESTCILIA): a multicentre, double-blind, randomised, placebo-controlled phase 3 trial. Lancet Respir Med 2020;8:493–505. https://doi.org/10.1016/s2213-2600(20)30058-8.