Infection and Drug Resistance

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SHORT REPORT

Identification of a Multidrug Resistant *Pseudomonas aeruginosa* Isolate Harboring Infrequent Red Fluorescence Plasmid from COPD Patient

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Abstract: *Pseudomonas aeruginosa* is a notorious Gram-negative opportunistic pathogen that normally causes acute and chronic infections in a wide range of hosts. In this study, a multi-resistant *P. aeruginosa* isolate L1a harboring an infrequent plasmid with red fluorescence was obtained from the bronchoalveolar lavage fluid of a patient with chronic obstructive pulmonary disease. The results of susceptibility testing and virulence-related phenotypic identification revealed that *P. aeruginosa* L1a was resistant to levofloxacin, cefepime, aztreonam, and imipenem and showed significantly stronger capacities for swimming and pyocyanin production than the reference strain PAO1. The genome of *P. aeruginosa* L1a was assembled into one circular chromosome (6,216,913 bp) and one circular plasmid (9111 bp). *P. aeruginosa* L1a was found to belong to the multilocus sequence type ST549, and serotype O5, and carried 8 drug resistance genes and 18 multidrug efflux pump-related genes in the chromosomal DNA. The plasmid pL1a harbored a tetracycline resistant gene *tetA* and a functional red fluorescence protein. This study reports a multidrug resistant *P. aeruginosa* clinical isolate harboring an infrequent red fluorescence plasmid for the first time.

Keywords: *Pseudomonas aeruginosa*, drug resistance, phenotypic identification, whole-genome sequencing, fluorescence

Chronic obstructive pulmonary disease (COPD) is one of the major public health problems that brings increasing morbidity and mortality worldwide and has become the third leading cause of death following cancers and cardiovascular diseases.[1–3] It is considered that smoking is the main causative factor of COPD, especially in elderly people over 60 years old. In addition to the common clinical manifestations such as dyspnea, cough and excessive phlegm, COPD is frequently accompanied by a series of complications, which greatly worsen the lung functions and impose massive healthcare and economic burdens on patients. Therefore, COPD airways are susceptible to being infected by bacterial pathogens because of their poor immune system.[4–7]

COPD can be classified into stable COPD and acute exacerbation of COPD (AECOPD) according to the progress of the disease and the clinical manifestation of the patients. Especially, AECOPD is mainly caused by bacterial infection and characterized by recurring attacks (1 to 3 times per year), high mortality (23–36% per year), poor prognosis and hard to be permanently cured, and is the critical incident in resulting the rapid decline of lung function and the deaths of patients. Clinical therapy of AECOPD is mainly dependent on the heavy, combined and continued use of antibiotics. Additionally, the treatment methods are differed in dependence of the presence of *Pseudomonas aeruginosa*, which is a notorious Gram-negative pathogen closely relevant to the recurrence of AECOPD and the readmissions of the patients.[1,7–10]

P. aeruginosa has a relatively larger genome (≈ 6.3 Mbp) than other common pathogenic bacteria and contains over 5500 genes, including dozens of two-component regulatory genes and membrane transport (efflux pump) systems.[11] The complicated and flexible transcriptional network and multidrug resistance mechanisms contribute *P. aeruginosa* a growth advantage to colonizing a variety of adverse environmental habitats and host tissues, causing acute and chronic infections in

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immunocompromised patients, and resisting the clearance of host immune system and antibiotics.[12,13] The carbapenem-resistant *P. aeruginosa* has been recognized as Priority 1 (critical) antibiotic-resistant bacteria in the latest global priority list released by the World Health Organization in 2017.[14] According to the latest report of China Antimicrobial Surveillance Network (CHINET) in 2021 (www.chinets.com), *P. aeruginosa* is the third (13.5%) most prevalent bacterial pathogen following *Klebsiella pneumoniae* (18.6%) and *Acinetobacter baumannii* (14.1%) among the 114,033 respiratory tract samples collected throughout the country. The resistance of *P. aeruginosa* isolates (n = 24,035) to imipenem reaches 23%, and to cefepime reaches 9.4%. Here, we report the complete genome of a multidrug resistant *P. aeruginosa* isolate L1a in China, in an effort to characterize its genomic and functional features, particularly the presence of an infrequent plasmid containing red fluorescence-encoding gene.

P. aeruginosa L1a was isolated from the bronchoalveolar lavage fluid of a female COPD patient (79-year-old) hospitalized in the Affiliated Hospital of Chengdu University (Sichuan, China). Species identification was performed according to the green pigment of the colony, followed by 16S rDNA sequencing and BLAST against the NCBI database. Susceptibility of *P. aeruginosa* L1a to the commonly used antibiotics (Sigma-Aldrich, USA) in the clinic was determined by the broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute.[15] The result showed that *P. aeruginosa* L1a was resistant to levofloxacin (16 μg/mL), cefepime (32 μg/mL), aztreonam (32 μg/mL), and imipenem (8 μg/mL) (Table 1). Compared to the reference strain *P. aeruginosa* PAO1, *P. aeruginosa* L1a had significantly stronger swimming motility (about 1.1 folds, $p < 0.01$) and pyocyanin production (about 2.7 folds, $p < 0.01$) abilities, but produced moderately lower amount of biofilm (Table 1). Moreover, *P. aeruginosa* L1a formed a proteolytic ring comparable to *P. aeruginosa* PAO1 on M9-skim milk plate and could grow on M9-adenosine plate (Table 1), indicating the intact quorum-sensing circuitry of L1a.[16] Moreover, the virulence of *P. aeruginosa* L1a was generally similar to that of PAO1 as determined by the fast-killing assay using *Caenorhabditis elegans* infection model, albeit L1a killed fewer nematodes than PAO1 between 26 and 45 hours post infection (Figure 1). These results collectively suggested that *P. aeruginosa* L1a was multidrug resistant and adapted to a planktonic lifestyle in causing acute infections. Correspondingly, *P. aeruginosa* L1a was isolated as soon as the patient was hospitalized, and the patient passed away in only one month due to severe pulmonary infection.

Genomic DNA of *P. aeruginosa* L1a was then extracted and subjected to whole-genome sequencing with the Nanopore PromethION platform and Illumina NovaSeq PE150 platform with over 100 × sequence depth. The sequence reads obtained from the two platforms were combined and assembled with Unicycler and SPAdes. Finally, the chromosomal DNA of *P. aeruginosa* L1a was assembled into one 0gap circular complete sequence with 6,216,913 bp in total length and 66.54% in GC-content. The prediction of genome components including coding sequences (CDSs), interspersed repetitive sequences, non-coding RNAs, Genomics Islands (GIs), Prophage, annotation of CDSs, and the prediction of resistance and virulence genes were performed as described elsewhere.[17] The results of genomic analyses revealed that *P. aeruginosa* L1a is a multilocus sequence type (MLST) ST549 strain according to the 7 loci scheme (identify of 100%). The genome contained 5672 predicted CDSs, 62 tRNA genes, 9 rRNAs, 7 genomic islands, 6 prophages, 198 tandem repeats, 148 minisatellite, and 8 microsatellites. Consistent with the results of susceptibility testing, there were two β-lactamase encoding genes *blaOXA-50* and *blaPAO*, one aminoglycoside resistance gene *aph(3')-IIb*, and one tetracycline resistance gene *tet(A)* in the genome of

Table 1 Minimum Inhibitory Concentrations (μg/mL) of Commonly Used Antibiotic Resistance and Virulence-Related Phenotypes of *P. aeruginosa* L1a

	CIP	LVX	GM	AM	TOB	PB	FEP	IPM	ATM	PIP
L1a	0.5	16	< 0.125	1	0.5	0.5	32	8	32	8
PAO1	0.5	0.5	0.25	0.5	0.125	2	4	1	4	4
	Swimming[a]		Twitching[a]		Pyocyanin[a]		Biofilm[a]		Skim-milk[a]	Adenosine
L1a	113.73 ± 3.40**		107.94 ± 11.00		270.56 ± 40.16**		79.20 ± 13.38		105.13 ± 4.2	+
PAO1	100 ± 3.31		100 ± 24.17		100 ± 8.14		100 ± 37.15		100 ± 4.44	+

Notes: [a]Percent values of L1a to those of PAO1. "+" Indicates the growth of colony on M9-adenosine plate. Data shown are the means ± standard deviation of three independent replicates. **Unpaired two-tailed *t*-test, $p < 0.01$.

Abbreviations: CIP, Ciprofloxacin; LVX, Levofloxacin; GM, Gentamicin; AM, Amikacin; TOB, Tobramycin; PB, Polymyxin B; FEP, Cefepime; IPM, Imipenem; ATM, Aztreonam; PIP, Piperacillin.

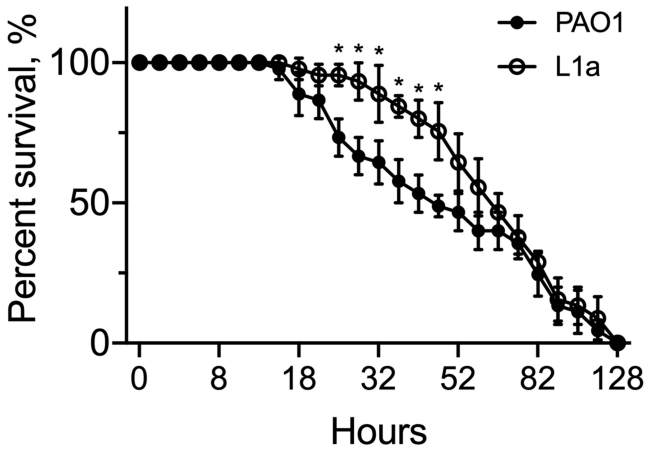

Figure 1 Pathogenicity of *P. aeruginosa* L1a against *C. elegans* models. Fifteen adult nematodes (L4 stage) were fed with *P. aeruginosa* L1a or the reference strain PAO1 in the fast-killing assays, and the survival of nematodes was monitored at different timepoints. Data shown are the means ± standard deviation of the numbers of survived nematodes from three independent experiments. Unpaired two-tailed *t*-test. *$p < 0.05$.

P. aeruginosa L1a. Additionally, there were also a substantial number of drug resistance determinants encoding resistance to chloramphenicol (*catB*), fosfomycin (*fosA*), penicillin (*pbp*), polymyxin (*arnA*), and vancomycin (*vanB*), and multidrug efflux pumps associated with the resistance to aminoglycoside, tigecycline, fluoroquinolone, β-lactam, and tetracycline (*mexA-mexB -oprM, emrE*), glycylcycline, fluoroquinolone, roxithromycin, and erythromycin (*mexC-mexD-oprJ*), chloramphenicol and fluoroquinolone (*mexE-mexF-oprN*), macrolide (*macB*), and others (*mexX-mexW-oprM, mexH-mexI-opmD*, and *acrA*).

Specifically, *P. aeruginosa* L1a harbored a circular plasmid (9111 bp in length and 58% in GC-content), designated pL1a, encoding nine proteins, such as TetR family transcriptional regulator, monomeric red fluorescent protein Bc-che1 (mCherry), two hypothetical proteins, replication protein RepA, chromosome-partitioning proteins ParA and ParB, resolvase, and RK2 tetracycline resistance protein TetA (Figure 2A). The result of plasmid classification using PlasmidFinder v1.3 and BLAST showed that pL1a had no significant similarity with any known plasmids or sequences. When the sequence of pL1a was subjected to the software SnapGene, the auto-generated common features of the plasmid

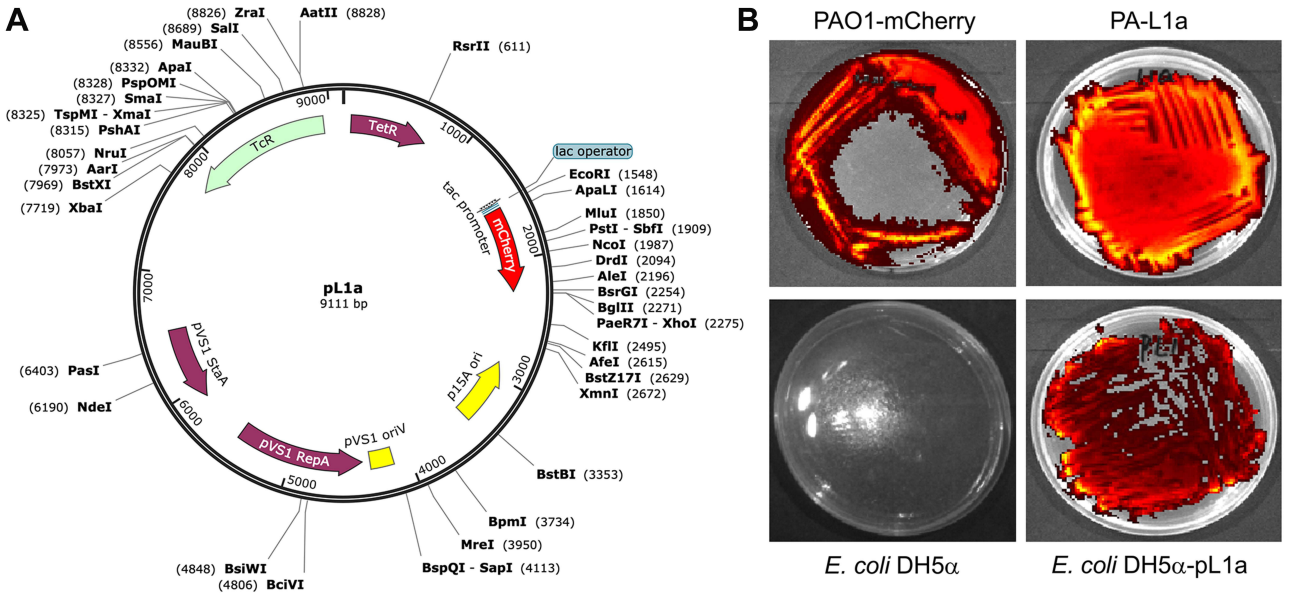

Figure 2 Genetic and functional features of the plasmid pL1a harbored by *P. aeruginosa* L1a. (**A**) Map and genetic feature of plasmid pL1a. (**B**) pL1a expresses the red fluorescence protein. Upper left, fluorescence of *P. aeruginosa* PAO1 harboring a mCherry encoding plasmid as positive control. Bottom left, fluorescence of *E. coli* DH5α as negative control. Upper right, fluorescence of *P. aeruginosa* L1a. Bottom right, fluorescence of *E. coli* DH5α harboring the plasmid pL1a extracted from *P. aeruginosa* L1a.

showed that pL1a might belong to the pVS1 family according to the backbone reported by Stanisich et al.[18] The result of fluorescence test revealed that *P. aeruginosa* L1a colonies showed red color, indicating the normal expression of pL1a (Figure 2B). When this plasmid was extracted from *P. aeruginosa* L1a and transformed into fluorescence-negative *Escherichia coli* DH5α on LB-tetracycline plate (50 μg/mL), the colonies of *E. coli* DH5α-pL1a successfully produced red fluorescence (Figure 2B). Intriguingly, although the genetic structure of pL1a showed some features of genetic modification for laboratory use, such as the *tac* promoter and ambiguous multiple cloning site (Figure 2A), there were no documented literatures reporting the application of this plasmid, to the best of our knowledge. Additionally, the patient carrying *P. aeruginosa* L1a routinely lived in a county of the Tibet Plateau without any laboratories around, and the isolate was identified as soon as she was hospitalized in the Affiliated Hospital of Chengdu University because of AECOPD. Therefore, the origin of the plasmid pL1a is still a mystery.

In conclusion, this is the first report of a multidrug resistant *P. aeruginosa* clinical isolate harboring an infrequent red fluorescence plasmid. Although it is hard to trace the source of *P. aeruginosa* L1a and to determine whether the plasmid pL1a is laboratory or wild origin, the present study highlights the susceptibility and complexity of COPD airways in terms of bacterial colonization, and provides a new small and convenient plasmid in labelling Gram-negative bacteria for further research.

GenBank Accession Number

The whole-genome sequence has been deposited in the NCBI database under accession no. PRJNA803822.

Ethical Approval

This study was approved by the Ethics Committee of the Affiliated Hospital of Chengdu University (PJ-2020-021-03). Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin. This study contains no identifiable features of patients, and complies with the declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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