In Silico Prediction and Comparison of Resistomes in Model *Pseudomonas* Strains by Resistance Gene Identifier (RGI)

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1. ABSTRACTS

Pseudomonas is a genus of bacteria including strains of human and plant pathogens, plant-growth promoting and biological control agents. While most *Pseudomonas* strains are known resistant to several antibiotics, their genetic elements conferring antimicrobial resistance (AMR) are largely unexplored systematically. The current study exploits a robust AMR gene predicting tool Resistance Gene Identifier of most recently updated version 5.2.0 based on newly curated database (the Comprehensive Antibiotic Research Database version 3.1.3) to detect AMR genes from thirteen genomes of *Pseudomonas* strains affiliated with seven species, including twelve pseudomonads as popularly studied model strains plus a well-known *Pseudomonas protegens* CHA0. A list of 281 AMR genes have been detected in five genomes of *Pseudomonas aeruginosa*, while 32 in the rest *Pseudomonas spp.* strains. Among the species, *P. aeruginosa*, *P. fluorescens*, *P. protegens* and *P. stutzeri* have the resistome of multi-drug resistance, while the rest is resistant to narrower spectrum of drugs. All *Pseudomonas spp.* investigated here have resistance genes to antibiotics classes of fluoroquinolone and tetracycline, which is consistent with an antibiotic resistance gene hit of adeF (ARO No. 3000777, resistant to fluoroquinolone, tet- racycline) has found in high redundancy in almost all *Pseudomonas* species except *P. aeruginosa* and *P. stutzeri*, implying the limit of these classes of drugs for treating pseudomonads. While inter-species data were focused here, further analysis will be conducted to reveal the features of inter-strain level features of pseudomonads. The *in silico* analysis will complement wet-lab research for designing treating strategies of these bacteria.

KEYWORDS: *Pseudomonas*; Antimicrobial Resistance; Genome Analysis; Drug Resistance Mechanism; Antibiotic Resistance Gene Ontology

ABBREVIATION: AMR: Antimicrobial Resistance; MDR: Multidrug-resistant; MIC: Minimum Inhibitory Concentration; WGS: Whole Genome Sequencing; RGI: Resistance Gene Identifier; CARD: Comprehensive Antibiotic Resistance Database; ARO: Antibiotic Resistance Gene Ontology; WHO: World Health Organization; NCBI: National Centre.

2. INTRODUCTION

The emergence and rapid evolution of ubiquitous bacterial antimicrobial resistance (AMR) is a noteworthy global threat to medical practice, public health, industrial and agricultural production, and environment. As widely accepted, the world has entered the post-antibiotic era accompanied by the increasing occurrence of multidrug-resistant (MDR) pathogens [1]. Generally, AMR bacteria, including the pathogenic species acquire AMR through two mechanisms: horizontal transfer of AMR genes and spontaneous genetic mutations [2]. Therefore, there is an urgent need to survey into AMR bacteria and their associated genes from various sources such as hospitals, soils, water etc.

Microbiological research approaches in tradition perform antimicrobial susceptibility testing to detect and identify AMR bacteria using phenotypic methods, such as the minimum inhibitory concentration (MIC). However, these methods standardly rely on cultured isolates and are less valid if pathogen is slow growing or unculturable. Molecular techniques thereafter are developed to complement traditional culture-based phenotypic AST. Specifically, the rapid development of whole genome sequencing (WGS) allows detection of AMR genes from bacteria genomes. As the number of identified AMR genes grows, numerous open-source tools for AMR gene prediction in high-throughput based on algorithms of gene sequence similarity have been created as novel bioinformatic tools and databases to improve our capability of screening and recognizing the prevalence of AMR from various niches. The most widely used tools for predicting AMR genes include ABRicate (<https://github.com/tseemann/abricate/>), AMRfinder [3], RGI [4], AMR++, GROOT [5], BabyGROOT [5], DeepARG [6], ARG-ANNOT [7], MEGARes [8] Resfinder [9] and their referred databases are NCBI, CARD, DeepARG, ARG-ANNOT, MEGARES, EcoH, PlasmidFinder, Ecoli_VF and VFDB. Among these available resources, the Resistance Gene Identifier (RGI) based on Comprehensive Antibiotic Resistance Database (CARD) is particularly of wide use for its high quality, frequent update, manually curated resistance detection

models derived from experimentally verified phenotype–genotype associations reported in the scientific literature. Based on homology models, CARD is adapted to homologs, sequence variants, and mutations to improve precision and accuracy. Besides, CARD has constructed Antibiotic Resistance Ontology (ARO) to include terms for harmonizing assays on AMR phenotypes [4]. RGI and CARD of previous versions have been used to identify AMR genes in human infectious pathogen *Staphylococcus aureus*, and a range of pathogenic *Pseudomonas* groups, validating their efficacy for disclosing bacterial resistomes from genomic sequences.

Pseudomonas is a large genus of Gram-negative aerobic Gamma- proteobacteria, consisting of diverse species which include plant pathogens, human pathogenic strains, biological control bacteria [10]. Attributing to its metabolic versatility and genomic plasticity, its members inhabit in various niches including soil, water, different hosts and most have intrinsic AMR. As they are spreading across a large conserved core region and highly diverse accessory regions including almost all terrestrial and aquatic environments, *Pseudomonas* spp. of numerous genomes and genetic elements for shaping their life style are constantly catching research interests [10]. The most research attention focuses on *P. aeruginosa* which is a species of opportunistic human infectious pathogen and ranked as top category ‘critical’ pathogenic bacteria by World Health Organization (WHO) global priority pathogens list for relating to high morbidity and mortality rates [1]. It is notorious for causing a large number of community-acquired infections such as folliculitis, puncture wounds leading to osteomyelitis, pneumonia, otitis externa, and nosocomial infections like ventilator-associated pneumonia, catheter-associated urinary tract infections [11]. Nowadays, it remains a medical treatment challenge due to its extreme versatility, various dynamics defence mechanisms, and importantly AMR capability. It has been discovered for a range of AMR mechanisms, including intrinsic resistance to antibiotics by restricting membrane permeability for drugs, drug efflux systems, and synthesizing antibiotic-inactivating enzymes [11]. Apart from most multi- drug resistant *P. aeruginosa*, other *Pseudomonas* species such as plant-associated *P. syringae* or *P. protegens* from soil sources also were also studied of their AMR for the merit of farmland protection and environment management. Soil is one of the habitats for most diverse microbes on earth inhabiting both antibiotic-producing and AMR bacteria [12]. Further, soil niches have been recognized as the origin of antibiotics production and a rich repository of bacteria undergoing evolution and dissemination of AMR.

Our study aims for profiling and comparing the magnitude of resistomes for AMR mechanisms and genetic diversities by RGI predicting in several most intensively studied *Pseudomonas* spp. of various features from several niches. The *Pseudomonas* spp. investigated here are clinical pathogens *P. aeruginosa* strains PAO1, LESB58, PA7, PAK, and UCBPP PA14), phyto-pathogens *P. syringae* pv.tomato DC3000 and *P. savastanoi* pv.phaseolicola 1448A, plant- protecting agents *P. fluorescens* SBW 25 and *P. protegens* Pf-5 and CHA0, plant-growth promoting bacteria *P. stutzeri* A1501, and industrial technological bacteria *P. putida* F1 and KT2440. The study will use the most recent version of RGI and CARD to improve the updated results. Analysis of the AMR profiles and mechanisms in these *Pseudomonas* strains of clinical and soil origins will help determine the epidemiology and improve infection control strategies for medical practice, and benefit the plant and soil health for natural eco-system as well as agricultural productivity. Inter-species comparison of resistomes will develop our understanding of resistant flexibility and metabolic versatility in *Pseudomonas*.

3. METHODOLOGY

3.1. Retrieval of complete genomes from NCBI

For predicting antimicrobial resistance genes, thirteen complete genomes of selected *Pseudomonas* strains were retrieved from NCBI database for their complete amino acid sequences. The thirteen *Pseudomonas* strains include the twelve popularly investigated pseudomonads as described by *Pseudomonas* Genome Database (<https://www.Pseudomonas.com/strain/browser>) plus a well-known biological control strain *Pseudomonas protegens* CHA0. The retrieved sequences were in FASTA format, which was download from the National Center for Biotechnology Information (NCBI) website (<https://www.ncbi.nlm.nih.gov>) as well as their accession numbers. The genomic analysis was performed at amino acid level to minimize false negative detection of AMR genes as RGI computes based on protein homology models for this study.

3.2. Phylogenetic analysis of *Pseudomonas* spp

To find the divergence and evolution of the *Pseudomonas* strains investigated in this study, phylogenetic analysis was conducted on the phylogenetic marker 16S rRNA gene sequences from the representative genomes for each species by PhyloT Version 2 to construct an evolutionary tree. The analysis included an *E. coli* sequence as an outlier. The genomic features were also summarized from NCBI website (Table 1).

The evolutionary tree was decorated by iTOL to visualize the strains of human pathogen, phyto-pathogenic bacteria, industrial use, and plant-growth promoting agents.

3.3. Predicting antibiotic resistance gene in amino acid sequence using RGI

Amino acid sequences of thirteen *Pseudomonas* strains were imported into the RGI analysis portal in bulk using custom software developed. Default set based on strict or perfect criteria only was used to detect AMR genes, AMR gene family, drug class and resistance mechanism data. The resistance genes, mechanism and drugs obtained from RGI platform were further analyzed.

4. RESULTS

The thirteen *Pseudomonas* strains selected for this investigation encompass species for industrial use agent, plant pathogens, clinical isolates, plant-growth promoting bacteria (Figure 1). Their genomic features and ecological habitats have been summarized (Table 1). The *P. protegens* strains have larger genome sizes (6.87 and 7.07 Mbs respectively) than the rest pseudomonads (6.21 Mbs).

Figure 1 The phylogenetic tree (Maximum Likelihood) of *Pseudomonas* species investigated in this study constructed by PhyloT Version 2 and decorated by iTOL. Branch colours indicate strain features: green, industrial use strain; red, plant-growth promoting strain biological control brown, phytopathogenic strain; black, *E. coli* used as a reference outlier.

A total of 313 AMR genes was detected by RGI, including 281 in five genomes of *P. aeruginosa*, and 32 for the rest six *Pseudomonas* species (Table 2). In each *P. aeruginosa* genome, the amount of AMR genes ranges from 49 to 59, while for other *Pseudomonas* strains, no genome has more than 10 AMR genes. The AMR genes from each genome of *P. aeruginosa* are affiliated with more than 10 families respectively, while these from the rest belong to no more than 3 families. According to the definition of multi-drug resistance [13] which is resistance to at least one antibiotic in three different antimicrobial categories, the strains of *P. aeruginosa*, *P. fluorescens*, *P. protegens* and *P. stutzeri* have sets of AMR genes for multi- drug resistance. As *P. aeruginosa* genomes have more diversified families of AMR genes (Table 2), they also have more resistance mechanisms conferred by AMR genes (Table 2, Figure 2).

Although antibiotics efflux is the main mechanism in *P. aeruginosa*, it also has other mechanisms as antibiotics alteration, antibiotics inactivation, antibiotics target replacement, while antibiotics efflux is the sole mechanism for almost all other *Pseudomonas* spp. except *P. fluorescens* (Figure 2), demonstrating more resilience in response to antibiotics treatment of *P. aeruginosa* than others.

The concept of Antibiotic Resistance Ontology (ARO) has been introduced to describe AMR genes and their sequence polymorphisms, encoding products, mechanisms, phenotypes, and molecular targets [4].

While *P. aeruginosa* has less extent of ARO redundancy in their genomes, the rest of the *Pseudomonas* genomes

have frequent repetitive adeF (ARO No. 3000777, resistant to fluoroquinolone, tetracycline) in most of them (Table 3). Resistance genes to fluoroquinolone and tetracycline have been found in every genome investigated in this study.

5. DISCUSSION

Our current study applied the most recent version of a frequently applied tool RGI based on a comprehensive database CARD using protein homology model to predict AMR genes from several intensively studied *Pseudomonas* genomes affiliated with 7 species originally isolated from various sources.

The clinical pathogenic species *P. aeruginosa* has a much larger number of AMR genes, more diverse AMR gene families and more classes of resisted drugs than other pseudomonads from environmental sources, suggesting the resistance to stronger selection by drug treatment under medical practice environments than in natural habitats. For *P. aeruginosa*, *P. fluorescens*, *P. protegens*, *P. stutzeri*, genes for multi-drug resistance have been detected in their genomes. Every strain demonstrates gene sets resistant to fluoroquinolone and tetracycline, indicating their potential mutual resistance of these drug classes.

The *P. aeruginosa* has been studied for their resistome in seven strains by a combination of wet-lab methods and previous version of RGI and CARD [27]. In consistency with previous data, a few core sets of genes such as Mex family (A-N, P-S, V-Z) have been detected in all the *P. aeruginosa* strains (data not shown), while the current study released more AMR genes due to the update of RGI and CARD used. Further analysis will be conducted into the resistomic comparison of *P. aeruginosa* at strain level.

Table 1: Summary of the *Pseudomonas* strains' genomic features

Species	Strain	Accession NO.	Number of Genes	Genome size (Mb)	%GC	Isolate source
<i>Pseudomonas aeruginosa</i>	PAO1 [14]	NC_002516.2	5697	6.26	66.6	Fibrosis cystic patients
	LESB58 [15]	NC_011770.1	6171	6.60	66.4	
	PA7 [16]	NC_009656.1	6163	6.59	66.5	
	PAK [17]	NZ_LR657304.1	5887	6.40	66.4	
	UCBPP					
	PA14 [18]	NC_008463.1	6025	6.54	66.3	
<i>Pseudomonas fluorescens</i>	SBW25 [19]	NC_012660.1	6123	6.72	60.5	Soil
<i>Pseudomonas protegens</i>	Pf-5 [20]	NC_004129.6	6392	7.07	63.3	Soil
	CHA0 [21]	NC_021237.1	6252	6.87	63.4	
<i>Pseudomonas putida</i>	F1 [22]	NC_009512.1	5390	5.96	62	Environment
	KT2440 [23]	NC_002947.4	5666	6.18	61.6	
<i>Pseudomonas savastanoi</i>	pv.phaseolicola 1448A [24]	NC_005773.3	5540	5.93	57.8	Plant soil

<i>Pseudomonas stutzeri</i> A1501 [25]	NC_009434.1	4247	4.57	63.8	Plantendosphere
<i>Pseudomonas syringae</i> pv.tomato DC3000 [26]	NC_004578.1	5891	6.54	58.3	Tomato

Table 2: Summary of the antimicrobial resistance genes identified in each strain for diversity of genes and mechanisms.*multi-drug resistance: defined by resistance to each drug from at least three classes of drugs.

Bacterial Strains	No. of AMR genes detected	Multi-drug Resistance by AMR genes	Types of Resistance mechanisms	No. of AMR gene family
<i>P. aeruginosa</i> PAO1	58	Yes	4	>10
<i>P. aeruginosa</i> LESB58	58	Yes	4	>10
<i>P. aeruginosa</i> PA7	49	Yes	4	>10
<i>P. aeruginosa</i> PAK	59	Yes	4	>10
<i>P. aeruginosa</i> UCBPP PA14	57	Yes	4	>10
<i>P. protegens</i> CHA0	4	Yes	1	1
<i>P. protegens</i> Pf-5	8	Yes	1	1
<i>P. fluorescens</i> SBW25	5	Yes	2	3
<i>P. putida</i> F1	3	No	1	1
<i>P. putida</i> KT2440	3	No	1	1
<i>P. savastanoi</i> pv. <i>phaseolicola</i> 1448A	4	No	1	2
<i>P. stutzeri</i> A1501	2	Yes	1	1
<i>P. syringae</i> pv. <i>tomato</i> DC3000	3	No	1	2

The redundancy of Antibiotic Resistance Gene Ontology (ARO) hits has been observed for *Pseudomonas* spp. except *P. aeruginosa* and *P. stutzeri*, particularly for adeF (ARO No. 3000777, resistant to fluoroquinolone, tetracycline). The gene AdeF is an Ade family multidrug resistance-nodulation-cell division (RND) transporter periplasmic adaptor subunit reported in *Acinetobacter baumannii* [28]. The role of redundant AROs of adeF detected in the *Pseudo- monas* genomes need further investigation.

Figure 2 The *P. aeruginosa* demonstrates for more diverse classes of resistance mechanisms demonstrated by resistance genes than other *Pseudomonas* species.

Table 3 The *Pseudomonas* spp. other than *P. aeruginosa* and *P. stutzeri* have redundancy of Antibiotic Resistance Gene Ontology (ARO) hits. *Background colour in grey indicates gene redundancy in each genomes.

	<i>P. protegens</i> CHA0	<i>P. protegens</i> Pf-5	<i>P. fluorescens</i> SBW25	<i>P. putida</i> F1	<i>P. putida</i> KT2440	<i>P. savastanoi</i> pv. phaseolicola 1448A	<i>P. syringae</i> pv. tomato DC3000
ARO Hits	adeF	adeF	<i>A. baumannii</i> AbaQ	adeF	adeF	<i>A. baumannii</i> AbaQ	adeF
	adeF	adeF	adeF	adeF	adeF	adeF	<i>A. baumannii</i> AbaQ
	adeF	adeF	adeF	adeF	adeF	adeF	adeF
	YajC	adeF	adeF			adeF	
		adeF	<i>P. aeruginosa</i> soxR				
		adeF					
		YajC					
		YajC					

6. CONCLUSION

The current study has used a bioinformatics platform RGI with CARD database to detect the AMR genes from thirteen frequently studied *Pseudomonas* strains. The strains from *P. aeruginosa* of clinical source have a large volume of AMR genes resistant to a broad spectrum of antibiotics via various mechanisms, while other species from natural habitats have much less AMR genes resisting a narrow range of drugs. The strains of *P. aeruginosa* will be compared for their resistome at higher resolution.

7. CONFLICT OF INTEREST

The authors declare no conflict of interests.

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