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Analysis of antibiotic resistance genes in *Pseudomonas* strains associated with plants: A computational investigation

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Abstract

The species of the genus *Pseudomonas* are an important part of the microbiota associated with plants. These species can be beneficial to the host plant by promoting its growth, and by protecting it against diseases, but they can also have a phytopathogenic effect. The genus *Pseudomonas* and especially the species *P. aeruginosa* is classified among the pathogenic species, that are multi-resistant to antibiotics due to the possession of a large number of antibiotic resistance genes (ARGs). Therefore, the risk of contamination of crops by these genes is real and likely to present a danger in terms of human health. In this study, the genomic sequences of 21 strains of *Pseudomonas* associated with plants were in silico analyzed to assess the number and diversity of ARGs. A number of 63 ARGs belonging to seven different species were detected among the studied gnomes. The phylogenetic and the physicochemical properties of the proteins encoded by these genes were analyzed. The interaction network of the studied genes has been established; it shows great connectivity between the genes involved in the different systems of antibiotic efflux in *Pseudomonas*.

Keywords: antibiotic resistance genes; in silico; plant-associated bacteria; *Pseudomonas*

Introduction

The use of antibiotics has increased significantly around the world. An increasing number of antibiotics administered to humans and animals has been widely released in the environment. As a consequence of this practice, new mechanisms of antibiotic resistance have been selected and accumulated in different bacterial species, which represents a major concern for public health (Berendonk *et al.*, 2015). Humans and animals disseminate bacteria of their commensal flora in the environment, among them, those carrying antibiotic resistance genes (ARGs) intrinsic or acquired by horizontal transfer. These ARGs can easily reach the agricultural system, either directly or indirectly (Forsberg *et al.*, 2012). Wastewater treatment plants, where wastewater linked to human activities is discharged, concentrate a large number of bacteria resistant to antibiotics and their resistance genes (Le-Minh *et al.*, 2010). In addition, sewage sludge can be used as fertilizer in crop fields (Kominko *et al.*, 2018), while effluents are discharged into the aquatic environment or used to irrigate crops (Pan *et al.*, 2014). On other hand, animal feces loaded with antimicrobial resistance genes are used as natural fertilizers to fertilize soils (Wichmann *et al.*, 2014; Zhang *et al.*, 2016). In all these cases, contamination of the agronomic system by new ARGs occurs.

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Furthermore, there are many horizontal genetic exchanges in bacteria, which can lead to transmission of ARGs to endogenous soil bacteria, leading to high dissemination of ARGs in the agricultural environment. Under these conditions of contamination, agricultural soils are filled with all kinds of resistant bacteria and their ARGs. Plants growing in these soils can themselves be contaminated by resistant bacteria that colonize their root or leaf tissues as endophytic or pathogenic bacteria (Hardoim *et al*., 2015; Brader *et al*., 2017).

Species of the genus *Pseudomonas* are ubiquitous bacteria with a preference for humid environments, they can be found in water, soil, plants, and biological tissues (Fernandez *et al*., 2015). In addition to including species pathogenic to humans such as *P. aeruginosa* and *P. stutzeri* (Noble and Overman, 1994), this genus includes species associated with plants that can be either pathogenic such as *P. syringae* (Tarkowski and Vereecke, 2014), or beneficial for plant growth such as *P. fluorescens* (Lally *et al*., 2017). The majority of research focuses on both the favorable and harmful effects of endophytic *Pseudomonas* species on plant growth and health, but few studies aim to assess the prevalence of resistance genes in *Pseudomonas* species associated with plants. In order to assess the risk of contamination of plants by resistant *Pseudomonas* species, the presence of ARG in the genomes of various *Pseudomonas* species associated with different plants was studied using bioinformatic tools allowing the identification and characterization of these ARGs.

Materials and Methods

Identification of antibiotic resistance genes (ARG)

The FASTA formats of the genomic sequences of 21 strains of *Pseudomonas* were retrieved from the NCBI Genome database. All the strains studied are isolates associated with different plant species. The GenBank identifier, the code given to each strain, as well as their host plant species are summarized in table 1. The online program Resistance Gene Identifier (RGI) (*https://card.mcmaster.ca/analyze/rgi*) (Alcock *et al*., 2020) was used for the identification of resistance antibiotics genes (ARG) for each genomic sequence.

Table 1. Details on the genomes of the studied *Pseudomonas* strains

Strains	Level	Identifier	Host plant	Code
Pseudomonas putida strain PC2	Complete	CP011789.1	*Seeds of Pistacia chinensis*	Pp1
P. putida strain W5	Complete	CP026115.2	*Medicago sativa*	Pp2
P. putida strain1290	Complete	CP039371.1	Phyllosphere of *Pyrus communis*	Pp3
P. putida strain E46	Complete	CP024086.1	Roots of *Sida hermaphrodita*	Pp4
P. putida strain JBC17	Complete	CP029693.1	Strawberry	Pp5
P. putida strain AA7	Complete	CP018846.1	*Zea mays*	Pp6
P. aeruginosa strain YD001	Complete	CP053922.1	*Morus alba*	Pa1
P. aeruginosa strain NCTC9433	Complete	LS483497.1	Tobacco plant	Pa2
P. aeruginosa strain ACR22	Complete	CP058331.1	Sugarcane	Pa3
P. fluorescens strain NEP1	Draft	CP022313.1	*Leonotis nepetifolia*	Pf1
P. fluorescens strain L321	Complete	CP015637.1	Leaf tissue of *Miscanthus x giganteus*	Pf2
P. fluorescens strain A506	Complete	CP003041.1	Pear tree leaf	Pf3
P. fluorescens strain CREA-C16	Draft	CP017951.1	Root of *Pisum sativum*	Pf4
P. syringae pv. *actinidiae* ICMP 18708	Complete	CP012179.1	*Actinidia*	Ps1
P. syringae pv. *actinidiae* P155	Complete	CP032871.1	Canker of kiwifruit	Ps2
P. syringae pv. *syringae* HS191	Complete	CP006256.1	*Panicum miliaceum*	Ps3
P. syringae pv. *pisi str.* PP1	Complete	CP034078.1	*Pisum sativum*	Ps4
*P.*syringae pv. *syringae* B728a	Complete	CP000075.1	snap bean leaflet	Ps5
P. viridiflava CFBP 1590	Complete	LT855380.1	*Prunus cerasus*	Pv
P. brassicacearum L13-6-12	Complete	CP014693.1	*Solanum tuberosum* cv. 'Desiree'	Pb
P. citronellolis strain P3B5	Complete	CP014158.1	Basil phyllospher	Pc

Phylogenic analysis

A phylogenetic analysis was carried out for the identified ARGs. The FASTA sequences of the genes were retrieved and aligned by the Clustal W multiple alignment tool. A phylogenetic tree depicting the relationships between all resistance genes was constructed using the maximum-likelihood method and Kimura 2-parameter model on MEGA-X version 10.2.2 software. Bootstrap consensus trees were inferred from 1000 replicates.

Physicochemical analysis

A study of different physicochemical properties was carried out for the identified resistance genes. LipoP 1.0 Server (*www.cbs.dtu.dk/services/LipoP/*) (Juncker *et al*., 2003) and SignalP (*http://www.cbs.dtu.dk/services/SignalP/*) (Armenteros *et al*., 2019) programs were used for lipoproteins and signal peptides prediction. TMHMM Server v.2.0 (*http://www.cbs.dtu.dk/services/TMHMM/*) was used for the prediction of transmembrane helices in proteins. The location of the different gene products has been determined from the website *Pseudomonas* Genome DB (*https://www.pseudomonas.com/*).

ARG-ARG interactions network

The online program StringDB version 10.5 (*https://string-db.org*) (Szklarczyk *et al*., 2019) was used to predict the interactions between the ARGs identified in this work. For this reason, the FASTA sequences of the proteins were introduced into the platform and the genus *Pseudomonas* was chosen as an organism. Proteins showing no interaction were neglected.

Results and Discussion

The soil is a reservoir of a wide variety of antibiotic resistance genes which could be mobilized to humans and cause real health risks. Plants growing in the soil harbor several types of resistant bacteria and may be the cause of the spread of antibiotic resistance genes, due to their consumption by humans. Bacteria of the genus *Pseudomonas*, are present in many natural ecological niches, such as water and soil, as they can colonize artificial niches in hospital settings (Moore *et al*., 2006) as catheters, breathing tubes, and ventilation systems. *Pseudomonas* spp. can be the cause of minor external infections, or can lead to serious illnesses, involving the life of patients. In addition to being a pathogenic agent for humans, certain species of *Pseudomonas* are considered to be highly pathogenic for plants, causing significant crop losses (Jagdalea *et al*., 2019). Other than their pathogenic capacity, *Pseudomonas* species form an important part of the microbiota of several plants (Lally *et al*., 2017). These bacteria are known for their potential to stimulate plant growth and protect crops against different phytopathogenic agents (Sivasakthi *et al*., 2014). Antibacterial resistance is the major problem in the treatment of *Pseudomonas* infections. *Pseudomonas* may have the highest incidence of resistance among all bacteria (Lister *et al*., 2009).

Identification of the ARGs in the studied Pseudomonas species

The examination of the genomic sequences of 21 *Pseudomonas* strains associated with plants by the RGI platform was allowed to identify a total number of 63 ARGs distributed over the genomes of the different studied strains. Figures 1 and 2 represent a heatmap and a histogram, respectively, summarizing the distribution of these genes in the 21 strains of *Pseudomonas.* Among the resistance genes, we can distinguish two types of genes, those present in a single species and those widely distributed among the studied species. The genes *APH(3')-IIb*, *arnA*, *basRS*, *bcr-1*, *catB7*, *cprSR*, *cpxR*, *emrE*, *mexA-Z*, *muxABC*, *nalCD*, *opmBDEH*, *oprJMN*, *oxa50*, *oxa-486*, *parRS*, *PDC-7*, *PDC-8*, *pmpM*, *rsmA*, *triABC*, and *nfxB* are only present in the three strains of *P. aeruginosa*. On the other hand, *abaQ* gene is present in all the studied species, except *P. aeruginosa,* where it is absent from the genomes of the three strains of this species. *adeF* gene appears to be the

most common gene, where it is detected in all strains except *P. aeruginosa* strain YD001 and *P. aeruginosa* strain ACR22.

In addition to being the most widespread, *adeF* gene is present more than one copy in certain strains as is represented in Figure 1. In the strains *P. putida* W5, *P. putida* JBC17, *P. fluorescens* A506 and *P. citronellolis* P3B5, *adeF* gene is present 4 times in their genomes, while three copies of this gene are identified in *P. putida* strain1290, *P. putida* strain E46, *P. putida* strain AA7, *P. fluorescens* strain NEP1, *P. fluorescens* strain L321, *P. syringae* pv. *syringae* B728a, *P. brassicacearum* L13-6-12, and two copies in *P. aeruginosa* strain NCTC9433, *P. syringae* pv. *actinidiae* ICMP 18708, *P. syringae* pv. *actinidiae* P155, *P. syringae* pv. syringae HS191, *P. syringae* pv. *pisi* str. PP1, and *P. viridiflava* CFBP 1590.

In comparison to *P. aeruginosa*, the other species studied in this work have only a few ARGs. Five ARGs (*abaQ, adeF, APH(3")-Ib*, *Pseudomonas aeruginosa soxR,* and *yajC*) were identified in the six strains of *P. putida*, four ARGs (*abaQ*, *adeF*, *fosA*, and *Pseudomonas aeruginosa soxR*) in *P. fluorescens*, three in *P. syringae* (*abaQ, adeF,* and *APH (3")-Ib*), three in *P. brassicacearum* and *P. citronellolis* (*abaQ, adeF,* and *Pseudomonas aeruginosa soxR*), and only two in *P. viridiflava* (*abaQ* and *adeF*). Unlike *P. aeruginosa*, *P. putida* and *P. fluorescens* are relatively less virulent for humans (Gilarranz *et al*., 2013; Mazurier *et al*., 2015). But besides their low pathogenicity, *P. fluorescens* and P. *putida* are especially recognized as rhizobacteria, which promote the growth of plants and protect them against diseases (Luczkiewicz *et al*., 2015). The species *P. syringae*, *P. brassicacearum,* and *P. viridiflava* are known to be pathogens for plants (Belimov *et al*., 2007; Sarris *et al*., 2012; Tarkowski and Vereecke, 2014). While *P. citronellolis* is recognized for its potential for bioremediation through the degradation of a variety of hydrocarbon compounds such as citronellol (Seubert, 1960).

Different mechanisms of action have been detected in resistance genes, the most common are antibiotic efflux pump (*mex, triABC, oprMNJ, yajC, cpxR, cprSR, muxABC, soxR, rsmA, basRS, emrE, opmDEH, bcr-1, parRS, pmpM, nalDC, nfxB, adeF,* and *abaQ*), and antibiotic inactivation (*PDC-7, PDC-8, APH (3')-IIb, APH (3") - Ib, fosA, catB7, oxa-50,* and *oxa-486*). Other resistance mechanisms should also be noted: antibiotic target alteration (*cprRS, soxR, basRS,* and *arnA*), and reduce permeability to antibiotic (*parR*).

The majority of ARGs belong to the resistance-nodulation-cell division (RND) antibiotic efflux pump family (*mex, triABC, oprMNJ, yajC, cpxR, muxABC, soxR, rsmA, opmDEH, parRS, nalDC, NfxB,* and *adeF*). The beta-lactamase family includes *PDC-7, PDC-8, oxa-50,* and *oxa-486,* and the pmr phosphoethanolamine transferase family includes *parSR, basRS,* and *arnA*.

Most of the genes identified in this work confer resistance to a wide range of antibiotic families, while others are resistant to only one type of antibiotic, like *bcr-1* which confers resistance to bicyclomycin, *fosA* confers resistance to fosfomycin, *catB7*, *mexM* and *mexN* confer resistance to phenicol antibiotic, *opmH* and *triABC* confer resistance to triclosan, *Acinetobacter baumannii abaQ* confers resistance to fluoroquinolone antibiotic, and *APH(3")-Ib* confers resistance to the aminoglycoside antibiotic.

Some identified genes included a Single Nucleotide Polymorphisms (SNP) type mutation leading to antibiotics resistance, these genes are *basR* with a single SNP (L71R), *nalC* with two SNPs (S209R, G71E) conferring resistance to a wide range of antibiotics like fluoroquinolone antibiotic, tetracycline antibiotic, macrolide antibiotic, peptide antibiotic, phenicol antibiotic, cephalosporin, sulfonamide antibiotic, and penem.

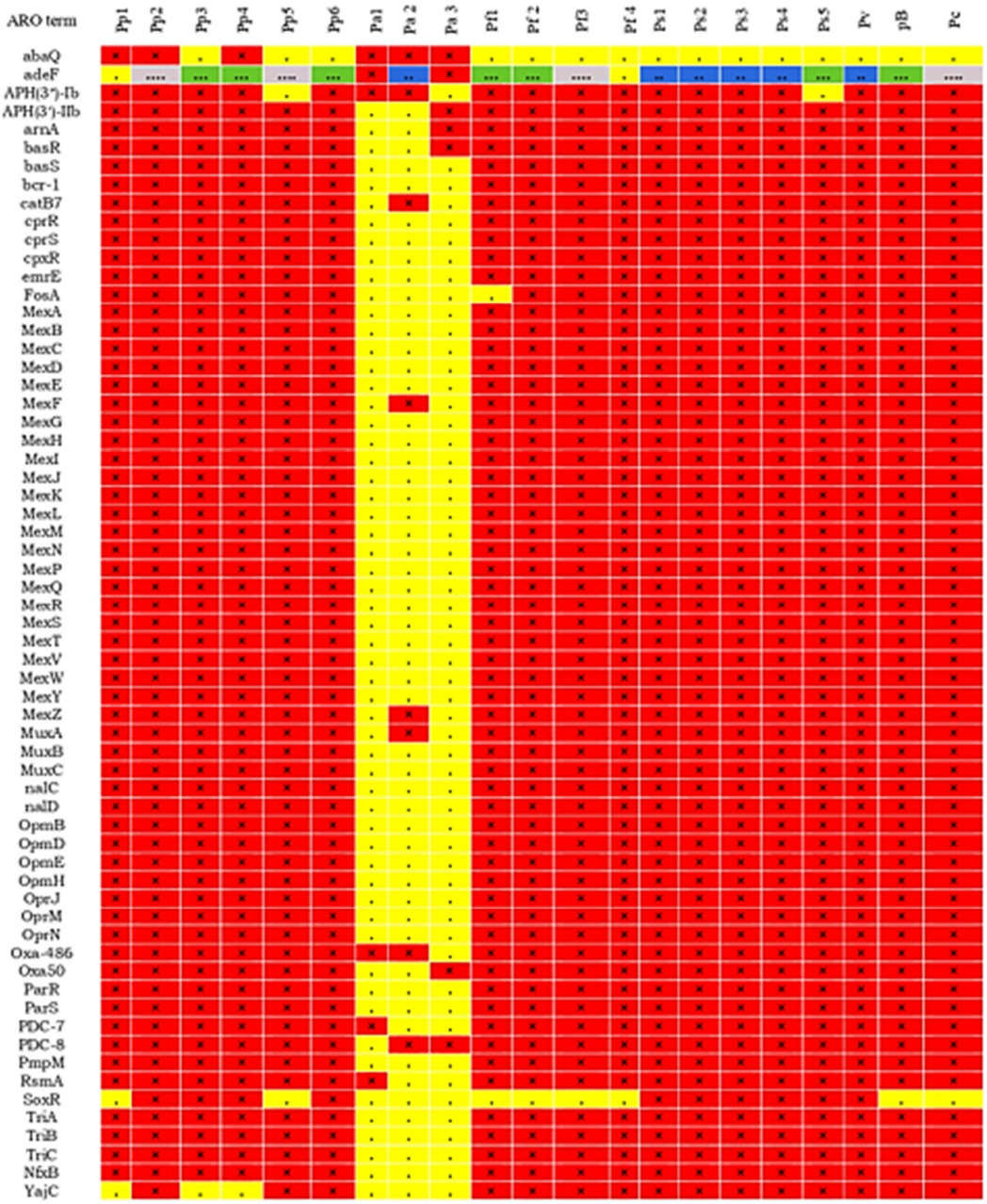

Figure 1. A heat map represents the number and distribution of ARGs in the genomes of the studied *Pseudomonas* strains. ARO, antibiotic resistance ontology

Red: no gene; yellow: a one ARG copy; blue: two ARG copies, green: three ARG copies; gray: four ARG copies.

In this study, the *abaQ* gene found in all strains of the *P. fluorescence* species and of *P. synringea*, the three strains of *P. putida* (strains1290, JBC17, and AA7), and the strains *P. viridiflava* CFBP 1590, *P. brassicacearum* L13-6-12 and *P. citronellolis* P3B5; is a gene identified for the first time in *Acinetobacter baumannii* as being a new efflux pump from the major facilitator superfamily (MFS), involved in the extrusion of quinolone-type drugs (Pérez-Varela *et al*., 2018). According to the authors, the *abaQ* gene found in clinical strains of *A. baumanii* is involved in the virulence of these strains. Based on the results of the CARD platform, the prevalence of *Acinetobacter baumannii abaQ* among sequenced genomes available at NCBI is 84.62% in

Pseudomonas fluorescens and 24.32% in *Pseudomonas putida* and 0% in *P. Pseudomonas aeruginosa*, which is consistent with our results.

The most dominant gene *adeF* is a membrane fusion protein of the multidrug efflux complex AdeFGH conferring resistance to tetracycline and fluoroquinolone antibiotic. This gene is widely distributed among various bacterial species. In *P. aeruginosa*, this gene is found in 33.33% of chromosomes and 1.92% of plasmids available at NCBI. 100% of the sequenced *P. putida* chromosomes available at NCBI include this gene. Despite the presence of several copies of the *adeF* gene in most genomes of the studied species in this work, but the other genes of the *ade* operon were not detected. The *adeGH* genes and the transcriptional regulator gene *adeL* are absent in all these strains; which suggests that the presence of *adeF* gene in *Pseudomonas* sp. is probably due to a horizontal transfer event via a transposon from the species *A. baumannii* (Coyne *et al*., 2011).

Among the 63 ARGs found in this study, the strains of *P. aeruginosa* have 62 genes, among them, 57 ARGs are exclusively present in this species (Figures 1 and 2), which make it the *Pseudomonas* species with the with the highest number of ARGs. *P. aeruginosa* or pyocyanic bacillus is an opportunistic pathogenic bacterium, mainly infecting individuals with weakened immune defenses (Barbier and Wolff, 2010). *P. aeruginosa* is considered to be one of the multi-resistant pathogenic species, belonging to the ESKAPE group responsible for potentially fatal nosocomial infections (Boucher *et al*., 2009). This bacillus exhibits natural resistance to many antibiotics with a great capacity to produce virulence factors. The presence of this species in edible plants can therefore present a real risk for human health via the horizontal transfer of ARGs to the natural bacteria of the human intestinal microbiota.

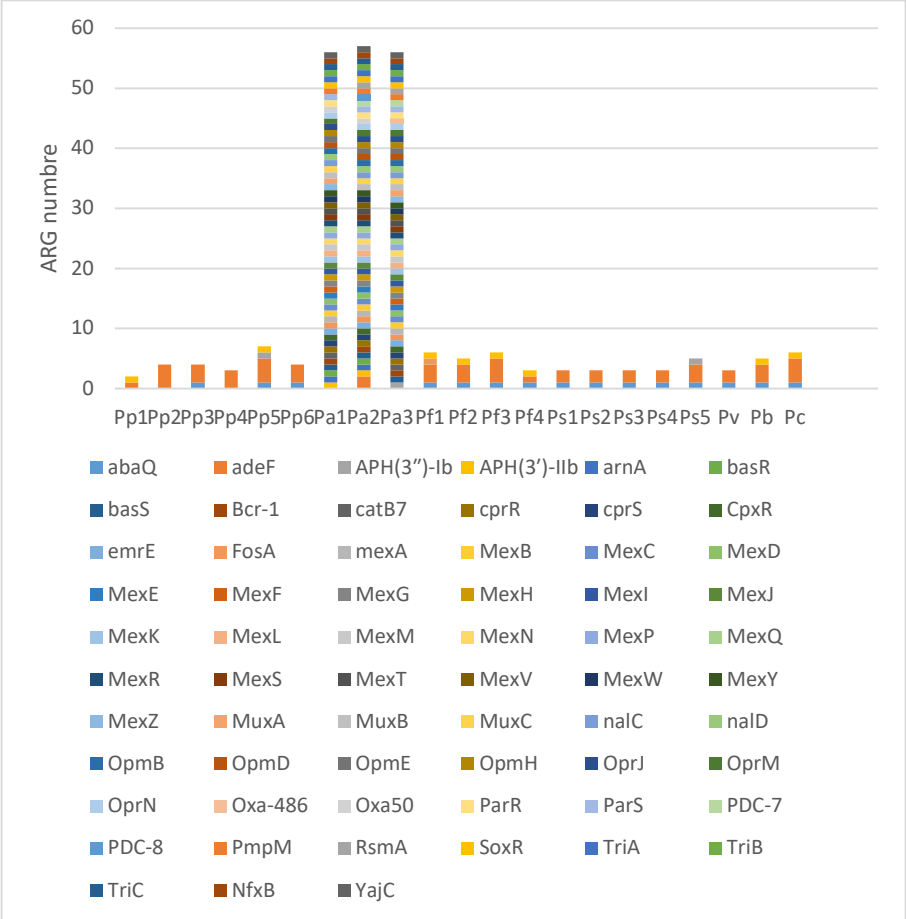

Figure 2. A histogram showing the number and distribution of ARGs in the genomes of the studied *Pseudomonas* strains

The great success of *P. aeruginosa* is mainly due to its intrinsic antibiotic resistance, which is superior to that of the majority of other pathogenic or environmental bacteria. On the other hand, resistance can be acquired by horizontal transfer or by gene mutations. This fact confers to *P. aeruginosa* a multidrug resistance to many classes of antibiotics and therefore limited treatment solutions. *P. aeruginosa* possesses several resistance genes carried on the chromosome of the species, the most widespread among them are the determinisms of numerous efflux systems, which consist of pumps belonging to the RND family. The results show that the three strains of *P. aeruginosa* have many efflux systems that are well known and documented. Among these efflux pumps, we find the MexAB-OprM pump, conferring resistance to ciprofloxacin and meropenem (Nehme and Poole, 2007), MexAB-OprM conferring resistance to aztreonam ciprofloxacin, and ceftazidime (Tian *et al*., 2016), MexEF-OprN a multidrug efflux protein, the triclosan efflux protein MexJK-OpmH (Chuanchuen *et al.,* 2005) and TriABC-OpmH (Mima *et al*., 2007), the multidrug efflux pumps MexMN-OprM, MexJK-OprM, MexVW-OprM and MexPQ-OpmE, the efflux complex MexGHI-OpmD that confers resistance to vanadium, acriflavin, and norfloxacin (Schweizer, 2003) and the MuxABC-OpmB, a multidrug efflux pump that confers resistance to novobiocin, aztreonam, tetracycline, erythromycin, and rokitamycin (Mima *et al*., 2009). Interestingly, we note the presence of the *parRS* genes in the three strains of *P. aeruginosa;* these genes are a two-component sensor that mediates MexXY/OprM efflux pump (Muller *et al*., 2011), but the *mexX* gene is absent in the three strains, probably by a deletion.

PmpM is another efflux pump family that is also encoded in the three genomes of *P. aeruginosa* strains. This pump is part of the multidrug and toxic compound extrusion (MATE) transporter, providing resistance against fluoroquinolones, fradiomycin, benzalkonium chloride, chlorhexidine gluconate, and ethidium bromide (He *et al*., 2004). The EmrE is also another class of efflux pump, it is a small multidrug transporter found in the three strains of *P. aeruginosa*. The *bcr-1* gene present in the three strains of *P. aeruginosa*, codes for a transmembrane protein, that expels bicyclomycin from bacteria, thus leading to resistance to bicyclomycin (Malik *et al*., 2014).

In addition to this efflux pump arsenal, which confers multidrug resistance to several families of antibiotics by their effective injection out of the cell, other resistance mechanisms consist of enzymes that modify antibiotics and render them inactive. This is the case of the *APH(3'')-Ib* gene found in *P. putida* JBC17, *P. aeruginosa* ACR22, *P. syringae* pv. *syringae* B728a and the gene *APH(3')-IIb* found in *P. aeruginosa* YD001 and *P. aeruginosa* NCTC9433. These two genes encode an aminoglycoside phosphotransferase conferring resistance against aminoglycoside antibiotics. This type of resistance is known to be acquired by plasmids, transposons, integrative conjugative elements, or carried on the chromosome (Scholz *et al*., 1989; Hainrichson *et al*., 2007). B-lactamase enzyme determinisms are also among the intrinsic resistance mechanisms of *Pseudomonas* species, among these *PDC-7* in *P. aeruginosa* strain NCTC9433 and *P. aeruginosa* strain ACR22, and the *PDC-8* gene in *P. aeruginosa* strain YD001, which are extended-spectrum beta-lactamase, conferring resistance against cephalosporin, carbapenem, and monobactam (Rodriguez-Martinez et *al*., 2009) and the *oxa-486* gene in *P. aeruginosa* strain ACR22. In addition, the gene *oxa-50* in *P. aeruginosa* strain YD001 and *P. aeruginosa* strain NCTC9433, confers decreased susceptibility to ampicillin and ticarcillin (Girlich *et al*., 2004) by antibiotic modification. Chloramphenicol acetyltransferase (CAT) is another enzyme encoded by the *catB7* gene conferring resistance to the phenicol antibiotic (White *et al*., 1999) present in the genomes of strains *P. aeruginosa* YD001and *P. aeruginosa* ACR22.

In addition to being present in the three strains of *P. aeruginosa*, the *fosA* gene is also detected in the genome of the *P. fluorescens* strain NEP1. Indeed, this gene belonging to the fosfomycin thiol transferase family encodes an enzyme that confers resistance to fosfomycin by catalyzing the conjugation of glutathione to carbon-1 of fosfomycin, which renders it inactive (Beharry *et al*., 2005). The *Pseudomonas aeruginosa soxR* gene is found in the genomes of the strains *P. putida* PC2, *P. putida* JBC17, in the genomes of the three strains of *P. aeruginosa*, in the four strains of *P. fluorescens,* and evenly in the strains *P. brassicacearum* L13 -6-12 and *P. citronellolis* P3B5. Indeed, the product of this gene is a transcriptional activator that induces expression of a regulon that includes the RND efflux pump-encoding operon *mexGHI-opmD* in *P. aeruginosa,* ensuring

resistance against several classes of antibiotics such as tetracycline antibiotic, fluoroquinolone antibiotic, glycylcycline, triclosan, rifamycin antibiotic, penam, cephalosporin, and phenicol antibiotic (Sakhtah *et al*., 2016). In other *Pseudomonas* species, the *soxR* gene appears to be involved in the defense against oxidative stress, but its exact role in this process is not well determined (Park *et al*., 2006). The *yajC* gene detected in the genomes of *P. putida* strain PC2, *P. putida* strain1290, *P. putida* strain E46 and the three strains of *P. aeruginosa* encodes a preprotein translocase subunit which interacts with the Sec secretory system and also with the AcrAB-TolC efflux pump in *E. coli*. In *Pseudomonas* species, the role of this gene is not clearly determined as an antibiotic resistance gene (Rundell *et al*., 2020).

Other than natural resistance, the acquisition of mobile genetic elements, the alteration of antibiotics' cellular targets by mutations, as well as the overproduction of antibiotics inactivating enzymes and efflux systems allows *Pseudomonas* to acquire higher resistance levels. The determinisms of efflux pump systems, for example, can be subject to different types of mutations (deletion, insertion, or substitution of nucleotides) resulting in overexpression. Among the mutations encountered in the three studied *P. aeruginosa* strains, the MexAB-OprM system with NalC mutation of SNP type, conferring acquired resistance to aztreonam (Braz *et al*., 2016), MexCD-OprJ with type A NfxB mutation, conferring acquired resistance to ofloxacin, cephems erythromycin and to the new zwitterionic (Masuda *et al.,* 1996). MexEF-OprN with MvaT deletion (the repressor of the pump) is another mutation conferring an acquired resistance to chloramphenicol and norfloxacin (Richardot *et al*., 2016). The absence of the RsmA regulator from *P. aeruginosa* strain YD00, possibly by deletion, can also lead to the overexpression of the mexEF-oprN pump (Burrowes *et al*., 2006).

The study of the physicochemical characteristics of the ARGs products including the number of transmembrane helices, the presence of a signal peptide, and the cellular localization, show that the proteins OpmB, OprJ, OpmH, OpmD, OpmE, OprM, and OprN are all located in the outer membrane, do not have any transmembrane helix and all have a lipoprotein signal peptide (Sec/SPII) except OpmH that has Sec signal peptide (Sec/SPI) which allow their excretion. All of these proteins are channels involved in the different RND efflux systems and are used to expel antibiotics out of the cell. The MexD, MexF, MexN, MexQ, MexW, MexB, MexK, MexY, TriC, MexI, MexG, MuxB, and MuxC proteins are the inner membrane transporters of RND efflux pumps, all these proteins have 12, 11, or 10 membrane helices and no peptide signal because they remain anchored in the cytoplasmic membrane. Furthermore, MexA, MexC, MexE, MexH, MexJ, MexP, TriA, and TriB are all periplasmic proteins lacking transmembrane helices and possessing a signal peptide, type (Sec/SPII) for most of them allowing their secretion into the periplasmic space. These proteins correspond to the periplasmic adaptors of the RND efflux systems. Surprisingly and unlike the first proteins, the three proteins MexV, MexM, and MuxA, which are also membrane fusion proteins components, all have a single transmembrane helix and no peptide signals.

MexL, MexR, MexS, MexT, MexZ, NalC, NalD, CpxR, SoxR, and rsmA are proteins having neither a transmembrane helix nor a signal peptide, located in the cytoplasm and are regulators of the RND system operons, therefore their mode of action consists of interaction with the gene promoter, which explains their location in the cytoplasm. MexZ, MexL, and MexR are the repressors of the *mexXY* (Vogne *et al*., 2004), *mexJK* (Chuanchuen *et al*., 2002) and *mexAB-oprM* operon (Srikumar *et al*., 2000), respectively. MexS, MexT regulate the expression of the *mexEF-oprN* pump (Sobel *et al*., 2005b). NalD is a repressor of *mexAB-OprM* (Sobel *et al*., 2005a), and NalC is a repressor of PA3720-PA3719, which are themselves positive regulators of *mexAB-OprM* (Braz *et al*., 2016). CpxR is an activator of *mexAB-OprM* efflux pump and enhances antibiotic resistance (Tian *et al*., 2016). The SoxR protein is a redox-sensitive transcriptional activator of efflux pump MexGHI-opmD (Sakhtah *et al*., 2016). In addition to being implicated in the virulence of *P. aeruginosa*, RsmA may cause overexpression of the MexEF-OprN efflux pump leading to resistance to a wide range of antibiotics (Mulcahy *et al*., 2006). Along with these RND efflux systems transcriptional regulatory proteins, the NfxB protein is a repressor of the efflux pump MexCD-oprJ (Higgins *et al*., 2003), but unlike the other regulators, this protein whose location is cytoplasmic, surprisingly has a transmembrane helix which does not go with its

function since it is a transcriptional regulator which reacts with DNA and must therefore be free in the cytoplasm.

Physicochemical analysis

The physicochemical properties of the ARG products studied in this work are summarized in Table 2.

Table 2. Physicochemical characteristics of the studied ARG products

Gene	N° of transmembrane helices (TMH)	Lipoproteins and signal peptides	Localization
AbaQ	12	none	Cytoplasmic Membrane
adeF	12	none	Cytoplasmic Membrane
APH(3″)-Ib	0	none	Cytoplasmic
APH(3′)-IIb	0	none	Cytoplasmic
arnA	0	none	Cytoplasmic
BasR	0	none	Cytoplasmic
BasS	2	none	Cytoplasmic Membrane
Bcr-1	12	none	Cytoplasmic Membrane
catB7	0	none	Cytoplasmic
cprR	0	none	Cytoplasmic
cprS	2	none	Cytoplasmic Membrane
cpxR	0	none	Cytoplasmic
emrE	4	none	Cytoplasmic Membrane
fosA	0	none	Cytoplasmic
MexA	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
MexB	11	none	Cytoplasmic Membrane
MexC	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
MexD	12	none	Cytoplasmic Membrane
MexE	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
MexF	12	none	Cytoplasmic Membrane
MexG	4	none	Cytoplasmic Membrane
MexH	1	Signal peptide (Sec/SPI)	Periplasmic
MexI	10	none	Cytoplasmic Membrane
MexJ	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
MexK	11	none	Cytoplasmic Membrane
MexL	0	none	Cytoplasmic
MexM	1	none	Cytoplasmic Membrane
MexN	12	none	Cytoplasmic Membrane
MexP	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
MexQ	12	none	Cytoplasmic Membrane
MexR	0	none	Cytoplasmic
MexS	0	none	Cytoplasmic
MexT	0	none	Cytoplasmic
MexV	1	none	Cytoplasmic Membrane
MexW	12	none	Cytoplasmic Membrane
MexY	11	none	Cytoplasmic Membrane

MexZ	0	none	Cytoplasmic
MuxA	1	none	Cytoplasmic Membrane
MuxB	10	none	Cytoplasmic Membrane
MuxC	10	none	Cytoplasmic Membrane
nalC	0	none	Cytoplasmic
nalD	0	none	Cytoplasmic
OpmB	0	Lipoprotein signal peptide (Sec/SPII)	Outer Membrane
OpmD	0	Lipoprotein signal peptide (Sec/SPII)	Outer Membrane
OpmE	0	Lipoprotein signal peptide (Sec/SPII)	Outer Membrane
OpmH	0	Signal peptide (Sec/SPI)	Outer Membrane
OprJ	0	Lipoprotein signal peptide (Sec/SPII)	Outer Membrane
OprM	0	Lipoprotein signal peptide (Sec/SPII)	Outer Membrane
OprN	0	Lipoprotein signal peptide (Sec/SPII)	Outer Membrane
Oxa-486	0	Signal peptide (Sec/SPI)	Periplasmic
Oxa-50	0	Signal peptide (Sec/SPI)	Periplasmic
ParR	0	none	Cytoplasmic
ParS	2	none	Cytoplasmic Membrane
PDC-7	0	Signal peptide (Sec/SPI)	Periplasmic
PDC-8	0	Signal peptide (Sec/SPI)	Periplasmic
PmpM	12	none	Cytoplasmic Membrane
RsmA	0	none	Unknown
SoxR	0	none	Cytoplasmic
TriA	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
TriB	0	Lipoprotein signal peptide (Sec/SPII)	Periplasmic
TriC	11	none	Cytoplasmic Membrane
NfxB	1	none	Cytoplasmic
YajC	1	none	Cytoplasmic Membrane

The two proteins ParR and ParS are the two-component sensors ensuring the regulation of efflux components such as the activation of the *mexEF-OprN* system (Wang *et al*., 2013) and the activation of the *mexXY* efflux genes, which confers resistance to polycationic antibiotics. ParS is a histidine kinase sensor composed of three parts comprising a periplasmic domain, two transmembrane segments, and a cytoplasmic transmitter domain, which explains the presence of two transmembrane helices and localization in the cytoplasmic membrane. When ParR, is a cytoplasmic regulatory protein (Gao and Stock, 2009).

The BasR and BasS proteins (also called PmrA and PmrB) are two regulators of the *arnBCADTEF* operon responsible for the chemical modification of lipopolysaccharide (LPS) of the outer membrane in Gram-negative bacteria leading to resistance against cationic antimicrobial peptides such as Polymyxin. BasS is a sensor kinase activated by the presence of a cationic peptide, and it is localized at the inner membrane level, which explains the presence of two transmembrane helices, while BasR is a cytoplasmic regulator (Ezadi *et al*., 2019). ArnA (or PmrL) involved in this resistance process, is a cytoplasmic protein necessary for the modification of the LPS by the addition of positively charged arabinosamine. Two other proteins called CprS and CprR are also involved in the regulation of the *arn* operon in the presence of other cationic antimicrobial

peptides. CprS is a sensor kinase, composed of two transmembrane helices and a periplasmic domain, while CprR is an interacting regulator with the *rna* operon and it is found in the cytoplasm (Fernández *et al*., 2012). But despite the presence of all these regulatory determinisms in the genomes of the *P. aeruginosa* strains studied in this work, we note that except for the *arnA* gene, the elements of the *arnBCADTEF* operon are all absent from the three genomes, which can be explained by a deletion mutation removing this operon.

PmpM, emrE, and Bcr-1 are proteins anchored in the cytoplasmic membrane possessing 12, 4, and 12 membrane helices, respectively. PmpM is a multidrug efflux pump belonging to the multidrug and toxic compound extrusion (MATE) transporter family functioning by expelling antibiotics belonging to the classes of aminoglycoside antibiotic, fluoroquinolone antibiotic, and benzalkonium chloride (He *et al*., 2004). Whereas the emrE protein is a small multidrug resistance (SMR) antibiotic efflux pump that couples the efflux of small polyaromatic cations from the cell (Ma and Chang, 2007). Bcr-1 is a transmembrane protein belonging to the major facilitator superfamily (MFS) antibiotic efflux pump family, it works by expelling bicyclomycin from the cell, leading to bicyclomycin resistance (Fonseca *et al*., 2015).

The AbaQ is another protein located at the level of the cytoplasmic membrane and possessing 12 transmembrane helices, this number corroborates with the study by Pérez-Varela *et al*. (2018) who carried out a prediction of the structure of AbaQ and found 12 transmembrane α-helices with N and C termini located in the cytoplasm. This protein is an MFS transporter involved in the extrusion of quinolone-type drugs (Pérez-Varela *et al.,* 2018). AdeF is another protein that has 12 transmembrane helices; it is the membrane fusion protein of the multidrug efflux complex AdeFGH (Coyne *et al*., 2011).

The proteins Oxa-486, Oxa50, PDC-7, and PDC-8 do not have any transmembrane helix and carry a peptide signal (Sec/SPI) allowing their secretion into the periplasmic space to exercise their role as inactivating B-lactamase enzymes antibiotics. The two proteins APH(3″)-Ib and APH(3′)-IIb are aminoglycoside phosphotransferase, cytoplasmic enzymes containing neither a transmembrane helix nor a signal peptide, they exert their action inside the cytoplasm where they inactivate the aminoglycosides antibiotic. Likewise, the Catb7 and FosA proteins located in the cytoplasm, are chloramphenicol acetyltransferase (CAT) and fosfomycin thiol transferase, respectively, acting on the phenicol antibiotic (White *et al*., 1999) and fosfomycin, respectively, rendering them inactive.

The YacJ protein is localized in the cytoplasmic membrane having a single transmembrane helix and no peptide signal. Indeed, this protein is involved in a membrane complex serving as a secretory system allowing translocation into and across the membrane (Schulze, 2014).

Phylogenic analysis

The phylogenetic analysis of the ARGs (Figure 3), demonstrates that the studied genes are grouped into two large groups; the first group contains most of the ARGs, while the second group considered as the most divergent group includes only five ARGs, which are *nfxB*, *abaQ*, *yajC*, *APH(3″)-Ib,* and *catB7*. In the first group, which is itself divided into three clades, we can notice that in general, genes are grouped together in clades according to their functional nature, as well as their physicochemical properties. The first clade includes all the genes encoding the inner membrane transporters of the different RND efflux pumps *(mexD mexF, mexN, mexQ, mexW, mexB, mexK, mexY, triC, mexI, mexG, muxB,* and *muxC*), and those encoding sensor components ensuring the regulation of system efflux (*parS, parR, basS, basR, cprS, cprR, soxR,* and *cpxR*). While the second clade, contains the *mexL, mexR, mexS, mexT, mexZ, nalC, nalD,* and *rsmA* genes, which encode the regulators of the RND system operons. The genes *mexJ, mexA, mexC, mexE, mexH, mexP, triA, triB, mexV mexM,* and muxA, encoding periplasmic adaptors of the RND efflux systems as well as the genes *opmB, oprJ, opmH, opmD, opmE, oprM, and oprN* encoding for channels involved in the different RND efflux systems are grouped together in the third clade. The rest of the genes are distributed over the three clades. Surprisingly, members of the genes family encoding the aminoglycoside phosphotransferase, are not grouped in the same clade, where APH (3′)-IIb was found in the first group, while the APH (3″)-Ib gene is located in the second divergent group.

ARG-ARG interactions network

In order to visualize the interactions between the studied ARGs, a network of interactions was built (Figure 4). Previous studies have demonstrated the value of network analysis between ARGs for better understanding their interactions and predicting the different expected functional partners of these genes (Li *et al*., 2015; Zhu *et al*., 2016).

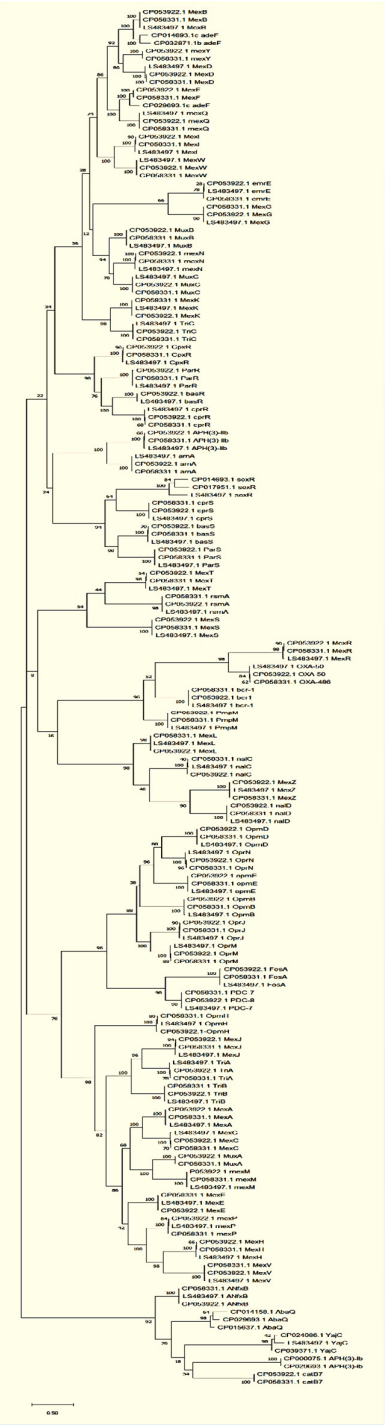

Figure 3. Phylogenetic tree showing the evolutionary links between the studied ARGs
The tree was constructed using the maximum likelihood method using MEGAX software. Bootstrap consensus trees were derived from 1000 repetitions.

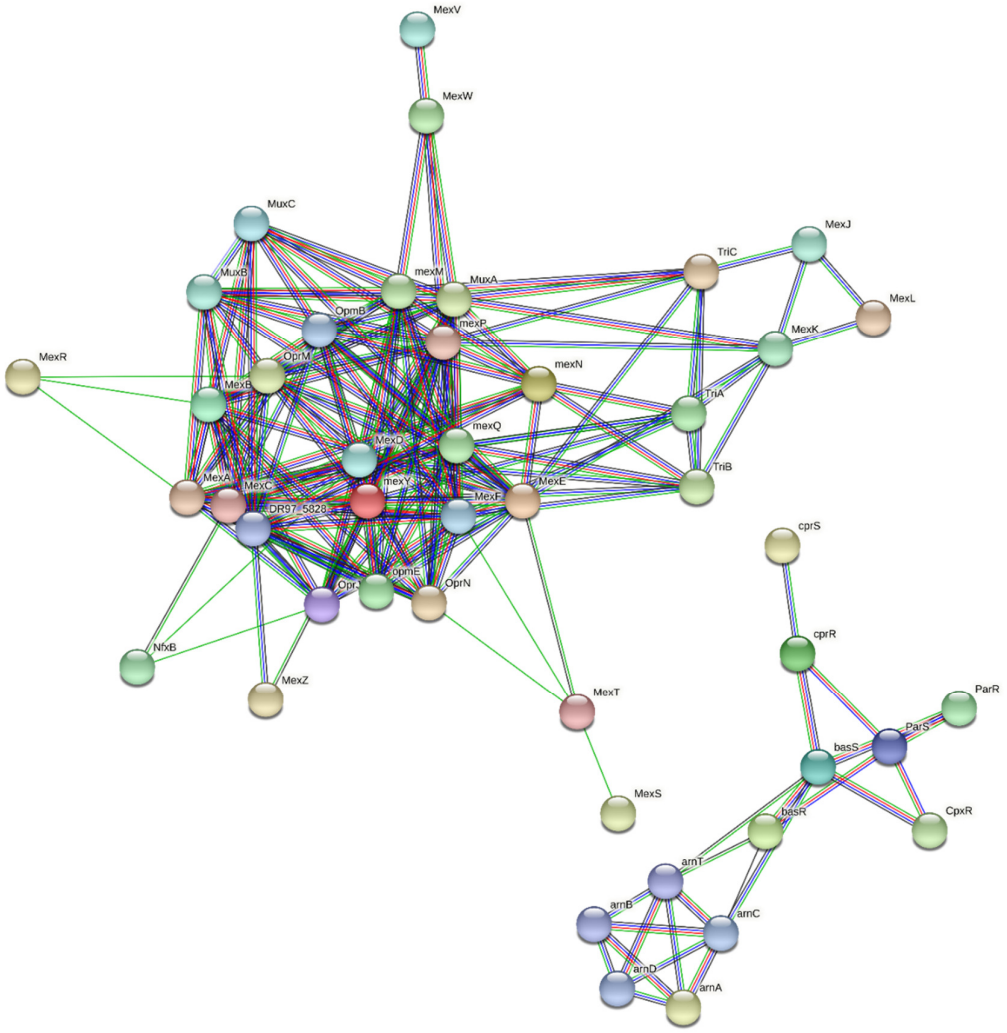

Figure 4. Analysis of the network interactions between the studied ARGs
The nodes are colored according to the types of ARG. The color of the edges represents the nature of the protein-protein associations; blue for gene co-occurrence, red for gene fusions, green for gene neighborhood, black for co-expression.

The established network includes 57 nodes, 54 between them are the genes studied in this work, the rest are predicted, functional partners. Two separate subgroups of genetic interactions are represented in the interaction network (Figure 4). The first subgroup, which is the main interactome, represents a complex network of interactions modulating resistance in *Pseudomonas*. This network is mainly composed of genes belonging to the RND antibiotic efflux pump family, which confers resistance to several families of antibiotics. Each of these genes establishes a large number of connections. The highest number is established by *mexE* and *mexP* with 16 interactions for each gene and the *mexD*, *opmB*, *mexF*, *mexM,* and *muxA* genes with 15 possible interactions for each gene. It was shown that in a gene interaction network study, the most connected genes can be used to detect the co-occurrence of genes, thus serving to save time and workload for the inspection of ARGs (Zhu *et al*., 2016). On the other hand, we also note that certain genes establish interactions with only a single gene, like *mexS,* which establishes a single interaction of the neighborhood on chromosome type with *mexT*, which is highly expected given that *mexS* is a repressor for *mexT* (Sobel *et al*., 2005b). Likewise, *mexV* gene establishes neighborhood on a chromosome and phylogenetic co-occurrence interaction with *mexW*. The

second subgroup has fewer complex interactions than the first, it encompasses the *arnA* gene and its transcriptional regulators *basR, basS, cprR, cprS* belonging to the family of pmr phosphoethanolamine transferase acting by an antibiotic target alteration mechanism, as well as, the *pasRS* genes ensuring the reduce permeability to antibiotics. The proteins encoded by the genes *arnC, arnD, arnB,* and *arnT* are predicted to have interactions with the *arnA* gene.

Conclusions

In the present study, an assessment of the number and diversity of ARGs belonging to *Pseudomonas* species associated with plants was performed. It was found that among the seven studied species, *P. aeruginosa* harbors the highest number of ARGs, this species is considered to be a nosocomial pathogen; mainly isolated from hospitals and patients with severe infections. *P. aeruginosa* is also known for its multi-resistance to antibiotics. Thus, the presence of this species in comestible plants can entail a serious threat to human health by participating in the transmission of a multitude of ARGs, which create a significant selection pressure, as a result, antibiotic therapies against a variety of pathogenic bacteria are severely compromised. This fact is, therefore, important for the monitoring of ARGs especially at the level of edible plants in terms of human health.

Authors' Contributions

Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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