Plant Soil (2024) 495:57–76
https://doi.org/10.1007/s11104-023-06320-z

RESEARCH ARTICLE

Antibiotic- and metal-resistant endophytes inhabit *Armeria maritima* hyperaccumulator

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Published online: 19 October 2023

Abstract

Background and aims Recent research has recognized the presence of metal-resistant bacteria in plants and their role in phytoremediation intensification. However, information on the antibiotic resistance profile of those bacteria remains scarce. This study, describes the first isolation of endophytic bacteria from green parts of *Armeria maritima* growing on mine-tailing soil in southern Poland, and presents the resistance profile of these microorganisms.

Methods Bacteria were isolated from internal tissues of *Armeria maritima* and characterized. Minimal Inhibitory Concentration (MIC) of metals was determined by the plate dilution method using $(CH_3COO)_2Pb$ and $ZnSO_4$ supplemented medium; antibiotic susceptibility was determined by disk diffusion method according to EUCAST version 11.0; the whole genome sequencing was performed using the MiSeq platform (Illumina). The physicochemical properties of soil were evaluated according to European Standards.

Results Toxic metal-resistant bacteria were isolated from the green parts of *Armeria maritima*. The endophytes were identified as *Pseudomonas* spp. The annotated bacterial genomes carried genes encoding numerous metal ion transporters, metal reducing enzymes and efflux pump components. The bacteria were resistant to streptomycin, fosfomycin and ß-lactams. Moreover, genome analysis revealed the presence of MacAB-TolC efflux pump genes conferring resistance to macrolides, the multidrug efflux pumps AcrAB-TolC and MexAB-OprM.

Conclusion *Armeria maritima* is inhabited by endophytic bacteria identified as *Pseudomonas* species that are resistant to metals and to antibiotics. Under the One Health concept the contamination of soil and plants with ARB and ARGs should be monitored and limited and a regulatory framework for safety use of bacterial bioinoculants should be established.

Keywords Antibiotic resistance · *Armeria maritima* · Endophytes · Metallophytes · *Pseudomonas*

Responsible Editor: Antony Van der Ent.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-023-06320-z.

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Introduction

Careless management of agricultural and industrial activities can result in serious contamination of soils by metals (He et al. 2015; Sharma et al. 2007; Walker et al. 2003). If unchecked, this can pose significant risk to public health. Therefore, new biosafety and effective technologies intended to reduce such contamination are needed. A commonly used technology to remove metals from soil is phytoextraction: a phytoremediation method based on the application of hyperaccumulating plants that can decrease metal level in contaminated areas (Kumar et al. 1995). Hyperaccumulators are capable of sequestering extremely high levels of metals in their tissues. Although phytoextraction is an eco-friendly, low-cost method, it tends to have low efficiency because of slow growth of the plants and the low mobility and bioavailability of the metals in soil (Khan et al. 2000; Liu et al. 2020). Hence, recent years have seen growing interest in developing new phytoextraction efficiency approaches.

Recently, one promising technology for enhancing phytoextraction based on the use of plant-growth promoting bacteria (PGPB) to increase plant biomass production and tolerance to metals has been approved (Ahemad 2015; Kong and Glick 2017; Silambarasan et al. 2020). These PGPB include rhizosphere microorganisms inhabiting plant roots (PGPR) and endophytes inhabiting internal plant tissues without causing them any harm (PGPE). PGPB protect plants and promote their growth mainly by producing antibiotics, and phytohormones, and by inducing the Induced Systemic Resistance (ISR) system of the plant; they also support the dissolution of mineral nutrients, such as phosphorus or potassium, and support iron chelation (Olanrewaju et al. 2017; Gamalero and Glick 2011). PGPB can also stimulate metal uptake and bacteria resistance by various mechanisms, such as metal sorption (Kloepper et al. 1980), enzymatic reduction (Glick 2012), oxidation or extracellular precipitation via active efflux pumping (Alves et al. 2022; Bargaz et al. 2018; Kong and Glick 2017). The most commonly used PGPB species are *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas* or *Rhizobium* (Alves et al. 2022).

The use of PGPB to enhance phytoremediation may well be a common biotechnology in the near future. Various strains of PGPB have been tested. Wu et al. (2018) confirmed that the endophytic strain *Buttiauxella* sp. SaSR13 significantly enhanced cadmium accumulation in *Sedum alfredii*. Inoculation with this bacterium resulted in root elongation and, stimulated the secretion of organic acids and increased Cd uptake by *S. alfredii* compared to controls during a seven-day pot experiment.

Endophyte assisted phytoremediation has also been studied in *Sedum alfredii* by Zhang et al. (2013). The findings indicate that the tested *Burkholderia* sp., *Sphingomonas* sp., and *Variovorax* sp. strains significantly promoted Zn and Cd-extraction and had plant growth promoting properties. The experiment was conducted in pots for 60 days (Zhang et al. 2013). Similarly, Wang et al. (2023) revealed that inoculation of *Miscanthus floridulus* with an endophytic strain *Bacillus cereus* BL4 significantly strengthen Cd phytoremediation.

Nowadays, PGPB are commonly used in agriculture as bioinoculants. However, it is important to note that such PGPB may enhance the spread of antibiotic resistance genes (ARGs) in soil and plants because they themselves very often harbour ARGs (Chen et al. 2019; Zhang et al. 2020; Mahdi et al. 2022). Furthermore, ARGs can be located on mobile genetic elements (MGE) and they can be easily transferred among indigenous soil bacteria by horizontal gene transfer (HGT) (Arber 2014; Forsberg et al. 2012). This can represent a potential threat to public health because agricultural soil and agricultural plants act as huge reservoir and propagation hotspot of ARGs (Cadena et al. 2018; Tan et al. 2018; Forsberg et al. 2012; Zhang et al. 2015). Plants and their associated bacteria can absorb ARGs from soil and threaten human health (Zhang et al. 2011; Buchholz et al. 2011). Despite this, little research has been performed of the ARGs present in PGPB used in agriculture, and no description yet exists of the ARGs in endophytes inhabiting green parts of metallophytes.

It has been proposed that a regulatory framework is needed for new bacterial-based biofertilizers (Mahdi et al. 2022). This should include *inter alia* better characterization of new biofertilizers (genome mining) regarding their antibiotic resistance (AR) profile, ARG content and ARG transfer potential. Moreover, multidrug resistant strains or human pathogens should be excluded. It has also been suggested that standard criteria, regulations and quality control procedures for biofertilizer

candidates should be established, so as to guarantee environmental and public health protection (Mahdi et al. 2022).

The present study describes the isolation and characterization of *Armeria maritima* subsp. *halleri* (Wallr.) Rothm. endophytes. It demonstrates that isolated *Pseudomonas* spp. endophytes were resistant to antibiotics and metal ions, and they harboured potential resistance genes. It also explores the possible resistance mechanisms present in the bacteria and attempts to explain the origin of the ARGs present in the isolated endophytes.

Materials and methods

Study site, sampling and soil physicochemical analysis

The studied area was located near the ZGH "Bolesław" mining and metallurgical plant in Bukowno village, in the south of Poland (50°16'40.7"N 19°28'13.8"E). ZGH "Bolesław" S.A. is a Polish company that has been operating since 1955 in Bukowno village, near Olkusz. Today, it is a modern mining and metallurgical complex, the main producer of zinc in Poland and a supplier of zinc to neighboring countries, mainly the Czech Republic, Slovakia, Austria and Hungary. In this plant zinc and lead ores are extracted and processed to produce electrolytic zinc, zinc alloys, sulfuric acid and zinc and lead concentrates.

Samples were taken during May 2015, during the flowering stage of the plants. The plant species selected for investigations was *Armeria maritima* subsp. *halleri* (Wallr.) Rothm. All collected plants were placed in polyethylene bags and transported to the laboratory in an ice cooler at 4 °C; all testing was performed within two days.

The total organic carbon, pH, calcium, magnesium, and metal content (Cr, Cu, Cd, Ni, Pb, Zn, Hg) were determined. Hg content was determined as described in DIN ISO 16,772. The other metals were tested according to the following: (ICP-OES/ICP-MS) – DIN EN ISO 11,885/DIN EN ISO 17294-2. pH was determined according to DIN EN ISO 10,390 and Total Organic Carbon (TOC) according to DIN EN ISO 15,936.

Isolation and purification of metal-tolerant bacteria

Any metal-tolerant endophytic bacteria were isolated using the Luria Bertani agar (LB) medium supplemented with filter-sterilized soluble salts of lead $(CH_3COO)_2Pb$ (Pb^{2+}) or zinc $ZnSO_4$ (Zn^{2+}) at a concentration of 20 mg/dm^3. To isolate endophytic bacteria, the green parts of plants were separated and subjected to surface sterilization in sterile conditions under a laminar chamber (Goryluk et al. 2009). Before starting the procedure, the ends of the stem sections were secured against the inflow of sterilization agents. The first stage of sterilization was to rinse the plant fragments in 70% ethanol for about 60 s; these were then transferred to 2% mercury (II) chloride solution for 10 s and rinsed three times in distilled water. After surface sterilization, the plant material was homogenized. The obtained homogenates were diluted 10-fold and 100-fold, and 0.1 cm^3 aliquots were plated on culture media. All plates were incubated at 30° C for 24–48 h. In order to determine the dry weight of the tested plants, each homogenate was poured onto a filter paper and weighed after complete drying. Based on these results, the numbers of colony forming units were then calculated per one gram of dry plant matter.

Individual bacterial colonies with different morphological characteristics were randomly selected and streaked on the LB agar medium supplemented with metal salts until pure cultures were obtained. A total of 100 bacterial isolates were selected for further studies and stored in 20% glycerol stock at -80 ºC.

Characterization of metal-tolerant bacteria

Identification

The morphological features of bacterial isolates (Gram staining) were recorded using light microscopy. Following this, biochemical analyses were performed, involved to determined oxydase and catalase activity, gelatin hydrolysis, citrate utilization, glucose fermentation and urease and fluoresceine production. All tests were prepared according to Bergey's Manual of Systematic Bacteriology and isolates were identified to genus level (Bergey 1994).

Five out of 100 Gram-negative bacterial isolates with different morphologies were selected for further analysis. To identify the species, isolates were plated

on LB agar and MALDI-TOF MS analysis was conducted by a commercial service (ALAB laboratory, Warsaw Poland). The standard Bruker interpretative criteria were applied. A score > 2.300 was used for certain species identification (Suppl. Tab. S1).

The toxic metal MIC assay

The Minimum Inhibitory Concentration (MIC) values were determined by the plate dilution method as adopted by Malik and Jaiswal (2000) with modifications. Luria Bertani LB medium supplemented with filter-sterilized soluble salts of $(CH_3COO)_2Pb$ (Pb^{2+}) and $ZnSO_4$ (Zn^{2+}) was used. The starting concentration for each metal was 10 mM. The inoculation was performed using 0.1 ml of bacterial suspension with a density of 10^6 CFU/ml. The MIC was taken as the lowest metal concentration that prevented the growth of the bacteria (Haroun et al. 2017). In this experiment *E. coli* 1655 strain was used as control (Spain and Alm 2003).

The antibiotic susceptibility test

Antibiotic susceptibility was determined by the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing EUCAST version 11.0, valid from 2023-01-01. All 13 antibiotics recommended for *Pseudomonas* spp. were tested. Two additional antibiotics not included in EUCAST breakpoints were tested, viz. fosfomycin (50 µg) and streptomycin (25 µg), based on the presence of resistance genes detected by genome sequencing (see below). The diameter of bacterial growth inhibition zone around each of the antibiotic discs was interpreted according to the EUCAST criteria for *Pseudomonas* spp. If the antibiotic was not included in the standard, then a lack of any inhibition zone was interpreted as no susceptibility to the given antibiotic.

Isolation of resistance genes

The genomic DNA of the selected bacterial isolates was extracted according to Kpoda et al. (2018), and then stored at -20 °C for subsequent use. The genes coding for the efflux pump were identified using PCR amplification, while *bla* genes were isolated using multiplex PCR.

The efflux pump genes *mexA* and *mex B* of the Mex AB-OprM pump were amplified by PCR as described by Ugwuanyi et al. (2021). *MexD*, *mexF* and *mexY* genes of the MexCD-OprJ, MexEF-OprN, MexXY-OprM efflux pumps were amplified according to Poonsuk and Chuanchuen (2014). In addition, the *czcA* and *czcR* genes encoding components of the CzcCBA efflux pump were amplified using primers proposed by Perron et al. (2004). The types of ß-lactamase coding genes present were determined by multiplex PCR (Colom et al. 2003; Dallenne et al. 2010; Piotrowska et al. 2019). Four multiplex PCR assays were performed for the detection of *bla* genes: bla_{TEM}, bla_{SHV} and bla_{OXA} genes (Multiplex I); bla_{CTXM} genes (Multiplex II); bla_{VER}, bla_{PES} and bla_{GES} genes (Multiplex III) and bla_{KPC}, bla_{IMP} and bla_{VIM} genes (Multiplex IV).

All the PCR amplicons were gel-purified (Gel-Out kit, AA Biotechnology) and submitted for sequencing by a commercial service (Institute of Biochemistry and Biophysics, Polish Academy of Sciences) using ABI 3730 Genetic Analyzer, Applied Biosystems (BigDye v3.1 sequencing chemistry). Sequences of obtained gene fragments were searched against the National Center of Biotechnology Information (NCBI) using a local BLASTX program. A gene was designated as a resistanc gene if it shared at least 98% identity with other resistance gene in the database.

The whole genome sequencing and bioinformatic analysis

Genomic DNA from five selected bacteria was extracted using a Genomic Mini® kit (A&A Biotechnology) as described by the manufacturer. DNA concentration and quality were checked with the Qubit™fluorometer (Invitrogen) and bacterial genomes were sequenced by a commercial service (Institute of Biochemistry and Biophysics, Polish Academy of Sciences).

The whole genome sequencing WGS was performed on MiSeq platform (Illumina) with 300 bp paired-end reads (Supp. Tab. S4). Only high quality reads after filtering using fastp (https://github.com/OpenGene/fast) were taken for assembly step. The Unicycler version 0.4.8 assembly method was used. This Whole Genome Shotgun BioProject was deposited at DDBJ/ENA/GenBank under the accession number PRJNA886618.

Phylogenetic affiliation analysis

The genome sequences of each strain were uploaded to the Type Strain Genome Server (TYGS), i.e. a bioinformatic platform for digital, highly-reliable estimation of the relatedness of genomes based on DNA-DNA hybridization (DDH) (available at the website: http://tygs.dsmz.de) (Meier-Kolthoff et al. 2013). Additionally, a phylogenetic tree was constructed based on the RNA polymerase sigma factor RpoD (*rpoD*) gene (Banasiewicz et al. 2021; Girard et al. 2020). Briefly, 37 environmental-type *Pseudomonas* spp. strain *rpoD* genes were uploaded from the NCBI database (accession numbers listed in Supp. Table 2). The *rpoD* sequences (650 bp) were aligned using Clustal W software. The multiple sequence alignments were then used to create phylogenetic trees by the Neighbor Joining method with complete deletion of gaps, implemented in MEGA7 software (Kumar et al. 2016; Saitou and Nei 1987; Tamura et al. 2004). The evolutionary distances between sequences were computed using the Maximum Composite Likelihood method (Tamura et al. 2004), represented as the units of the number of base substitutions per site. The tree itself was drawn to scale, with branch lengths given in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Resistance genes screening

Genomes were annotated in the Rapid Annotation using Subsystem Technology (RAST) server (available at the website: http://rast.nmpdr.org) (Brettin et al. 2015). The annotation process enables the prediction of protein-coding genes, like ARG and HMRG, as well as other important elements, like direct and inverted repeats, insertion sequences, transposons and plasmids. ARGs were predicted using the Resistance Gene Identifier (RGI) application, available at the Comprehensive Antibiotic Resistance Database (CARD) (available at the website: http://card.mcmaster.ca/analyzer/rgi). In addition, Antibacterial Biocide and Metal Resistance genes Database (BacMet) was used to find resistance genes (Pal et al. 2014; available at the website: http://bacmet.biomedicine.gu.se). Finally, manual annotation was performed.

BLASTX analysis

The obtained gene fragments were searched against the National Center of Biotechnology Information (NCBI) using a local BLASTX program. A gene was designated as an ARG or MRG if it shared at least 98% identity with the best hit in the database.

Results

Physico-chemical properties of soil

At the test site, the total concentrations of Pb and Zn were 1100 mg/kg soil and 3620 mg/kg soil, respectively (Table 1). Hence, the tested soil was classified as highly contaminated (Trafas et al. 2006).

Biochemical characterization of metal tolerant bacteria

The mean total count of metal-tolerant bacteria isolated from *A. maritima* endosphere varied from 5.73 log CFU/g of plant material on lead supplemented medium to 5.46 log CFU/g on the zinc supplemented material. All 100 isolates selected for further studies were Gram-negative, catalase positive and glucose fermentation negative. Some differences in urease and fluoresceine production were noted between selected isolates (Table 2). According to Bergey's Manual of Determinative Bacteriology, the isolates were identified as *Pseudomonas* spp. MALDI-TOF-MS analysis failed to identify the isolates down to the species level (Supp. Tab. S1).

The antibiotic susceptibility test, performed according to EUCAST, revealed different resistance profiles among selected isolates (Table 3, Supp. Tab. S3). All of the isolates were resistant to aztreonam (ATM), and meropenem (MEM) while four out of five isolates were resistant to ceftazidime (CAZ), cefepime (FEP) and streptomycin (S). Only two isolates showed resistance to imipenem (IPM). Regarding metal resistance, three isolates demonstrated a maximum MIC of 60 mM for Pb (II) (AM4, AM8, AM14) and one isolate a maximum MIC of 220 mM for Zn (II) (AM14; Table 3). The

Table 1 Soil properties and metal concentrations

Soil properties		Total concentration of metals (mg kg^{-1})								
pH	TOC (g kg^{-1})	Ca	Cd	Cr	Cu	Hg	Mg	Ni	Pb	Zn
7.0 ± 0.75	28.3 ± 0.09	4530 ± 850	35.00 ± 10.3	8.40 ± 1.75	18.50 ± 2.50	0.15 ± 0.02	2670 ± 150	8.25 ± 4.75	1100 ± 710	3620 ± 2220

All values represented mean ± standard deviation

lowest MIC (30 mM) was observed for isolate Z18, for both metals tested.

Resistance determinants detection

The tested bacteria were screened for genes encoding multidrug efflux pumps known to be common in various *Pseudomonas* strains, such as the Resistance Nodulation Cell Division family pump genes (RND). However, none of the Mex-type pumps genes were detected and only one of two tested CzcCBA system genes were detected in any tested strain. The PCR amplicons of the *czcR* gene were shorter (315 bp) than the expected *czcR* gene (880 bp) and sequence analysis did not confirm membership of any known resistance gene.

Regarding antibiotic resistance multiplex PCR amplify any selected variants of the *bla* genes.

Genome characterization of endophytic *Pseudomonas* sp. strains

Multidrug-resistant endophytic *Pseudomonas* sp. strains were sequenced on Illumina platform (AM4, AM8, AM14, Z13, Z18). The draft whole genome sequence length varied from 6.1 Mb to 7.4 Mb (Table 4, Supp. Tab. S4). Mean G + C content ranged from 60 to 61%. Annotation performed using RAST server predicted between 5,700 and 7,059 coding sequences. The analysis revealed the presence of between 374 subsystems with 65 RNA genes and 395 subsystems with 63 RNA genes. The entire Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the following accessions: SAMN31135831, SAMN31136163, SAMN31136268, SAMN31137609, SAMN31137624 (Table 4). The version described in this paper is the first version of the WGS project.

A whole-genome based taxonomic analysis based on DNA-DNA hybridization (in TYGS) found that isolate AM8 shared high homology with *Pseudomonas marginalis* species (dDDH above 80%; Supp. Tab. S5). In addition, the species demonstrating the greatest homology to the rest of the tested bacteria were, as follows: *P. paracarnis* (strain AM4, dDDH = 78.6%); *P. koreensis* (AM14, dDDH = 78.7%) and *P. yamanorum* (Z13, dDDH = 72.2%; Z18, dDDH = 70.7%) (Supp. Tab. S5). The *rpoD* phylogeny confirmed the species

Table 2 Biochemical characteristics of endophytic metal-tolerant bacteria (positive result +, negative result -)

Isolate	Biochemical characteristics							
	Gram-staining	Oxidase	Catalase	Glucose fermentation	Gelatin liquefaciens	Citrate utilization	Urease	Fluoresceine production
AM4	negative	+	+	-	+	+	+	-
AM8	negative	+	+	-	+	+	+	-
AM14	negative	+	+	-	+	+	+	+
Z13	negative	+	+	-	+	+	+	-
Z18	negative	+	+	-	+	+	-	+

Table 3 Antibiotic resistance profile and metal minimum inhibitory concentration (MIC) of endophytic bacteria isolated from *Armeria maritima (R-resistant, S- sensitive)*

Isolate	Antibiotic resistance profile						MIC (mM of metal ions)	
	ATM	CAZ	FEP	IPM	MEM	S	Pb (II)	Zn (II)
AM4	R	R	R	R	R	R	60	160
AM8	R	R	R	S	R	R	60	160
AM14	R	R	R	R	R	S	60	220
Z13	R	S	S	S	R	R	30	40
Z18	R	R	R	S	R	R	30	30
E.coli 1655							5	1

Table 4 General features for *Pseudomonas* spp. strain draft genomes

Isolate	GenBank accession no.	Genome size (bp)	No. of contigs	No. of coding sequences	G+C content (%)	No. of subsystems	No. of RNAs genes
AM4	SAMN31135831	6,189,201	119	5,700	60.0	374	65
AM8	SAMN31136163	7,381,324	64	6,953	60.4	391	65
AM14	SAMN31136268	6,182,403	54	5,713	60.3	375	67
Z13	SAMN31137609	7,401,235	160	7,059	60.1	395	63
Z18	SAMN31137624	7,381,527	121	6,785	61.0	394	62

identification of three isolates, viz. AM4, Z13, and Z18; however, the species of AM8 and AM14 remain unclear (Fig. 1).

Prediction of metal resistance genes

The annotated draft genomes confirmed the presence of genes conferring resistance to arsenic, cadmium, chromium, cobalt, copper, lead, tellurium and zinc (Table 5). All tested endophytic bacteria genomes demonstrated different *ars*, *cus* and *teh* genes. Moreover, all the genomes carried *copC*, *copD*, *copG* and *cueO*. The *Pseudomonas* sp. AM8 genome lacked the *cadA*, *cadR* and *czcD* genes. The *czcB*, *czcR* and *czcS* genes were absent from the *P. paracarnis* AM4 and *Pseudomonas* sp. AM14 genomes, while *chrA* and *copB* genes were not detected in *P. yamanorum* species (Z13, Z18).

The detected genes encode components of various metal resistance mechanisms (Table 6). Gene *arsB* encodes an inner membrane polypeptide of the ArsAB efflux pump. ArsB confer resistance

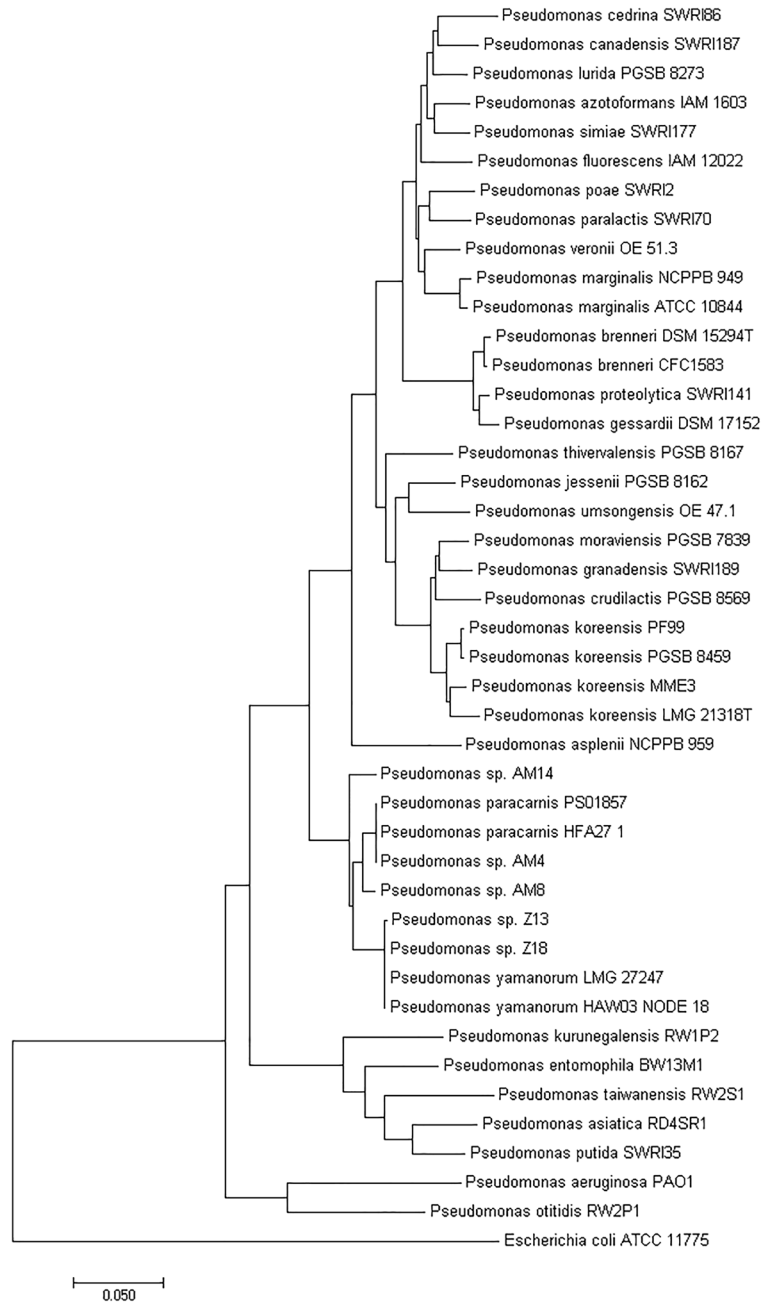

Fig. 1 Neighbor-Joining phylogeny of *rpoD* partial gene sequences (650 bp), comprising type strains of 30 *Pseudomonas* species and 5 unknown *Pseudomonas* spp. (AM4, AM8, AM14, Z13, Z18). *Escherichia coli* ATCC11775 was used as an outgroup. The optimal tree with a sum of branch length = 1.73015111 is shown. The analysis involved 43 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 617 positions in the final dataset

to As(III) and it provides sufficient arsenic resistance, even if the bacterium lacks ArsA (Bhattacharjee and Rosen 2007). Gene *arsC* encodes arsenate reductase, which reduces As(V) to As(III), and gene *arsR* encodes transcriptional regulator of the *ars* operon. Moreover, the *arsH* gene encodes a product that strengthens bacterial resistance to arsenate and arsenite; however, its function remains unclear (Rosen 2002).

Metal-translocating P-type ATPase genes, such as *cadA*, were present in the genomes of *Armeria maritima* endophytes. The CadA protein catalyzes the active efflux of zinc, cadmium and lead ions (Rossbach et al. 2000). Additionally, genes coding

Table 5 Putative metal resistance genes detected in tested *Pseudomonas* sp. genomes (positive result +, negative result -)

Metal resistance gene	Bacterial strains				
	P. paracarnis AM4	*Pseudomonas sp.* AM8	*Pseudomonas* sp. AM14	*P. yamanorum* Z13	*P. yamanorum* Z18
arsB	+	+	+	+	+
arsC	+	+	+	+	+
arsH	+	+	+	+	+
arsR	+	+	+	+	+
cadA	+	-	+	+	+
cadC	+	+	+	+	+
cadR	+	-	+	+	+
chrA	+	+	+	-	-
copB	+	+	+	-	-
copC	+	+	+	+	+
copD	+	+	+	+	+
copG	+	+	+	+	+
cueO	+	+	+	+	+
cusA	+	+	+	+	+
cusB	+	+	+	+	+
cusR	+	+	+	+	+
cusS	+	+	+	+	+
czcB	-	+	-	+	+
czcD	+	-	+	+	+
czcR	-	+	-	+	+
czcS	-	+	-	+	+
tehA	+	+	+	+	+
tehB	+	+	+	+	+

transcriptional regulators were detected in our studies, like *cadC* and *cadR*. Other P-type ATPase genes conferring resistance to copper were detected, such as *copB*, *copC*, *copD* and *copG* genes, which encode periplasmic proteins that bind and/or transport Cu ions. Genes confering other copper resistance mechanisms were also observed, such as *cueO* encoding multicopper oxidase, and the *cusR* and *cusS* genes encoding regulatory proteins of the CusCFBA system, involved in periplasmic copper detoxification. CusS is a sensor histidine kinase while CusR is a regulatory protein (Nies 1999).

Another resistance mechanism detected in bacteria was the chemiosmotic pump protein ChrA, a membrane transporter protein responsible for the efflux of chromium out of the cell cytoplasm. It is encoded by the chromium resistance gene *chrA* (Branco et al. 2008).

The CzcCBA system functions as a cation-proton antiporter by transferring Cd^{2+}, Co^{2+} and Zn^{2+} ions out from the bacterial cell (Wang et al. 2017). The *czcD* gene encodes cation diffusion facilitator transporter CzcD, while *czcB* encodes one of the CzcCBA efflux transporter system as genes *czcR* and *czcS* encode the two-component regulatory system CzcRS. Those genes form part of the Czc system, comprising the CzcCBA transporter lying across the inner and outer membrane, regulated by the CzcRS two-component system, and the CzcD cation diffusion facilitator protein (CDF).

Additionally, the *cusR* and *cusS* genes encoding regulatory proteins of the CusCFBA system involved in periplasmic copper detoxification were detected. CusS is a sensor histidine kinase while CusR is a regulatory protein (Bondarczuk and Piotrowska-Seget 2013). The proteins of the CusCFBA efflux pump,

Table 6 Characterization of selected metal resistance genes

Detected gene	Gene product	Function	Resistance mechanisms	Toxic metal removed
arsB	Membrane protein	Metal efflux transport	ArsAB RND-type efflux pump	As(III)
arsR	Transcription factor	Regulation of the *ars* operon expression		
arsC	Cytoplasm protein	Arsenate reduction	ArsC system	As(V)
arsH	Arsenical resistance protein	Still not clear	Still not clear	As(III)
cadA	Membrane protein	Cation transport out of the cell	P-type ATPase	Cd(II), Pb(II), Zn(II)
cadC	Transcription factor	Regulation of the *cadA* gene expression		
cadR	Transcription factor			
chrA	Membrane protein	Metal efflux transport	ChrA pump	Cr(VI)
copB	Membrane protein	Cation transport out of the cell	P-type ATPase	Cu(I)
copC	Periplasmic protein	Cu ions bind or transport		
copD	Periplasmic protein	Cu ions bind or transport		
copG	Periplasmic protein	Cu ions bind or transport		
cueO	Periplasmic protein	Multicopper oxidase	Cue system	Cu(I)
cusA	Membrane protein	Cation transport	CusCFBA RND-type efflux pump	Cu(I)
cusB	Periplasmic protein	Cation transport		
cusR	Cytoplasmic protein	Regulation of the cusCFBA operon expression		
cusS	Membrane protein	Regulation of the cusCFBA operon expression		
czcB	Membrane fusion protein	Divalent ion transport across the inner and outer membranes	CzcCBA RND-type efflux pump	Co(II)/Zn(II)/ Cd(II)
czcR	Transcription factor	Regulation of the czcCBA operon expression		
czcS	Transcription factor	Regulation of the czcCBA operon expresion		
czcD	Cytoplasmic protein	Cation diffusion facilitator transporter	CDF protein family transporter	
tehA	Integral membrane protein	Cation transport	TehAB system	Te(IV)
tehB	Membrane-associated protein	Cation transport		

CusA and CusB, were also detected (Bondarczuk and Piotrowska-Seget 2013).

Finally, the *tehA* and *tehB* genes, which may conferr resistance to tellurium, were detected. *TehA* encodes an internal membrane protein, while *tehB* encodes the membrane associated protein (Turner et al. 1995).

Prediction of antibiotic resistance genes

Tested *Pseudomonas* genomes carried genes conferring different antibiotic resistance mechanisms (Table 7). All five tested genomes contained genes believed to encode components of efflux pumps, such as: *acrA, acrB, macA, macB, mexT, pmpM, lysR, soxR, tolC*. All sequenced genomes were found to contain genes encoding components of the antibiotic inactivation system, such as *ampC*, *ampR*, and those coding for MBL (metallo-ß-lactamase encoding gene) and the GNAT enzyme family. In addition, all tested genomes demonstrated point mutations in the *gyrA* and *gyrB* gene. The *Pseudomonas yamanorum* genomes lacked *fosA* and *oprM* genes, while those of the rest of the tested *Pseudomonas* spp. lacked *oprN*. The presence of genes believed to encode components of various Mex pumps varied among the tested

Table 7 Putative antibiotic resistance genes detected in tested *Pseudomonas* sp. genomes (positive result +, negative result -)

Antibiotic resistance gene	Bacterial strains				
	P. paracarnis AM4	*Pseudomonas sp.* AM8	*Pseudomonas* sp. AM14	*P. yamanorum* Z13	*P. yamanorum* Z18
acrA	+	+	+	+	+
acrB	+	+	+	+	+
ampC	+	+	+	+	+
ampR	+	+	+	+	+
fosA	+	+	+	-	-
GNAT family gene	+	+	+	+	+
gyrA	+	+	+	+	+
gyrB	+	+	+	+	+
lysR	+	+	+	+	+
macA	+	+	+	+	+
macB	+	+	+	+	+
MBL	+	+	+	+	+
mexA	+	-	-	-	-
mexB	+	-	-	-	-
mexE	+	+	+	-	+
mexH	-	+	-	+	+
mexI	-	+	+	-	-
mexT	+	+	+	+	+
mexX	+	+	-	+	+
norM	+	+	+	+	+
oprM	+	+	+	-	-
oprN	-	-	-	+	+
pmpM	+	+	+	+	+
soxR	+	+	+	+	+
tolC	+	+	+	+	+

Pseudomonas strains (Table 7). The following genes were detected: *mexA*, *mexB*, *mexE*, *mexH*, *mexI* and *mexX*.

Genes coding the components of three antibiotic resistance mechanism types, viz. antibiotic inactivation, antibiotic target alteration and antibiotic efflux pumps, were identified in the tested genomes (Table 8). Five genes responsible for antibiotic inactivation were found in the tested genomes. Metallo-ß-lactamase genes (MBL), *amp*C and *ampR* genes confer resistance to ß-lactam antibiotics. Resistance to ß-lactams is largely mediated by enzymes called ß-lactamases encoded by *bla* genes. These enzymes are divided into four classes (A, B, C, D) and form two groups: metallo-ß-lactamases (MBL) and serine ß-lactamases. Metallo-ß-lactamases belong to Class B. Class B is divided in three subclasses: chromosomally-encoded genes of B2 or B3 subclasses, and MGE-encoded genes of the B1 subclass (Behzadi et al. 2020). In the present study, all tested genomes contained *bla* genes (Table 7). This result was consistent with the antibiotic resistance profile, as the tested bacteria were resistant to ß-lactam antibiotics (Table 3). A comparison of the sequences of identified genes with some other *bla* genes in the NCBI database confirmed that they coded for metallo-ß-lactamases. There is therefore a high probability that the *bla* genes belong to the chromosomally-encoded B2 or B3 subclass ß-lactamases.

Another antibiotic inactivation gene detected in the tested genomes was *fosA*. *FosA* encodes the FosA protein, and is the most common fosfomycin resistance mechanism in Gram-negative bacteria. FosA is a Mn^{2+}, K^{+} dependent metalloenzyme that catalyzes

Table 8 Characterization of selected antibiotic resistance genes

Detected gene	Gene product	Function	Resistance mechanisms	Antibiotics removed
acrA	Periplasmic membrane fusion protein	Component of the AcrAB-TolC pump	RND family efflux pump	Tetracyclines, rifamycin, fluoroquinolones, cephalosporins, phenicols,penams, glycylcycline
acrB	Membrane protein	Component of the AcrAB-TolC pump	RND family efflux pump	
ampC	ß-lactamases	Hydrolysis of the ß-lactam ring in antibiotic molecule	Antibiotic inactivation	Cephalosporins, penams
ampR	Transcription activator	Positive regulation of ampC gene expression	Antibiotic inactivation	
MBL	Metallo-ß-lactamases	Hydrolysis of the ß-lactam ring in antibiotic molecule	Antibiotic inactivation	β-lactams
fosA	FosA protein that is glutathione transferase	Addition of glutathione to antibiotic	Antibiotic inactivation	Fosfomycins
gyrA	Subunit A of the DNA gyrase	Point mutation in *gyrA*	Antibiotic target alteration	Fluoroquinolones
gyrB	Subunit B of the DNA gyrase	Point mutation in *gyrB*	Antibiotic target alteration	
GNAT family gene	N-acetyltransferase	Acetylation of aminoglycoside antibiotic	Antibiotic inactivation	Aminoglycosides
lysR	Transcription regulator LysR	Positive regulation of efflux pump gene expression	RND family efflux pump	Tetracyclines, fluoroquinolones
macA	Periplasmic membrane fusion protein MacA	Component of the MacAB-TolC pump	ABC family efflux pump	Macrolides
macB	ATP-binding cassette transporter	Export of macrolides	ABC family efflux pump	Macrolides
pmpM	Transmembrane protein	H^+ driven antiporter	MATE family efflux pump	Aminoglycosides, Fluoroquinolones
soxR	Redox-sensitive transcriptional activator SoxR	Positive regulation of efflux pump gene expression	RND family MFS family ABC family efflux pumps	Tetracyclines, rifamycin, fluoroquinolones, cephalosporins, phenicols,penams, glycylcycline
tolC	Type I secretion outer membrane protein TolC	Component of efflux pumps	RND family MFS family ABC family efflux pumps	
mexA	Membrane fusion protein	Component of the MexAB-OprM efflux pumps	RND family efflux pump	Peptide antibiotics, sulfonamides, cephalosporins,cephamycins monobactams, carbapenems, macrolides, penams, phenicols, tetracyclines, penems, fluoroquinolones
mexB	Inner membrane transporter	Component of the MexAB-OprM efflux pumps	RND family efflux pump	
mexH	Membrane fusion protein	Component of the MexGHI-OmpM efflux pump	RND family efflux pump	Fluoroquinolones, tetracyclines
mexI	Inner membrane transporter	Component of the MexGHI-OmpM efflux pump	RND family efflux pump	

Table 8 (continued)

Detected gene	Gene product	Function	Resistance mechanisms	Antibiotics removed
mexT	LysR-type transcriptional activator	Positive regulation of MexEF-OprN, OprD, MexS pump genes expression	RND family efflux pump	Fluoroquinolones, phenicols
mexX	Membrane fusion protein	Component of the MexXY-OmpM efflux pump	RND family efflux pump	Aminoglycosides, fluoroquinolones,phenicols, macrolides, carbapenems, tetracyclines, penams, cephalosporins, cephamycins
mexE	Membrane fusion protein	Component of the MexEF-OprN efflux pumps	RND family Efflux pump	Fluoroquinolones, phenicols
oprM	Outer membrane protein	Component of RND efflux pumps	RND family Efflux pump	Aminoglycosides, peptide antibiotics, sulfonamides, cephalosporins,cephamycins monobactams, carbapenems, macrolides, penams, phenicols, tetracyclines, penems, fluoroquinolones
oprN	Outer membrane protein	Component of the MexEF-OprN pump	RND family efflux pump	Phenicols, fluoroquinolones

the addition of glutathione to fosfomycin, thus resulting in antibiotic inactivation. In the tested genomes, the *fosA* gene was detected in *Pseudomonas* spp. AM4, AM8 and AM14 strains (Table 7). These results were in accordance with antibiotic resistance profile, as these three strains demonstrated a much narrower inhibition zone around the antibiotic disk (< 22 mm) compared to the other two strains (> 40 mm) (Supp. Table 3).

Finally, the GNAT-family gene was detected in tested genomes The gene encodes GCN5-related N-acetyltransferase (GNAT) responsible for acetylation and inactivation of aminoglycoside antibiotics (Burckhardt and Escalante-Semerena 2019).

Two genes responsible for antibiotic target alteration, *gyrA* and *gyrB*, were detected in all tested strains. Antibiotic target alteration is driven by single point mutations in *gyrA* and *gyrB*, which reduce the affinity between an antibiotic and its target. This is a very common mechanism of fluoroquinolone resistance detected in Gram-negative bacteria. Mutations in quinolone-resistance determining regions, such as *gyrA* or *gyrB* in DNA gyrase, are chromosomally encoded. Research indicates that high-level resistance to fluoroquinolones requires mutations in at least two genes with quinolone-resistance determining regions (Zhang et al. 2015). In the present study, all tested genomes contained single point mutations in the *gyrA* and *gyrB* genes. Our phenotypic antibiotic susceptibility test found the inhibition zone around fluoroquinolones (CIP, LEV) to be narrower for all strains (< 30 mm) than the susceptibility zone defined by EUCAST (> 50 mm) (Supp. Tab. S3).

The tested *Pseudomonas* genomes were found to encode numerous efflux pump components and regulatory protein genes. They included all genes of three efflux pumps, viz. MacAB-TolC, AcrAB-TolC and MexAB-OprM. Moreover, genes encoding components of other efflux pumps were detected: *mexE* and *oprN* of the MexEF-OprN pump, *mexX* and *oprM* of the MexXY-OprM pump, *mexH* and *mexI* of the MexGHI-OpmM pump.

The redox-sensitive protein SoxR is a global regulator of various efflux pump genes. It belongs to the MerR-family transcriptional regulators and it is common among both Gram-negative and Gram-positive bacteria. In enteric bacteria, SoxR mediates resistance to oxidative stress caused by nitric oxide or superoxide, and induces the expression of the *soxS* gene. SoxS activates the transcription of more than 100 genes encoding products that repair cellular damage. In nonenteric bacteria like *Pseudomonas* spp.,

SoxR directly activates the transcription of several multidrug efflux pump genes known to confer resistance to antibiotics (Park et al. 2006).

Discussion

Since the beginning of the XXI century, endophytes inhabiting metallophytic plants have gained increasing attention (El-Deeb et al. 2006; Idris et al. 2004; Stepanauskas et al. 2005; Ma et al. 2015); indeed, by the end of 2022, about 25 metallophytic plants had been tested for bacterial endophytes (Alves et al. 2022; Goryluk-Salmonowicz and Popowska 2019). All tested plants were found to harbor such endophytes, and all bacteria were resistant to high metal concentrations (He et al. 2013; Idris et al. 2004; Ma et al. 2015). Therefore, it was proposed that all metallophytic plants harbor endophytes resistant to metals. Even so, the presence and origin of ARGs in the bacteria, and their antibiotic resistance mechanisms, remain unclear. Seeing that endophytes isolated from hyperaccumulators have recently been used as beneficial biofertilizers, it is important to monitor the presence of ARGs in the genomes to prevent uncontrolled spread of ARGs in the environment.

Several studies have found endophytes inhabiting metallophytes to demonstrate antibiotic resistance. In 2005, *Pseudomonas fluorescens* and *Microbacterium* sp. endophytes isolated from *Brassica napus* (Stepanauskas et al. 2005) were found to be resistant to the antibiotics ampicilin, kanamycin and spectinomycin. In 2006, *Enterobacter* sp. endophytes resistant to ampicilin, kanamycin and tetracycline were isolated from *Eichhornia crassipes* (El-Deeb et al. 2006), while in 2015, a *Stenotrophomonas* sp. strain inhabiting *Sedum plumbizincicola* resistant to ampicilin, kanamycin and chloramphenicol was detected (Ma et al. 2015). While all these bacteria were found to be resistant to lead, zinc and cadmium, their antibiotic resistance genes were not researched.

In the present study, *Pseudomonas* spp. endophytes were isolated from the green parts of the hyperaccumulator plant *Armeria maritima*. The bacteria were resistant to ß-lactam antibiotics, fosfomycin, streptomycin and toxic metals, and demonstrated genes conferring possible resistance to arsenic, cadmium, chromium, cobalt, copper, lead, tellurium and zinc (Tables 5 and 6). Additionally, genes responsible for antibiotic inactivation, antibiotic target alteration, and genes coding efflux pumps were identified (Tables 7 and 8).

Bacteria are known to employ various mechanisms to provide metal resistance (Bruins et al. 2000; Ji and Silver 1995; Niño-Martínez et al. 2019), some of which were observed in the present study; for instance, some strains were found to harbor genes of the CzcCBA system, which are believed to confer resistance to cadmium, cobalt and zinc. Genes of the CzcCBA system can be detected in other *Pseudomonas* spp. endophytic genomes (Supp. Tab. S6). Previously, these genes were reported in the *P. poae* A2-S9 genome isolated from *Panicum virgatum* plant, the *Pseudomonas* sp.382 genome isolated from *Paullinia cupana* seeds (Xia et al. 2013, 2019; de Siqueira et al. 2018; Liotti et al. 2018) and the *P. putida* GM4FR genome isolated from the green parts of *Festuca rubra* L. (Wemheuer et al. 2016, 2017) (Supp. Tab. S6).

Interestingly, it has been proposed that the CzcRS two-component regulatory system may also be responsible for carbapenem antibiotic resistance (Wang et al. 2017). In the presence of Zn(II) ions, CzcS autophosphorylates and transmits a signal to the response regulator CzcR. CzcR up-regulates the expression of the CzcCBA efflux pump and represses the expression of the OprD porin responsible for the entry of carbapenem antibiotics (Perron et al. 2004). This is an example of a co-regulation system that act as a co-selection mechanism between the metal and antibiotic resistance mechanisms (Baker-Austin et al. 2006; Goryluk-Salmonowicz and Popowska 2019). It is possible that a co-regulation system also operates in the endophytes tested in our present study, as these were found to be resistant to both zinc ions and carbapenem antibiotics (Table 3).

Further, possible chromium resistance genes were detected, one of which codes for ChrA, a membrane potential dependent transporter. The gene has previously been detected in a variety of bacterial genera, including *Arthrobacter* spp., *Bacillus* spp., *Lysinibacillus* spp. and *Pseudomonas* spp. (He et al. 2010, 2011; Henne et al. 2009; Mondaca et al. 1998). The *ChrA* gene has also been found in environmental *Pseudomonas* genomes, like *P. chlororaphis* GP72 isolated from green pepper rhizosphere, *P. viridiflava* CDRTc14 isolated from the roots of *Lepidium draba* and *P. fluorescens* UM270 isolated from the roots of

Medicago truncatula (Supp. Tab. S6) (Hernández-León et al. 2015; Samad et al. 2016; Liu et al. 2006; Shen et al. 2012). Interestingly, the *chrA* gene is commonly detected on plasmid or Tn DNA. In 1990, it was detected on the *Pseudomonas aeruginosa* plasmid pUM505, and on the *Alcaligenes eutrophus* pMOL28 (Cervantes et al. 1990; Nies et al. 1990). The ChrA protein was also found to be encoded by a gene detected on transposon Tn*OtChr* from *Ochrobactrum tritici* (Branco et al. 2008). Similarly, genes encoding CzcCBA components can be located on mobile elements; these genes have been detected on the plasmid pMOL30 of *C. metallidurans* (Nies et al. 1990).

Finally, we identified genes associated with three copper resistance mechanisms (CusCFBA efflux system, P-type ATPases and multicopper oxidase CueO) and one zinc/cadmium/lead transporter (P-type ATPase CadA).Arsenic (ArsAB efflux pump), chromium (ChrA transporter) and tellurium (TehAB transporter) metalloid resistance genes were detected in all tested bacterial genomes (Table 5). These genes were also detected in other genomes of environmental *Pseudomonas* strains (Supp. Tab. S6).

The broad spectrum of metal tolerance demonstrated by the isolated endophytes makes them interesting candidates as bioremediation enhancing agents. However, this raises the important question of whether the presence of metal resistance genes promotes antibiotic resistance. Numerous studies have confirmed that metal-resistant environmental bacteria isolated from soil and water environments harbor antibiotic resistance genes (Barker-Reid et al. 2010; Cycoń et al. 2019; Forsberg et al. 2014; Su et al. 2020). There is a growing concern that metal contamination acts as selective agent in the spread of antibiotic resistance, especially when the metal resistance genes are located on mobile elements (Gorylu-Salmonowicz and Popowska 2019, 2022; Seiler and Berendonk 2012; Zhang et al. 2020; Baker-Austin et al. 2006; Perron et al. 2004). Therefore, the present study examined the locations of any potential resistance genes in the genomes of the tested bacteria.

The *Pseudomonas* spp. genomes were searched for genes encoding components of mobile genetic elements (MGE). Interestingly, numerous transposase genes were detected in the tested genomes. Transposases are needed for efficient transposition of insertion sequences (IS) or transposon DNA (Tn) (Beuzón et al. 2004). Transposases encoding genes of different Insertion Sequence (IS) families were detected, including IS5, IS66, IS110, IS200like, IS630, InsE, InsO and ISL3. However, it is unlikely that potential ARGs detected in the sequenced *Pseudomonas* genomes are a part of their mobilome, as the detected ARGs are commonly located on the chromosomes of other *Pseudomonas* spp. (CARD database). Further research is needed to confirm the presence of the detected ARGs on bacterial chromosomes. If so, the isolated endophytes can be used in laboratory experiments to evaluate their potential to promote plant growth and increase metal pollution remediation capacity. An interesting question is whether all detected potential metal resistance genes encode functional metal resistance proteins.

Conclusion

The present study demonstrated that *Armeria maritima* subsp. *halleri* (Wallr.) Rothm. is inhabited by *Pseudomonas* endophytes that are resistant to metals and antibiotics. Genome analysis confirmed the presence of genes conferring resistance to arsenic, cadmium, chromium, cobalt, copper, lead, tellurium and zinc. Genes encoding components of efflux pumps, extracellular sequestration proteins, P-type ATPases or metal detoxification proteins were detected. Moreover, the bacteria were resistant to antibiotics: streptomycin, fosfomycin and ß-lactams. Genes that may confer resistance to macrolides (MacAB-TolC efflux pump) and multidrug efflux pumps genes (AcrAB-TolC and MexAB-OprM) were identified, as well as some genes that may promote antibiotic inactivation and antibiotic target alteration.

The spread of antibiotic resistance genes (ARGs) in the environment is a global problem, and their main reservoir is considered to be soil. The bacteria inhabiting soil and plants are recipients of ARGs and hence form part of their transmission routes. Therefore, while the bacterial endophytes inhabiting hyperaccumulators may be beneficial for the host plant, they can also hasten the rise of antibiotic resistance.

In the available literature, little attention has been given to the problem of antibiotic resistance in bacteria used to promote plant growth in agriculture, and the resistance profiles of endophytes used in phytoremediation have not been addressed at al.

Noteworthily, such biological control agents and biofertilizers form important parts of new agricultural management systems. Such growth in the number of biopreparations available on the market, and the consequent large-scale and long-term usage of biopreparations containing ARGs may further increase the dissemination of antibiotic resistance.

Acknowledgements We are grateful to Professor Mieczysław Błaszczyk for his assistance in the fieldwork. This work was supported by the National Science Centre and the national Ministry of Education and Science.

Author contribution A.G-S. – Conceptualization, Investigation, Formal Analysis, Writing, Funding acquisition, Original Draft, Visualization; AW.M. – Investigation; M.P. – Writing, Review & Editing, Supervision, Funding Acquisition, Project Administration.

All authors read and approved the final manuscript.

Funding This work was supported by the National Science Centre (NCN), Poland (UMO-2019/32/Z/NZ8/0011), international project in the frame of the Biodiversity and its influence on human, animal and plant health (BiodivERsA Call 2018): project "ANTIVERSA – Biodiversity as an ecological barrier for the spread of clinically relevant antibiotic resistance in the environment" to MP and partially in the frame of the 'Excellence Initiative-Research University (2020–2026)' Program at the University of Warsaw. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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