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Research Article

The *cadCA* and *cadB/DX* operons are possibly induced in cadmium resistance mechanism by *Frankia alni* ACN14a

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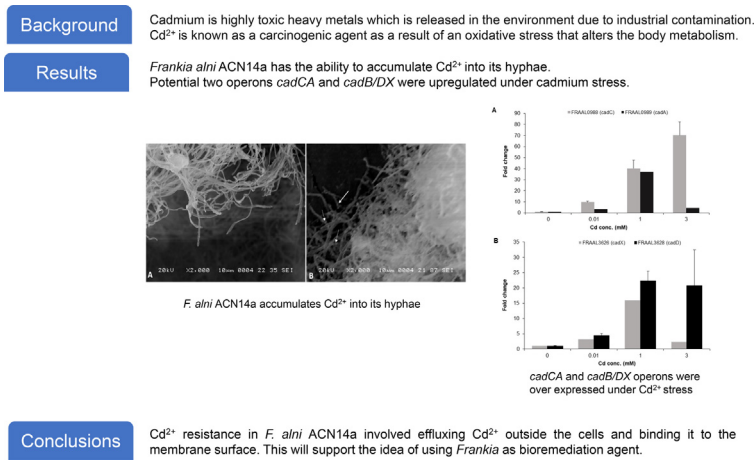
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Cadmium (Cd^{2+}) is one of the highly toxic heavy metals and is considered as a carcinogenic agent. Our aim was to confirm the ability of *Frankia alni* ACN14a to resist Cd^{2+} and to determine the genes involved in the resistance mechanism.
Results: *F. alni* ACN14a and *Frankia casuarinae* CcI3 hyphae showed up to 10 and 22 times Cd^{2+} accumulation when exposed to 1 mM Cd^{2+}, respectively. Scanning electron microscopy (SEM) exhibited a stable Cd^{2+} precipitate on the cell surface, and the increase in Cd^{2+} weight level reached 16.45% when evaluated with SEM-EDAX analysis. The following two potential Cd^{2+} operons were identified: 1. *cadCA* operon, which encodes a copper-transporting P-type ATPase A (*cadA*, FRAAL0989) and an ArsR family regulator (*cadC*, FRAAL0988), with 37- and 70-fold increase in their expression by qRT-PCR, respectively and 2. *cadB/DX,* which encodes a putative cobalt-zinc-cadmium resistance protein (*cadD*, FRAAL3628) and heavy metal-associated domain protein (*cadX*, FRAAL3626), with 22- and 16-fold upregulation when exposed to Cd^{2+} stress.

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Conclusions: Cd^{2+} tolerance by *F. alni* ACN14a involved efflux of Cd^{2+} outside the cells and binding it to the membrane surface. Our results indicate the existence of two cadmium-resistance mechanisms in *Frankia* strains, which support the idea of using them as a bioremediation agent.

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1. Introduction

Cadmium is used industrially in chlorine-alkali batteries, pigment in paints, engraving, cadmium vapor lamps, metal alloys, electroplating, fossil fuels such as coal and oil, fertilizers, and zinc refining [1].

Cadmium is considered as one of the highly toxic heavy metals with an atomic weight of 112.41 g/mol. Cadmium is released in the environment due to industrial contamination and is known as a carcinogenic agent [2,3]. It has toxic effects on higher organisms, causing oxidative stress that alters the body metabolism through free radical formation and inhibition of glutathione peroxidase formation, leading to reduced defense against lipid peroxidation. At high concentrations (> 10 mg per kg body weight), kidney and cardiac tissue damage can occur, while long-term exposure to Cd^{2+} may cause bone defects, including osteoporosis [4]. In microorganisms, the toxic effects of Cd^{2+} cause the disruption of protein function through binding to sulfhydryl groups in enzymes and binding to DNA leading to single-strand breaks [5,6].

Two well-known cadmium resistance mechanisms have been described in *Staphylococcus aureus* [7]. The *cadCA* operon located on plasmid pI258 (3.5 kbps) consists of two genes (*cadA* and *cadC*). The *cadA* gene encodes a 727-amino-acid protein with similarity to P-type ATPases that can protect cells from Cd^{2+} accumulation by functioning as an energy-dependent efflux ATPase across cell membranes. The *cadC* gene, encoding a protein of 122 aa, is the transcription regulator of the *cadA* gene [8,9,10,11,12]. This resistance mechanism works via efflux of Cd^{2+} outside the cell with ATPase activity (*cadA*). This protein contains six predicted membrane-spanning regions. The fourth membrane span contains a conserved Cys-Pro-Cys tripeptide and is thought to be involved in the cation translocation pathway [13]. CadC is a regulatory protein encoded downstream of *cadA,* and it is associated with resistance to Cd^{2+} in *Staphylococcus aureus* plasmid pI258. CadC is a member of the ArsR/SmtB regulator family protein with repressor activity that can bind to the promoter operator area in the *cadA-cadC* system [9,14].

The second resistance mechanism is the *cadB* system that differs significantly from the *cadCA* system. The *cadB/DX* system of cadmium resistance contains two genes (*cadB/D* and *cadX*). It has been suggested that resistance to Cd^{+2} is not based on a cation efflux but may offer protection to the cells by binding cadmium to the exterior of the cell membrane. In Staphylococcus lugdunensis, plasmid pLUG10 contains *cadB* and *cadX* genes that confer high-level resistance to cadmium. The *cadX* gene (a positive regulator of resistance) is similar to the *cadC* gene in S. aureus with 40% similarity [11]. Crupper et al. [15] reported that cadmium resistance in plasmid pRW001 from *Staphylococcus aureus* involved the *cadD* gene. This gene (*cadD*) showed a high degree of sequence similarity to *cadB*- like operon in *S. lugdunensis*.

Another cadmium resistance mechanism includes metal detoxification and homeostasis, which is mediated by metallothionein-encoding genes. Metallothioneins are cysteine-rich proteins with low molecular weight that can be synthesized in the presence of heavy metal stress in both prokaryotes and eukaryotes. The bacterial metallothionein system designated Smt in *Synechococcus* spp. consists of two genes (*smtA* and *smtB*), and it confers resistance to zinc and cadmium [16].

The Actinomycetales Gram-positive bacteria *Frankia* shows a pattern of resistance and degradation ability to diverse environmental pollutants. Frankia can resist a wide range of heavy metals and metalloids [17,18]. *Frankia inefficax* EuI1c showed copper resistance by five potential cop genes: copA, copZ, copC, copCD, and copD. These genes exhibited increase in mRNA levels reached 25-fold [19,20]. *Frankia sp.* EAN1pec showed lead resistance, and two metal transporters (a Cu^{2+}-ATPase and cation diffusion facilitator) were upregulated under lead stress. Otherwise, precipitation from lead was observed, and the expression of polyphosphate kinase, undecaprenyl diphosphate synthase, and inorganic diphosphatase genes increased significantly under stress [21]. *Frankia* strains CeSi1, CgIT3 L2, CgIS3 N2, CgIS1 N1, CgIT7N2, and G5 exhibited cobalt, cadmium, and zinc resistance [20,22]. *Frankia inefficax* EuI1c exhibited selenite detoxification and reduction of colorless sodium selenite ($Na_2SeO_3^{2-}$) to the nontoxic and red colored elemental selenium, with activity reaching 86.5 μg/ml from selenite after 8 d [23]. Furthermore, *Frankia* can degrade many aromatic toxic compounds such as atrazine. *Frankia alni* ACN14a and *Frankia inefficax* EuI1c degraded atrazine via dechlorination and dealkylation through trzN (FRAAL1474 and FraEuI1c_5874), atzB (FRAAL1473 and FraEuI1c_5875), atzR (FRAAL1471), AtzD/TrzD (FraEuI1c_3137), and AtzE (FraEuI1c_3136) genes [24,25]. Furthermore, *Frankia* sp. strain QA3 showed growth on naphthalene as a sole carbon source, and an operon for aromatic compound degradation in addition to ring-hydroxylating dioxygenases was upregulated under naphthalene stress [26]. Some *Frankia* strains show salt tolerance under salt-stressed conditions and can be used as bio-fertilizers in hypersaline biotopes [27,28]. Our research goal was to confirm the ability of *Frankia* to resist cadmium stress and to identify the cadmium resistance mechanism in *F. alni* ACN14a.

2. Materials and Methods

2.1. Frankia strains and growth conditions

Frankia alni ACN14a (NCBI Ref Seq: NC_008278 - DSM 45986 - cluster 1a) [29], *Frankia inefficax* EuI1c (NCBI Ref Seq: NC_014666 - DSM 45817 - cluster IV) [30], and *Frankia casuarinae* CcI3 (NCBI Ref Seq: NC_007777 - DSM 45818 - cluster 1c) [31], were grown and maintained in MPN medium containing MOPS (20 mM), K_2HPO_4 (10 mM), and NH_4Cl (5.0 mM) with pH adjusted to 6.8. MPN growth medium was supplemented with trace metal mixture containing ($MnCl_2.2H_2O$ (5.0), $CuCl_2.2H_2O$ (0.25), H_3BO_3 (0.5), $CaCl_2$, $2H_2O$ (10.0), $ZnSO_4.7H_2O$ (1.0), $CoCl_2.2H_2O$ (0.2) (g/l)) in addition to $Na_2MoO_4.2H_2O$ (1.0 mM), $MgSO_4$ (2.0 mM), and $FeCl_3$ (20 μM) dissolved in nitrilotriacetic acid (100 μM). Additionally, an appropriate carbon source was used (propionate and succinate (5 mM each) for *F. alni* ACN14a; glucose (20 mM) for *F. inefficax* EuI1c; and propionate only (5 mM) for *F. casuarinae* CcI3 at 30°C

as incubation temperature [32]. For cadmium (Cd^{2+}) assays, cadmium sulfate ($CdSO_4$) at the concentration of 0; 0.01; 0.1; 0.5; 1; 3; 5; and 10 mM was added.

2.2. Total cellular protein assay

The three tested *Frankia* strains were grown in MPN in a 24-well plate system, with selected Cd^{2+} concentrations (0; 0.01; 0.1; 0.5; 1; 3; 5; and 10 mM) for two weeks. The mycelium was collected in Eppendorf tubes, centrifuged at 10,000 rpm for 5 min at room temperature, and the total cellular protein content was measured by Roti®-Nanoquant protein quantification assay (CARL ROTH company, K880) with Bradford quantification method [33]. The protein yield was calculated by subtracting the initial inoculums from the final protein content.

2.3. Cadmium accumulation measurement

Frankia cells were grown for two weeks under cadmium exposure (0, 0.01, 0.1, and 1 mM). Cells were collected by centrifugation and washed with 0.01 M HCl to remove nonspecific surface-bound metals, suspended in distilled water (1 ml), and lyophilized by freeze drying (FreeZone 4.5, Labconco, USA). The metal content of the culture was measured after acid dissolution by atomic absorption spectrophotometry (AA-6200, Shimadzu, Japan) at the Soil Analysis Lab in Qassim University, SA, according to the method of Chapman and Pratt as modified by Reitemeier [34]. Data represent the average of 3 independent biological replicates.

2.4. Scanning electron microscope (SEM) - energy dispersive X-ray spectroscopy (EDX)

For SEM experiment, *F. alni* ACN14a was grown in MPN for two weeks in the presence of 1 mM cadmium. Cultured cells were harvested via centrifugation at 10,000 x g for 10 min, and the collected samples were subjected to critical point drying [35]. Cells were washed twice with phosphate buffered saline (pH 7.4) and fixed in modified Karnovsky's fixative mixture (2.5% w/v glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 and 2% wt/vol paraformaldehyde) by incubation for 4h at room temperature [35] Fixed samples were washed twice more with phosphate buffered saline and distilled water. The washed fixed samples were dehydrated via critical point drying by transferring samples through an alcohol concentrations series (30%, 50%, 70%, 90%, and 100% vol/vol). Finally, cells were covered with t-butanol for freeze drying, coated with titanium, and viewed under SEM (AMRAY 3300FE, Heritage Global Partners, Inc.). For SEM-EDX, areas from SEM sections were selected for elemental composition analysis using an EDX microanalysis system [36].

2.5. Bioinformatic analysis

The amino acid sequences (FASTA format) of selected *Frankia* genomes (*F. alni* ACN14a (NCBI Ref Seq: NC_008278). *F. inefficax* EuI1c (NCBI Ref Seq: NC_014666) and *F. casuarinae* CcI3 (NCBI Ref Seq: NC_007777)) were searched on the Integrated Microbial Genomes and Microbiomes (IMG/M) database https://img.jgi.doe.gov/ [37]. The deduced amino acid sequences of several known cadmium resistance genes and motifs [9,13,38,39,40] were used for a Blastp search of *Frankia* genomes at IMG/M [41] with an E-value of 1e-5 and 500 as a number of hits. For phylogenetic tree construction, CadA, CadC, CadD, and CadX amino acid sequences from *Frankia* strains ACN14a, EuI1c, CcI3, Dg1, and EAN1pec were aligned with homologous protein sequences recovered from the GenBank database by using ClustalW in MEGA11 software [42]. The following parameters were applied in tree construction: Maximum Likelihood, Jones-Taylor-Thornton (JTT) Model, and Nearest-Neighbor-Interchange (NNI) with 1000 bootstrap replicates [42].

2.6. RNA extraction and cDNA synthesis

All solutions and materials were RNase, DNase, and Protease free or treated with DEPC to prevent RNA degradation. For RNA experiments, *F. alni* ACN14a was grown under Cd^{2+}-stress (0, 0.01, 1, and 3 mM) for 6 d. The RNA was extracted by the Triton X-100 method [43]. After RNA extraction, DNA contamination was removed by treatment with DNase I (PureLink DNase, Invitrogen™) according to the manufacturer's instructions. The extracted and purified RNA was converted to cDNA using random hexamer primers, 300 ng RNA, and QuantiTect® Reverse Transcription kit (Qiagen, Germany) according to the manufacturer's instructions. The generated cDNA was diluted to 10 ng/µl in RNase- free water and stored at −20°C for qRT-PCR analysis. RNA and cDNA concentrations in samples were measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.7. Gene expression experiments

Analysis of *Frankia* gene expression was performed by qRT-PCR using SYBR Green PCR Master Mix (QuantiTect SYBR® Green PCR Kits, Qiagen, Germany) and specific primers (Table S1) according to the manufacturer's instructions. Each reaction (25 µL) involved 50 ng template Cd^{2+}-cDNA, 300 nM from forward and reverse primers (Table S1), and SYBR Green PCR Master Mix. Parameters for Applied Biosystems™ 7500 Real-Time PCR Systems were as follows: (1) initial heat activation at 95°C for 15 min, (2) 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The Comparative Threshold-cycle (Ct) method was selected to quantify gene expression by calculating the $2^{-\Delta\Delta CT}$ [44] as a fold change. Two separate biological samples with three reactions each were performed. The results were normalized with *rpsA,* a housekeeping gene, and calibrated with untreated samples.

2.8. Statistical analysis

Total cellular protein data and gene expression levels were presented as mean ± standard error of mean by using Student's-t test. SE = Standard Error; when the difference between two means was $\geq$ SE, a significant difference was considered.

3. Results

3.1. Data mining and bioinformatics analysis

Data mining, amino acid sequences, and motifs of Cd^{2+} resistance genes from literatures [9,14,39,40,45] were collected. These obtained sequences were used to scan published *Frankia* genomes (especially *F. alni* ACN14a, *F. casuarinae* CcI3, and *F. inefficax* EuI1c) at the protein level for possible genes involved in Cd^{+2} resistance. Four genes with two different potential resistance mechanisms were identified in selected *Frankia* genomes. The first resistance mechanism *cadCA* includes copper-transporting P-type ATPase A (FRAAL0989; Francci3_0490; and FraEuI1c_6307 - proposed *cadA*), which acts as an energy-dependent efflux ATPase through the cell membranes, and an ArsR family regulator (FRAAL0988; Francci3_0489; and FraEuI1c_6308 - predicted *cadC)* that works as transcriptional regulator for *cadA*. The second resistance mechanism system containing two genes (*cadD* and *cadX*) was also identified. A putative cobalt-zinc-cadmium resistance protein (FRAAL3628; Francci3_2598; and FraEuI1c_4783 - proposed *cadD*), which may

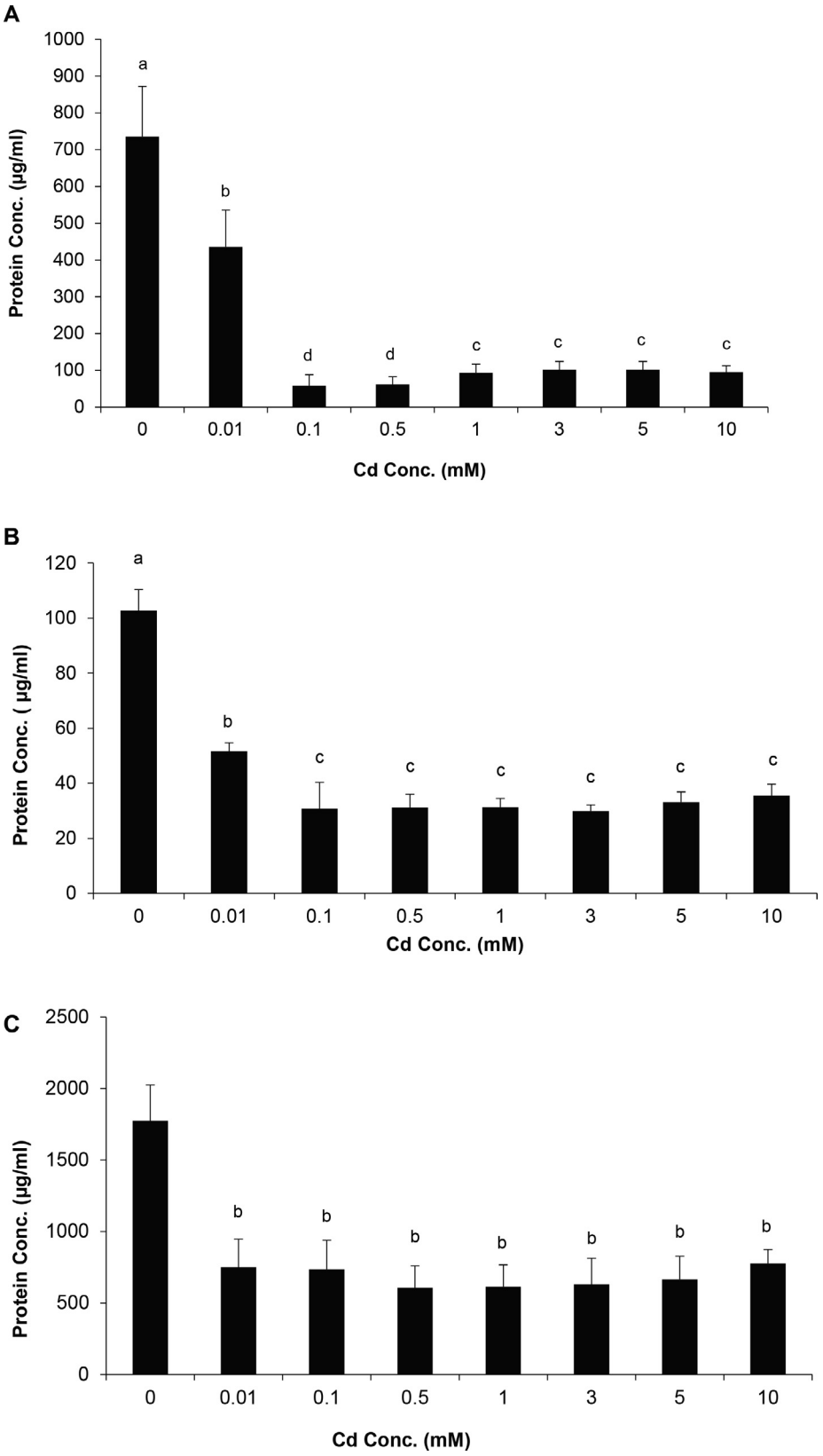

Fig. 1. Total cellular protein of strain ACN14a grown for two weeks by the 24-well plates system (1 ml per well). *Frankia* strains ACN14a, Ccl3, and Eul1c were grown in the presence of different concentrations from Cd^{+2} (0.01, 0.1, 0.5, 1, 3, 5, 10 mM) in comparison to nontreated cells; cells were harvested, and total protein contents was measured as described in Methods. Each value represents the mean of three biological replicates. Columns have their SD values at the top, and letters indicating the significant differences between treatments.

Table 1
Cadmium accumulation by three selected *Frankia* strains (ACN14a, CcI3, and EuI1c) measured by atomic absorption spectrophotometer after acid wash with 0.01 N HCl.

$CdSO_4$ Concentration in mM	Cd^{2+} accumulation (mg/g dry weight of cells)		
	Strain ACN14a	Strain CcI3	Strain EuI1c
0	0.73 ± N.D.	0.50 ± 0.03	1.14 ± N.D.
0.01	4.45 ± 0.46	4.54 ± 0.10	1.53 ± 0.41
0.1	42.65 ± 0.10	100.83 ± 0.67	23.98 ± 0.07
1	9.37 ± 0.15	19.77 ± 0.30	13.48 ± 0.17

N.D. not determined.

protect cells by binding with Cd^{2+} outside the cell surface, and heavy metal-associated domain protein (FRAAL3626; Francci3_2599; and FraEuI1c_4785 – *cadX*) as a putative regulator. The deduced amino acid sequence of the *cadA* gene from gram-positive *Listeria monocytogenes* showed 34%, 32%, and 28% identities and 55%, 53%, and 48% positives with the proposed *cadA* gene in *F. alni* ACN14a, *F. casuarinae* CcI3, and *F. inefficax* EuI1c, respectively. Otherwise, *cadD in Rhodobacter* sp. SW2 gene shared 42%, 40%, and 35% identities and 57%, 55%, and 53% similarity with *cadD* in *F. alni* ACN14a, *F. casuarinae* CcI3, and *F. inefficax* EuI1c, respectively.

3.2. Frankia growth under Cd^{2+} stress

In a 24-well plate system, *Frankia* strains were exposed to different Cd^{+2} stress concentrations (0; 0.01; 0.1; 0.5; 1; 3; 5; 10 mM, Fig. S1). The 3 tested *Frankia* strains showed the same growth pattern under Cd^{+2} exposure (Figs. 1, S1A, S1B, S2, and S3). Growth was markedly inhibited at low Cd^{+2} concentration (0.1 and/or 0.5 mM) but yield increased with higher Cd^{2+} exposure (1 to 10 mM, Fig. 1**)** in the three tested strains. This increase in growth yield was significant in strain ACN14a when comparing growth yield under 0.1 and 10 mM from Cd^{2+} (Fig. 1A). Otherwise, no significant increase in growth yield was observed in strains CcI3 and EuI1c when exposed to high concentrations from Cd^{+2} (Fig. 1B and C). The MIC and MTC for the three tested strains were > 10 and < 0.01 mM from Cd^{2+}, respectively. Previous studies on copper and lead resistance in *Frankia* had shown the same trend in growth pattern with a decrease in the beginning and an increase in protein content under higher concentrations [19,21], which may suggest two different genetic systems are involved in heavy metals resistance. The first mechanism will work with low concentrations from the heavy metal (Cd^{2+}), while the second resistance system will work when cells are exposed to high levels of the metal.

3.3. Cd^{+2} accumulation by Frankia

Cd^{+2} addition to the 3 tested *Frankia* strains lead to Cd^{+2} accumulation and reached a maximum with 0.1 mM. Upon exposure to Cd^{+2} (0.1 mM), 10 to 22 times more Cd^{+2} remained bound when compared with 0.01 mM in *F. alni* ACN14a and *F. casuarinae* CcI3, respectively (Table 1). The accumulation trend was decreased with a higher concentration of Cd^{+2} solution (1 mM) in all tested strains. These results suggest that Cd^{2+} resistance mechanism may be similar in all tested *Frankia* strains.

3.4. SEM–EDX and morphological changes in Frankia cells under Cd^{2+} stress

SEM photomicrographs of *F. alni* ACN14a grown with $CdSO_4$ (1 mM) yielded a precipitate associated to hyphae (Fig. 2). These results are similar to those observed in *Frankia* strain EAN1pec when grown under Pb^{+2}-stress [21]. SEM-EDX was used to determine the elemental composition of these precipitates (Fig. 3). An elevated cadmium content was detected with *Frankia* hyphae with intensity level reaching 0.85% and 16.45% increase in Cd^{2+} weight against no detection in the control. A 3-fold increase in phosphate weight was also detected in 1 mM Cd^{2+}-treated cells more than controls (4.19% and 1.45%, respectively).

3.5. Molecular phylogenetic tree

The constructed phylogenetic trees of the *cad* genes from selected *Frankia* genomes and other bacteria obtained from GenBank are presented in Figs. 4, 5, S4, and S5. Phylogenetic analysis revealed that the *cadD* gene (FRAAL3628) from *F. alni* ACN14a, *F. casuarinae* CcI3, and EAN1pec were all grouped together in one node, whereas the *cadA* gene from *F. symbiont of Datisca* Dg1 and strain EuI1c were separated in another subgroup, with nearest to *Corynebacterium variabile*. It's clear that the *cadA* gene in the four *Frankia* clades locate in the same subgroup, while the *cadD* gene is located in two different subgroups, which indicates slight evolution. By screening all available *Frankia* genomes located at IMG/M (54 genomes), almost all genomes had the two Cd^{2+} resistance mechanism in their genomes.

3.6. Gene expression of detected Cd+2 resistance genes

F. alni ACN14a grown under Cd^{+2} -stress for 6 d, exhibited dose-dependent gene expression changes for the two-cadmium resistance systems. *cadC* (FRAAL0988) gene showed a 70-fold change

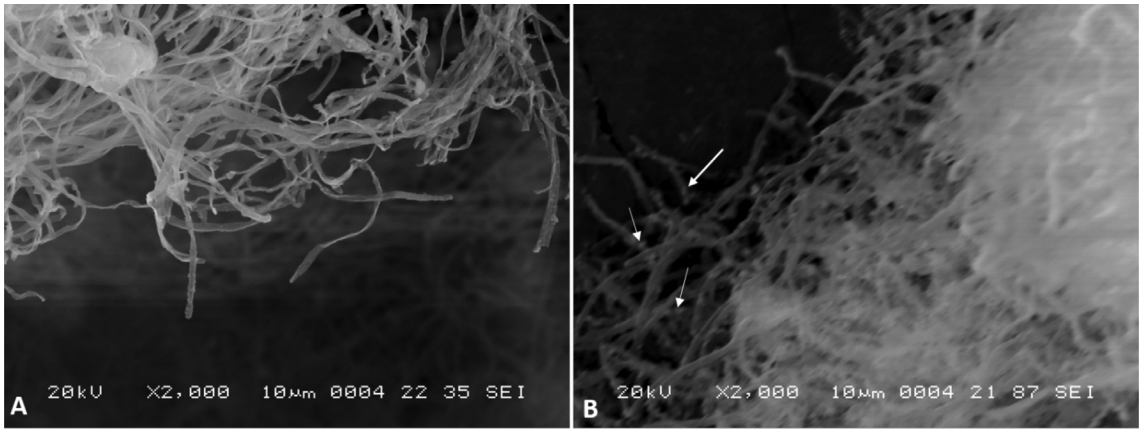

Fig. 2. SEM of *Frankia* strain ACN14a grown under Cd^{2+}-stress. Strain ACN14a was grown for 15 d in MPN medium with or without 1 mM from $CdSO_4$ as described in **Materials and Methods section**. (A) Control condition, (B) 1 mM $CdSO_4$.

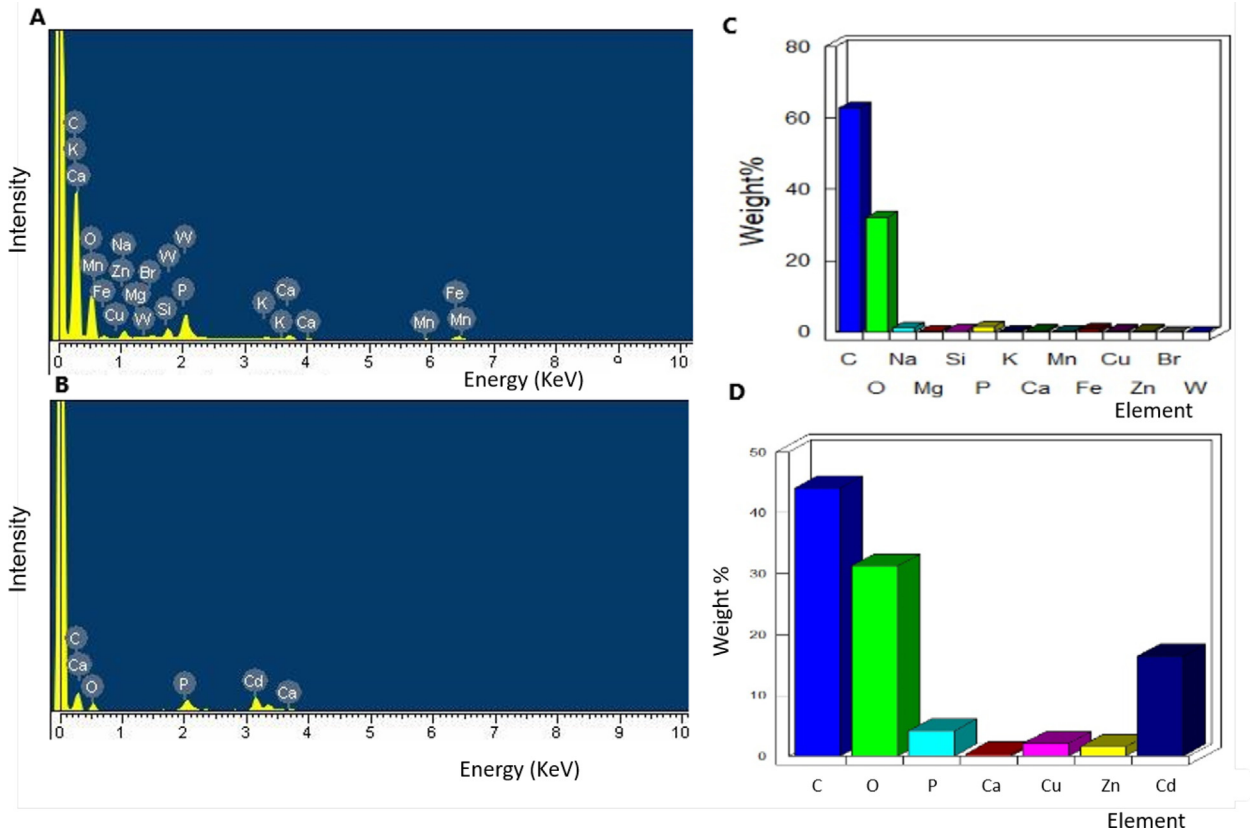

Fig. 3. SEM-EDX of *Frankia* strain ACN14a grown under Cd^{+2}- stress. *Frankia* strain ACN14a was grown for two weeks in the basal growth medium in the presence or absence of 1 mM CdSO4, as mentioned in the Materials and Methods section. Panels (A and C) EDX spectra and corresponding element analysis for control cells. Panels (B and D) represent the same data from Cd^{+2}-exposed cells. Cd weight and intensity were detected in Cd^{2+}-stressed cells as compared to nontreated cells with increase reaching 16.45% and 0.85, respectively.

increase in mRNA levels with increase Cd^{2+} exposure (3 mM), while *cadA* gene (FRAAL0989) upregulated and showed an mRNA 37-fold change increase when exposed to 1 mM Cd^{+2} (Fig. 6A).

The expression of the second proposed the Cd^{+2}-resistance operon (*cadB/DX*) in *F. alni* ACN14a grown under Cd^{+2}-stress was also measured by qRT-PCR (Fig. 6B). The *cadX* (FRAAL3626) gene exhibited a 16-fold increase in expression with 1 mM Cd^{+2}, but the expression decreased with higher Cd^{+2} exposure (3 mM). The level of *cadD* (FRAAL3628) mRNA peaked at 1 mM in stressed cells (22-fold) and remained relatively constant upon high exposure to Cd^{+2}-stress. These results indicate that the two different resistance mechanisms (*cadCA* and *cadB/DX*) genes were all upregulated by Cd^{+2} exposure, and *cadC* gene showed more highly expression than the other *cad* genes.

4. Discussion

Frankia can resist a diverse array of heavy metals and metalloids in addition to their capability to degrade different types from organic compounds and herbicides [17,19,21,23,24,25,27]. Growth pattern of microorganisms under heavy metals are affected significantly even in the presence of low concentrations. *Frankia* growth under copper and cadmium showed decrease in total cellular protein content with low metal concentrations (0.1 mM) and a slight increase under higher concentrations (1 to 10 mM). This decrease and increase may reflect the presence of two different resistance mechanisms in their genomes. Qin et al. [46] reported the minimum inhibitory concentration (MIC) in *E. coli* is 8 mM from cadmium, while Yu et al. [9] isolated two *Bacillus vietnamensis,* which could grow in the presence of 0.3–0.8 mM from Cd^{2+}. Following lead exposure, the *Frankia* growth in general increased for up to 2 mM, while with selenite stress, growth decreased gradually with a higher concentration [21,23].

4.1. Cd^{2+} binding to cell surface

F. alni ACN14a was shown to adsorb significant amounts of Cd^{2+} from the surrounding environment and precipitate it on the cell surface (Fig. 2, Table 1). This finding may indicate the presence of specific metal-binding proteins that strongly adsorb metal upon the cell surface. *Frankia* strains ACN14a and CcI3 showed Cd^{2+} accumulation reached 42.65 and 100.83 mg/g dry weight of cells, respectively. Copper accumulation in *F. inefficax* EuI1c was increased up to 5 times when cells were exposed to 1 mM in comparison to 0.25 mM from Cu^{2+} [19]. Otherwise, *Frankia* sp. strain EAN1pec showed saturation binding with 1256 ± 171 mg/ g dry weight of cells [21]. Moreover, *Bacillus infantis* and *Pseudomonas fluorescens* accumulated about 90 and 81 µg/mL of cadmium after 24h [47]. A novel strain of *Micrococcus* showed a significant absorption of cadmium reached 38% [48]. Yu et al. [49] isolated three *Burkholderia* sp. strains (ha-1, hj-2, and ho-3) with high Cd^{2+} removal rate (81.78, 79.37, and 63.05%), whereas Khan et al. [50] isolate Salmonella enterica 43C with Cd^{2+} removal efficiency reached 57%.

The EDX results under following exposure to 1 mM (Fig. 3) showed an increase in Cd^{2+} contents. Furthermore, the phosphate concentration was 3-fold higher in treated cells than non Cd^{2+}-stressed cells. Functional groups such as phosphate, amine, hydroxyl, and carboxyl facilitate Cd^{2+} binding to the bacterial cell surface such as chemisorption [51]. Similar SEM-EDAX patterns were

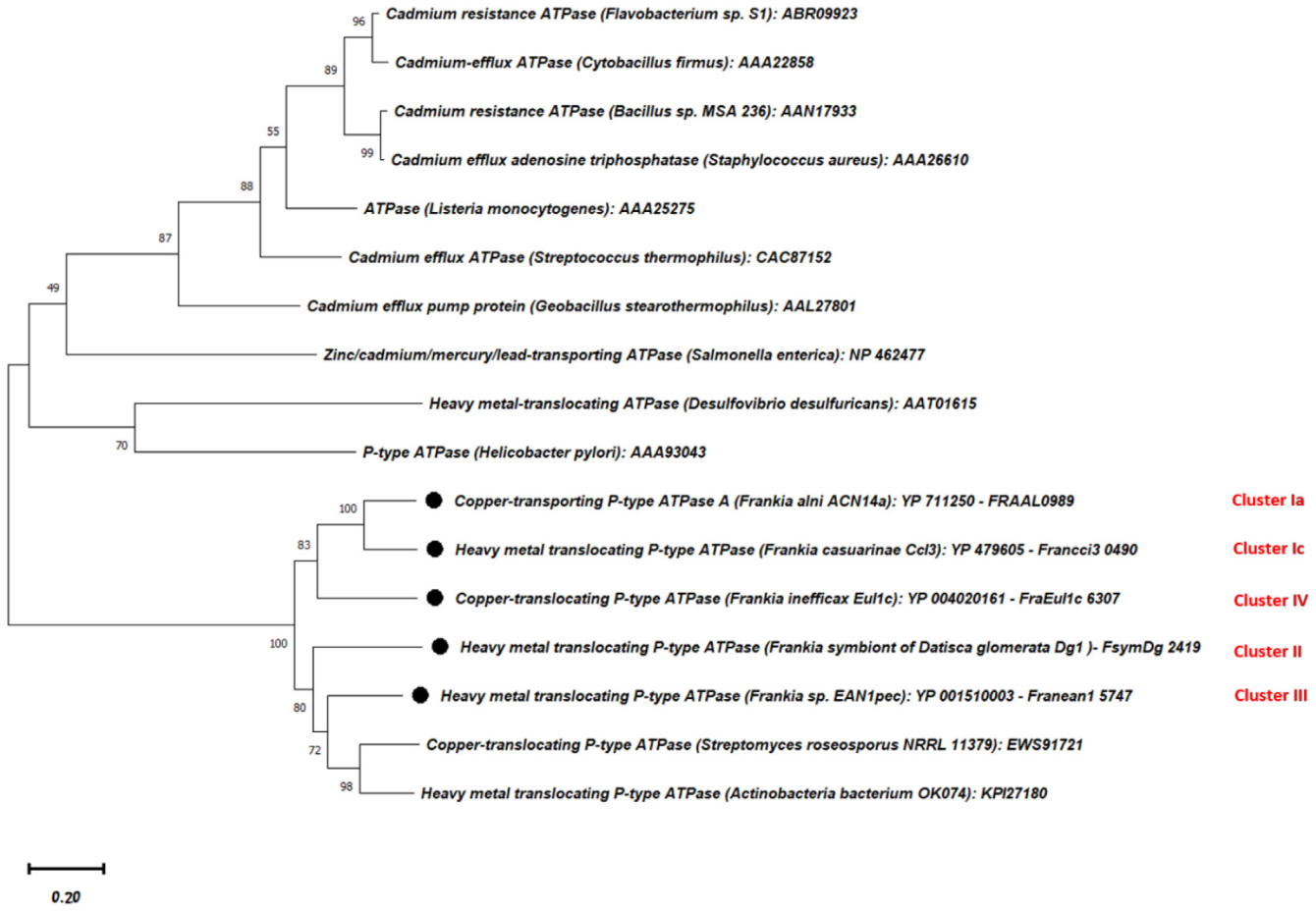

Fig. 4. Constructed phylogenetic tree of the proposed *cadA* gene sequences from five selected *Frankia* genomes plus seventeen protein sequences from other bacteria. The dendrogram was generated by Maximum Likelihood, Jones-Taylor-Thornton (JTT) Model, and Nearest-Neighbor-Interchange (NNI) with 1000 bootstrap replicates (Methods). The accession numbers of previously *cadA* published sequences are given.

observed in *F. inefficax* EuI1c with copper and strain EAN1pec with lead [19,21]. In *Pseudomonas aeruginosa* JP-11, EDX data showed an increase in Cd^{+2} accumulation with 11.64% bound to their cell wall [4]. These data suggest that the formed cadmium phosphate was bound to the cell surface.

4.2. Cd^{2+} export and precipitation mechanisms

In *F. alni* ACN14a, two different resistance mechanisms were identified. The first mechanism involves the *cadCA* operon (FRAAL0988 and FRAAL0989), which encodes ArsR family regulator and copper-transporting P-type ATPase A.

The second proposed Cd^{2+}-resistance operon (*cadB/DX*) in *F. alni* ACN14a comprises FRAAL3628 and FRAAL3626 that encode a putative cobalt-zinc-cadmium resistance protein and putative regulator, respectively. The putative protein has a transmembrane helix domain with Cation efflux ability. It probably functions as a cation sequestration mechanism by enabling cadmium to bind on their cell surface membranes [52]. In the SEM-EDAX experiment, increase in Cd^{+2} and phosphate concentrations could suggest that a cadmium phosphate compound formed and became bound to the *Frankia* cell surface. Similar results were observed in *Frankia* resistant to copper, with the probability of copper phosphate formation [19]. Increasing the energy-independent Cd^{+2} binding between the two strains from *Staphylococcus aureus* (sensitive strain 6538P and $cadB^{+}$ resistant strain AW16) reflects that this resistance is due to an inducible Cd^{2+}-binding factor [53]. Furthermore, the combination of multiple cad regulator and transporter genes contributes to the full resistance of cadmium in *Staphylococcus spp*., via including three major operons *cadCA*, *cadXD,* and *cadCB* in addition to four rare operons (*cadXB*-like operon. *CadB* and *CadD* represent the lower level of cadmium resistance when compared to *CadA,* which exhibited high-level resistance of Cd^{+2} in *staphylococci* [11,15].

Of the available sequenced 54 *Frankia* genomes and by *in silico* analysis, almost all screened genomes carried the four resistance Cd^{2+}-resistance determinants. *Frankia* genomes have both Cd^{2+} resistance systems (*CadCA* and *CadB/DX*) in their genomes, which indicates that heavy metal resistance is widespread in soils, especially in *Frankia* that are frequently seen in pioneer or anthropized sites that contain high levels of metals such as mine spoils [39], coal combustion ashes, [54] or pig manure-amended plots [55].

The deduced amino acids sequences of *cadCA* from *L. monocytogenes* showed similarity to cad operon in plasmid p1258 in *S. aureus* [56]. About 11 isolates from *Staphylococcus* species in addition to bacteria belonging to *Micrococcus* and *Halobacillus* were found to harbor the *cadA* gene. A cluster containing orf4111, orf4112, and orf4113 from *Bacillus vietnamensis* 151–6 was found to encode an ATPase transporter, a cadmium efflux system accessory protein, and a cadmium resistance protein, respectively [9].

The topology of the two genes indicates that there have been losses and reacquisitions from related actinobacteria. Such gains and losses that have been seen in *Klebsiella* [57] and *Mycobacterium*

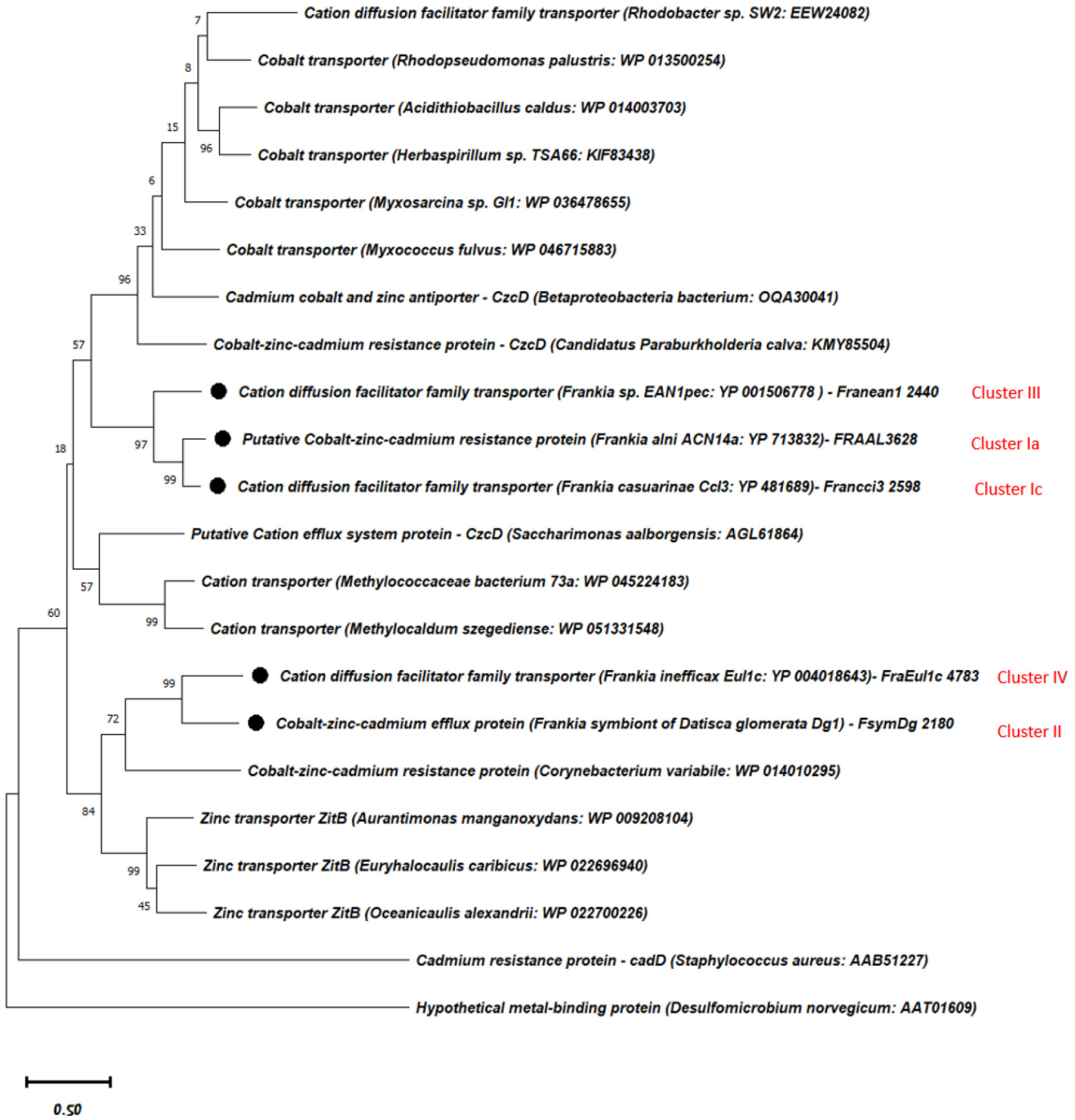

Fig. 5. Molecular phylogenetic analysis of *cadD* gene sequences from five selected *Frankia* genomes plus seventeen protein sequences from other bacteria. The dendrogram was generated by Maximum Likelihood, Jones-Taylor-Thornton (JTT) Model, and Nearest-Neighbor-Interchange (NNI) with 1000 bootstrap replicates (Methods). The accession numbers of previously *cadD* published sequences are given.

[58] are evocative of fluctuations in the selection pressure on these determinants.

4.3. Relative cad genes expression

A significant change in *CadA, C, D, and X* expression under Cd^{2+} stress was observed in *F. alni* ACN14a. The increase in expression reached 70-fold in *cadC* (FRAAL0988). This high expression under 3 mM can be explained through the repressor function of this regulator protein that control the expression of *CadA* gene. With high Cd^{2+} stress, *CadC* will be overexpressed to reduce the *CadA* expression, which is an energy-dependent efflux ATPase. Otherwise, *cadX* can also work as a transcriptional repressor; in case of its low expression, the *CadD* gene will be overexpressed to let the bacterial cells gain the resistance with high Cd^{2+} exposure. It could be concluded that the *CadCA* system is responsible for Cd^{2+} resistance under low concentrations, whereas *CadB/DX* will gain bacteria with resistance at high concentrations (*CadD* over expressed under 3 mM Cd^{2+}). The periplasmic sequestering (CopA) and RND type heavy metal efflux *czcA* genes in *Pseudomonas aeruginosa* strain J007 were upregulated under low concentrations and short time exposure to Zn, Cu, and Cd, based on expression analysis by

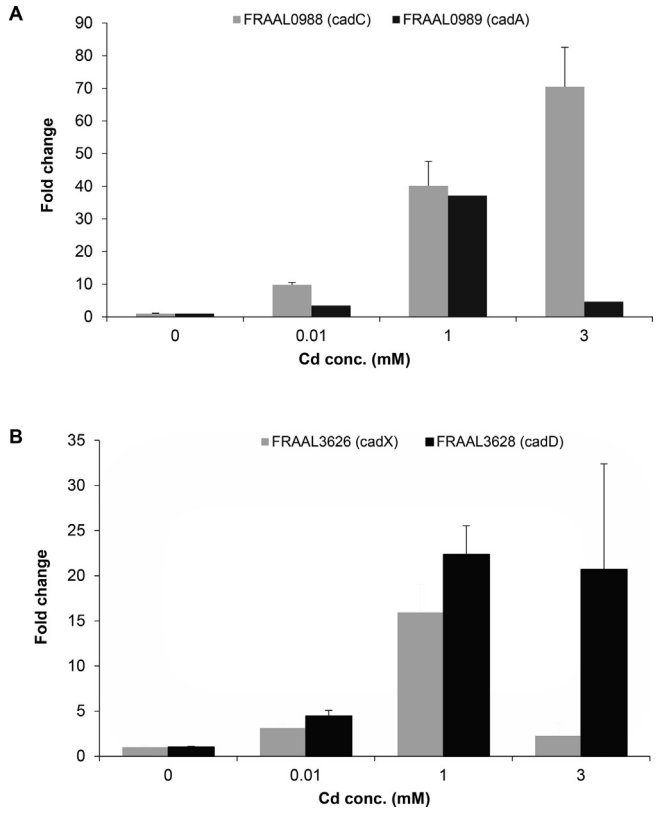

Fig. 6. Transcriptional changes of selected *Frankia* genes (FRAAL0988, FRAAL0989, FRAAL3626, and FRAAL3628) in response to Cd^{+2} stress. Values represent fold changes in mRNA levels of *Frankia* strain ACN14a exposed to Cd^{+2} (0.01, 1, and 3 mM) compared to nontreated cells. The level of gene expression was normalized using the housekeeping gene (*rpsA*). Error bars represent SD of three replicates (n = 3).

real-time PCR [59]. In *Enterobacter cloacae* strain EC01, the DUF326-like domain (a cysteine-rich protein) was over-expressed up to 220 fold when studied by proteomic analysis and had a 14-fold induction in its transcription level with evaluation by qRT-PCR [60].

5. Conclusions

Our *in silico* analysis revealed that two cadmium resistance mechanisms exist in the Gram-positive actinobacterium *Frankia*. The first resistance mechanism is CadB/DX, which is encoded by *cadB/DX*, and it is a putative periplasmic cobalt-zinc-cadmium resistance protein and its transcription regulator factor. The resistance mechanism implies the binding of Cd^{2+} to the cell surface as shown by Cd^{2+} accumulation measured by atomic absorption spectrophotometer and SEM-EDX analysis. The second mechanism is coded by the *cadCA* operon that produces an ArsR family regulator and an efflux P-type ATPase from the cell. The two resistance mechanisms genes exhibited a dose response in their expression in cells challenged with Cd^{2+}. Our results support the existence of two cadmium-resistance mechanisms that function through efflux of Cd^{2+} and binding it on the external cell-surface membrane. Furthermore, future studies such as knockout to produce strains deficient in these proposed cad genes should be conducted to demonstrate the direct function of Cd^{2+} resistance genes.

Author contribution

- Study conception and design: M Rehan, A Alhusays
- Data collection: M Rehan, A Alhusays
- Analysis and interpretation of results: M Rehan, H Boubakri, P Pujic, P Normand
- Draft manuscript preparation: M Rehan, AM Serag, P Normand
- Revision of the results and approved the final version of the manuscript: M Rehan, AM Serag, H Boubakri, P Pujic, P Normand

Conflict of interest

The authors declare that they have no conflicts of interest.

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Supplementary material

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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