Molecular characterization of cadmium-resistant *Cupriavidus* spp. and *Ralstonia solanacearum* isolated from soil and plants in Taiwan

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Abstract*—Cadmium is a natural heavy-metal element. It is highly toxic and a vital industrial pollutant to the environment. In order to survive in heavy-metal polluted environments, some bacteria can withstand high concentrations of heavy metals in environment due to their specific mechanisms, including the transport of heavy metal ions out of the cell. Three kinds of membrane-bound-proteins are known to participate in this transport. The objective of this study was to determine the cadmium-resistant properties and possible mechanism among strains of Cupriavidus metallidurans (= Ralstonia eutropha), C. taiwanensis (=R. taiwanensis) and Ralstonia solanacearum isolated from soil and plants in Taiwan. Strains tested include six strains, rcd 6, 8, 19 (C. metallidurans) and rcd 12, 14, 21 (C. taiwanensis), from cadmium-polluted soil, seventeen strains, nod 1 to nod 5 and nod s1 to s12 (C. taiwanensis) from root nodules of two Mimosa species (M. pudica and M. diplotricha), and ten strains of R. solanacearum isolated from different diseased plants. Sequence analysis of 16S-23S rDNA ITS and nifH gene regions were used to confirm the taxonomic classification of these bacteria. However, the 624-bp PCR product of nifH gene was only amplified from strains nod 1 to nod 3 and nod s1 to s12, but not from other tested strains of Cupriavidus and Ralstonia spp. These tested strains could tolerate cadmium within the range from 0.67 to 6.70 mM. The gene, czcC, thought to encode the outer membrane factor (OMF) of czc efflux system, was found in fifteen tested strains (rcd 6, 8, 12, 14, 19, 21; nod s1 to s6, s9, s11; PSS161) by PCR. Especially, strains rcd 12, 14, and 21 which could tolerate higher cadmium concentration (6.70 mM) harbored the entire czc operon. In addition, the PCR products revealed that cadmium-tolerant strains contained at least a portion of the czc operon and efflux mechanism was confirmed by cadmium uptake test. The results here indicated that cadmium resistant capability in Cupriavidus spp. and R. solanacearum was related to the presence of czcC or czc operon.*

***Keywords*—cadmium-resistant genes (czc operon), Cupriavidus spp., efflux system, Ralstonia solanacearum.**

I. INTRODUCTION

Heavy metals most commonly found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni). Soils may become contaminated by the accumulation of heavy metals through emissions from the rapidly expanding industrial areas, disposal of high metal wastes, and sewage sludge (Khan et al., 2008). Some bacteria use heavy metals for respiration, and some have evolved mechanisms to detoxify them. Microbes have evolved several mechanisms to tolerate the presence of heavy metals (by either efflux, complexation, or reduction of metal ions) or to use them as terminal electron acceptors in anaerobic respiration. Under normal conditions, essential and non-essential metals are transported by nonspecific entry systems. However, when metal ions are in excess, specific ion efflux protein complexes may be synthesized to aid in the elimination of non-essential metals. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals also in cleaning up or remediating metal-contaminated environments (Nies and Silver, 1995; Spain and Alm, 2003).

The sources of cadmium pollution include industries, such as those producing television screens, lasers, paints, cosmetics, batteries, and zinc refining. It is widely distributed in humans, cigarette smoke, welding, and contaminated food and beverages is also the major source of cadmium contamination (Bernhoft, 2013). Cadmium is highly toxic to animals, plants, microorganisms, and humans even at quite low concentrations (Belimov et al., 2005). It has been widely accepted in the model bacterium *Cupriavidus metallidurans* (=*Ralstonia eutropha*, *R. metallidurans*) that these metal resistance mechanisms appear to be cooperative, not metal specific, and are controlled by a complex regulatory network involving several clusters of genes and functions (Maynaud et al., 2013). Cadmium-resistant bacteria, *Cupriavidus metallidurans* and *C. taiwanensis* (=*R. taiwanensis*) were isolated from cadmium-polluted and tainted soil by a waterink factory in Yunlin, Taiwan. According to cadmium tolerance these bacteria were separated into two groups: lower than 400 mg/kg and ranges from 650 to 900 mg/kg,

respectively. These cadmium-resistant strains use *czc* operon, which is located on plasmid or chromosome, to release cadmium off the organism (Chen et al., 2005).

Ralstonia solanacearum is a devastating, soil-borne plant pathogen with a global distribution and an unusually wide host range. The complete genome sequence of *R. solanacearum* strain GMI1000 has been recently determined and annotated. It is organized in two replicons: a 3.7 Mb chromosome and a 2.1 Mb megaplasmid. The megaplasmid appears to encode numerous genes that might play a role in the overall fitness of the bacterium or that may provide advantages in diverse environments (for example: flagellum biosynthesis, many essential pathogenicity genes, catabolism of aromatic compounds, copper and cobalt–zinc–cadmium resistance gene clusters (Salanoubat et al., 2002). The objectives of this study were to examine the cadmium tolerance of *Cupriavidus* species (*C. metallidurans* and *C. taiwanensis*) and *Ralstonia solanacearum* collected from soil and plants in Taiwan and to determine the possible mechanism of theses bacteria resistance to cadmium.

II. MATERIAL AND METHOD

2.1 Bacterial strains

Three strains of *Cupriavidus metallidurans* (= *Ralstonia eutropha*; rcd 6, 8, and 19) and 3 strains of *C. taiwanensis* (= *R. taiwanensis*; rcd 12, 14, and 21) isolated from cadmium-polluted soil in Yunlin, Taiwan (Chen et al., 2005) and maintained in our laboratory. A total of 17 strains of *C. taiwanensis* (nod 1, 2, 3, 4, 5, s1, s2, s3, s4, s5, s6, s7, s8, s9, s10, s11, and s12) isolated from root nodules of sensitive plant (*Mimosa pudica* L.) and giant sensitive plant (*Mimosa diplotricha* C. Wright ex Sauvalle) in Chiayi, Taiwan. Ten tested *Ralstonia solanacearum* strains isolated from different host plants and locations in Taiwan, PSS36 (peanut, Taichung), PSS161 (strawberry, Taichung), PSS189 (bitter guard, Taichung), PSS225 (hot pepper, Taichung), PSS253 (potato, Kaohsiung), PS99 (eggplant, Changhua), GB03 (ginger, Nantou), Ps-Au-11 (anthunrium, Taichung), PsL-11 (loofah, Touliu), and CLWl579 (rice flat sedge, Hsinchu), were provided through the courtesy of The World Vegetable Center, Shanhua, Tainan, Taiwan.

All tested strains were grown on nutrient broth (0.3% beef extract and 0.5% peptone) for 24 hr, then 35% glycerol are added for the preservation of bacteria at -80°C. Partial 16S rDNA gene sequence analysis following polymerase chain reaction (PCR) with a primer pair, fD1/rP1, was used for preliminary identification of tested strains (Weusburg et al., 1991). Identity of 16S rDNA sequences between tested strains in this study and published data from NCBI GenBank was conducted (Table 1). Part of the *nifH* gene, encoding dinitrogenase reductase, a key enzyme in nitrogen fixation, *nifH* gene sequence analysis following polymerase chain reaction (PCR) with a primer pair, nifH3/nifH4 (Table 2), was used for preliminary identification of *C. taiwanensis* (=*R. taiwanensis*) strains (Chen et al., 2003). The representative soil samples were collected from root zone of sensitive plant and giant sensitive plant in Chiayi, Taiwan. Soil properties analysis was conducted by the Soil Survey and Testing Center, National Chung Hsing University, Taichung, Taiwan.

TABLE 1
IDENTITY OF 16S RDNA SEQUENCES BETWEEN TESTED STRAINS IN THIS STUDY AND PUBLISHED DATA FROM NCBI GENBANK

Strains	Sampling location	Bacterium (GenBank accession number)	Identity (%)
rcd 6, 8, 19[a]	Yunlin, Taiwan	*Cupriavidus* sp. KU-26 (AB266608)	98-100
rcd 12, 14, 21[b]	Yunlin, Taiwan	*Ralstonia taiwanensis* strain MS1 (AY303977) *Ralstonia taiwanensis* strain LMG 19425 (AF300325)	98-99
nod 1 to nod 5[b]	Chiayi, Taiwan	*Ralstonia taiwanensis* strain MS1 (AY303977) *Ralstonia taiwanensis* strain LMG 19425 (AF300325)	99
nod s1 to nod s12[b]	Chiayi, Taiwan	*Cupriavidus taiwanensis* strain PAS15 (AY752959) *Ralstonia taiwanensis* strain LMG 19424 (AF300324)	99
PSS36, Ps-Au-11[c]	Taichung, Taiwan	*Ralstonia solanacearum* strain LMG 17138 (EF016364)	99

[a] Cupriavidus metallidurans; [b] Cupriavidus taiwanensis; [c] Ralstonia solanacearum

2.2 Determination of cadmium tolerance and antibiotic resistance

For testing the tolerance to cadmium ion, bacterial suspension (A_{600} = 0.8-0.9) of tested bacterial strains was spread on nutrient agar plates containing 0.5 to 10 mM of cadmium chloride ($CdCl_2$), and incubated at 30°C for 3 to 6 days. In order to understand the relationship between cadmium-tolerance and drug resistance, the antibiotic resistance of the tested strains was also determined. A loop of tested bacterial colony was inoculated to nutrient broth contained ampicillin (Amp, 50 μg/ml; Sigma, USA), chloramphenicol (Chl, 20 μg/ml; Sigma, USA), kanamycin (Kmi, 30 μg/ml; Sigma, USA), nalidixic acid (Nal,

15 µg/ml; Sigma, USA), streptomycin (Str, 30 µg/ml; Sigma, USA), or tetracycline (Tet, 12 µg/ml; Sigma, USA) at 30°C for 72 hr (Ausubel et al., 2002).

TABLE 2
PRIMERS USED IN PCR ANALYSIS

Primers	Sequence (5'-3')	Position	Expected size (bp)	Reference
fD1	AGAGTTTGATCCTGGCTCAG	7-26		
rP1	ACGGTTACCTTGTTACGACTT	1505-1485	1499	Weusburg *et al.*, 1991
nifH3	ATCGGCAAGTCGACTACCTC	2-21		
nifH4	TTCTGCATGCTGGACTACGTT	625-605	624	This study
phsC1	AATACTATCTGGGGAGCGGAA	1-21		
phsC2	TCAGACGGCGGACTTATCC	762-744	762	Chen et al., 2005
Smt A1	AAGCATTCTTGGGCATGACA	1-20		
Smt A2	TTGATTCAGGTATGGTGGGTG	492-472	492	Chen et al., 2005
czcNf	CTTGCTAGGCATTCTCGGACTAGG	312-335		
czcNr	ATGGAACAGATCAAACGACTCCAC	612-589	301	This study
czcIf	GTTCTGATCTTCGTGCTGCTCATT	13-36		
czcIr	GGTCACTTCTACCCGATTCGCTAT	270-247	258	This study
CzcC1	ATGCGAAGACTATTTCTGCCG	32-52		
CzcC2	TTAACGTCCCAGAATGCGAT	1285-1266	1254	Chen et al., 2005
CzcB1	CAAACAAAAGGCTGCCATTG	15-34		
CzcB2	GTGTTCGGCGCTGGATTT	1554-1537	1540	Chen et al., 2005
CzcA1	AACCAGATCTCGCGCGAGAAC	2458-2478		
CzcA2	CGGCAACACCAGTAGGGTCAG	3090-3070	633	Burnley, 2000
CzcD1	AGCCTGGCGTTGATCTCC	118-135		
CzcD2	CCAGATGTGGAGGTCATG	717-700	600	Burnley, 2000
czcRf	GCGGGTACTTGTTGTAGAAGACGA	3-26		
czcRr	CTTGGATCGAATGGACTTGATGAC	207-184	205	This study
czcSf	AAAGTCATCGCTCATGTTCCAGTC	357-380		
czcSr	CAATGTAAAGCGTGTCTTCCCATC	1350-1327	994	This study
czcEf	GCTTCGTATGCTTTGGAAATGACC	58-81		
czcEr	AAGGTCCACACTCGTATCCCTGAC	315-292	258	This study
cadAf	AGAGAACCTCCGGCTAAAGAAGTT	399-422		
cadAr	GGTCAAGCTTTGGAGATGAGAC	1437-1416	1039	This study
zntAf	GCAAGGGCTGGATCGCAG	779-796		
zntAr	CCACGCCATCGGTTTCGG	1742-1725	964	Legatzki *et al*., 2003

2.3 Assay of Cd^{2+} uptake and efflux

Four gram dry weight of mid-exponential phase bacterial strains, rcd 6 (*C. metallidurans*), rcd 12, nod 1, nod s1, nod s7 (*C. taiwanensis*), PSS161, PSS36, PSS189, PSS225, and Ps-Au-11 (*R. solanacearum*), were resuspended in 100 ml deionized water contained 53 ppm (0.47 mM) and 390 ppm (3.48 mM) of cadmium ion and incubated at 25°C for 0, 20, 60, 120, 180, 240, 300, 360 mins, respectively. The bacterial suspension was centrifuged at 13,000 rpm for 5 min at 25°C and the residual cadmium concentrations were measured by Fast Sequential Atomic Absorption Spectrometer (AA240FS, VARIAN, USA) at different incubation time (Chen et al., 2005). Experiments were repeated three times and the mean value was calculated.

2.4 Amplification of cadmium resistance related genes

In order to determine the possible cadmium tolerance mechanism of tested strains, PCR with the specific primer was used to detect the genes. The cadmium resistance related genes include czcA and other eight components of efflux pump, *czc* operon (Burnley, 2000), *znt*A and *cad*A (P-type ATPases) (Legatzki et al., 2003), metallothionein protein gene (*smt*A), thiosulfate reductase gene (*phs*ABC) (Bang et al., 2000; Bruins et al., 2000; Stoffels et al., 2012). Primers used for PCR are listed in Table 2. PCR mixture (25 µl) contained 0.12 µM of each primer, 0.1 mM dNTPS, 1.0 U *Taq* DNA Polmerase, PCR buffer (1.5 mM $MgCl_2$) supplied with the enzyme and 1 µl (10 µg) of template DNA. The total volume of the reaction mixture was maintained with sterilized double distilled water. PCR was performed in GeneAmp PCR system 2700 (Applied Biosystems, Inc., USA) and was carried out as follows: a single denaturation step at 94°C for 5 min followed by a 30-cycle program

which included denaturation at 94°C for 1 min, annealing at 60°C for 30 s (phsC1/phsC2, cadAf/cadAr, zntAf/zntAr), 56°C for 30 s (SmtA1/SmtA2), 67°C for 15 s (czcNf/czcNr, czcIf/czcIr, czcRf/czcRr, czcSf/czcSr, czcEf/czcEr), 50°C for 30 s (CzcC1/CzcC2), 56°C for 15 s (CzcB1/CzcB2), 63.8°C for 30s (CzcA1/CzcA2), 53°C for 15s (CzcD1/CzcD2) and extension 72°C for 1 min and a final extension at 72 °C for 10 min. The amplification products were electrophoresed on a 1.5% agarose gel buffer with 0.5 X TBE at 100 V for 25 min along with standard DNA (Gen 100-3000 DNA ladder, GeneMark Biotechnology). The amplified DNA fragments were stained with ethidium bromide and photographed with a Gene Genius Bioimaging System.

2.5 Cloning and sequencing of PCR products

PCR products were extracted with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and the purified DNAs were then sequenced by using an automated sequencer at the Minsheng Biotechnology Co. (Taipei, Taiwan). The sequence thus obtained was analyzed using BLASTn search (http://www.ncbi.nlm.nih.gov/Blast).

2.6 Southern hybridization

In order to confirm the existence of *czcA* and *czc*C gene, total DNA from bacterial strains, rcd 6, rcd 8, rcd 12, rcd 21, nod 1, nod s1, nod s2, nod s7, PSS36, PS99, PSS161 and PSS189 were digested with 2 µl *Bam*HI (2 U/µl; New England BioLabs, Beverly, MA, USA), digoxigenin (DIG)-labelled as probes as described by the manufacturer (Boehringer, Mannheim, Germany) and used in subsequent southern hybridization procedures (Sambrook et al., 1989).

III. RESULTS AND DISCUSSION

3.1 Comparison of 16S rRNA gene sequences of tested strains

Based on the results of amplification with primer pair, fD1/rP1, a 1500-bp band was generated. Following the sequencing of theses PCR products, 16S rDNA sequence alignment among 25 tested strains in this study and published data from NCBI were made using the BLASTn program. The data revealed that the levels of sequence identity among all of the tested strains and published data from NCBI were relatively high (identity 98-100%). The results suggested that strains rcd 6, 8, and 19 were *Cupriavidus* (=*Ralstonia*) spp.; strains rcd 12, 14, and 21 were *Cupriavidus taiwanensis* (=*Ralstonia taiwanensis*)-like organisms; strains nod 1 to nod 5 and nod s1 to nod s12 and 9 were similar to *Cupriavidus taiwanensis* (=*Ralstonia taiwanensis*); the remaining strains PSS36 and Ps-Au-11 were *Ralstonia solanacearum* (Table 1). Chen et al. (2005) reported strain rcd 6 appeared to be *Ralstonia eutropha* tested by BIOLOG bacterial identification system and 16s rRNA gene sequence comparison. Their results also suggested that strain rcd 8 was similar to *Cupriavidus metallidurans* (=*Ralstonia eutropha*) and strain rcd 19 was *Cupriavidus taiwanensis* (=*Ralstonia taiwanensis*)-like organism.

Nitrogen fixation is carried out by the nitrogenase enzyme encoded by the genes *nifH*, *nifD*, and *nifK*. Of the three, *nifH* (encoding the nitrogenase reductase subunit) is the most sequenced and has become the marker gene of nitrogen-fixing microorganisms. Thus, many PCR primers have been developed for *nifH* gene with the purpose of amplifying this gene sequence (Gaby and Buckley, 2012). In order to understand whether the test strain has a nitrogen fixation ability to confirm the identity of the strain, a specific primer pair, nifH3/nifH4, was designed according to the *R. taiwanensis nifH* gene sequence. PCR amplification results showed that only strains nod 1 to 3 and nod s1 to s12 isolated from root nodules of *Mimosa* spp. with the expected size of 624 bp (Fig. 1).

These *nifH* gene sequences have more than 95% identity compared with GenBank database using the BLASTn program of NCBI. However, no products were amplified from the strains nod 4, 5 (*C. taiwanensis*), rcd 6, 8, 19 (*C. metallidurans*), rcd 12, 14, 21 (*C. taiwanensis*) and all strains of *Ralstonia solanacearum*. The specific primer pair nifH3/nifH4 has detected 15 from 20 (75%) of *R. taiwanensis*-like strains. Gaby and Buckley (2012) found that there were 15 universal *nifH* primers that targeted 90% or more of nitrogen fixers, but that there were also 23 *nifH* primers that targeted less than 50% of *nifH* sequences.

3.2 Cadmium tolerance and antibiotic resistance

Cadmium-resistant organisms were isolated from cadmium-polluted soil in Yunlin, Taiwan. The concentration of cadmium in soil was 79.6 mg/kg. Whereas the concentration of cadmium in root zone soils of *Mimosa* spp. sampled from Chiayi, Taiwan, was 0.14 to 0.23 mg/kg (Table 3).

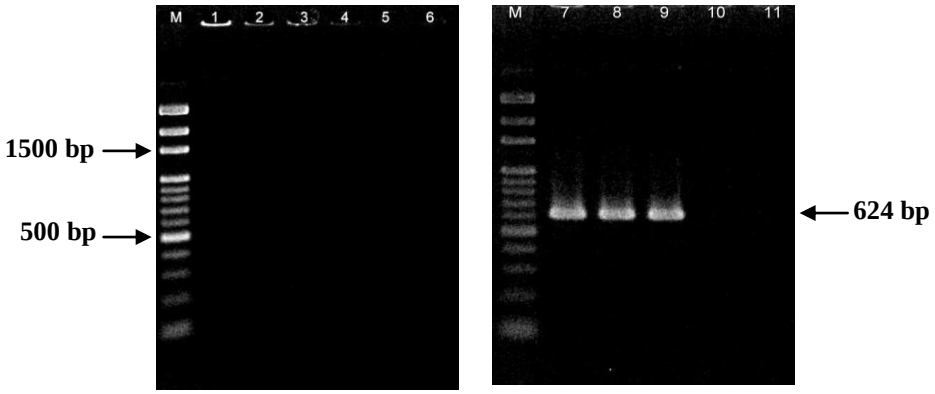

FIG. 1. PCR amplification of *nifH* gene from tested strains. M: 100 bp DNA Ladder Marker; Lanes 1-6: strains rcd 6, 8 (*Cupriavidus metallidurans*), rcd 12, 14 (*C. taiwanensis*), rcd 19 (*C. metallidurans*), rcd 21 (*C. taiwanensis*); Lanes 7-11: strains nod 1 to nod 5 (*C. taiwanensis*).

TABLE 3
CHARACTERISTICS OF TESTED SOIL

Character	Description		
	Huwei, Yunlin[1]	Holland Lake, Chiayi	Pa Cha River, Chiayi
Bacterial strains	rcd 6, 8, 12, 14, 19, 21	nod 1 to nod 5	nod s1 to s12
pH	7.5	5.3	5.3
Conductance (dS/m)	2.1	0.35	0.59
Water (%)	12	22	9.4
Texture	Loam	Silty clay loam	Sandy loam
Sand (%)	41.2	8	42
Silt (%)	44	61	43
Clay (%)	14.8	31	15
Organic matter (%)	1.58	3.3	0.4
Cadmium (mg/kg)	79.6	0.23	0.14

[1] ***Data from Chen et al. (2005).***

According to cadmium tolerance, these organism were then separated into three groups: (i) strains rcd 12, 14, 21 (*C. taiwanensis*) and PSS161 (*Ralstonia solanacearum*) higher than 5 mM; (ii) strains rcd 6, 8, and 19 (*C. taiwanensis*), nod s1-s6, s9 and s11 (*C. taiwanensis*), PS99, Ps-Au-11, CLWl579, PSS36 and PSS253 (*R. solanacearum*) ranges from 2.1 to 4.9 mM; and (iii) strains nod 1 to nod 5, nod s7, s8, s10, s12 (*C. taiwanensis*), PSS225, PSS189, GB03 and PsL-11c (*R. solanacearum*) lower than 2 mM, respectively (Table 4). Soil environment of sensitive plant and giant sensitive plant was not cadmium-contaminated and the isolated strains of *C. taiwanensis* have lower cadmium tolerance. The results were consistent with previous report that strains rcd 12, 14, 21 could be tolerant to cadmium ion at 900 mg/kg and strains rcd 6, 8, and 19 could reach 650 mg/kg (Chen et al., 2005).

TABLE 4
CADMIUM SUSCEPTIBILITY OF STRAINS OF *CUPRIAVIDUS* AND *RALSTONIA* SPP.

Strains	Cadmium concentration (mM)
rcd 12, 14, 21[b]	6.70
PSS161[c]	5.81
rcd 6, 8, 19[a]; nod s1 to s6, s9, s11[b]; PS99, Ps-Au-11, CLWl579[c]	3.13
PSS36, PSS253[c]	2.68
nod 1 to nod 5[b]; nod s7, s8, s10, s12[b]; PSS225[c]	1.34
PSS189, GB03, PsL-11[c]	0.67

[a] ***Cupriavidus metallidurans***; [b] ***Cupriavidus taiwanensis***; [c] ***Ralstonia solanacearum***

TABLE 5
ANTIBIOTIC SUSCEPTIBILITY TEST OF STRAINS OF *CUPRIAVIDUS* AND *RALSTONIA* SPP. BY BROTH METHOD

Strains	Antibiotic[1]					
	Amp	Chl	Kmi	Nal	Str	Tet
rcd 6[a]	+*	+	-	+	-	-
rcd 8[a]	+	+	-	+	-	-
rcd 19[a]	+	+	-	+	-	-
rcd 12[b]	-	+	-	-	-	-
rcd 14[b]	-	+	-	-	-	-
rcd 21[b]	-	+	-	-	-	-
nod 1[b]	+	+	-	+	-	-
nod 2[b]	+	+	-	+	-	-
nod 3[b]	+	+	-	+	-	-
nod 4[b]	+	+	-	+	-	-
nod 5[b]	+	+	-	+	-	-
nod s1[b]	+	+	-	+	+	-
nod s2[b]	+	+	-	+	+	-
nod s3[b]	+	+	-	+	+	-
nod s4[b]	+	+	-	+	+	-
nod s5[b]	+	+	-	-	+	-
nod s6[b]	+	+	-	+	+	-
nod s9[b]	+	+	-	+	+	-
nod s11[b]	+	+	-	+	+	-
nod s7[b]	+	+	-	-	+	-
nod s8[b]	+	+	-	-	+	-
nod s10[b]	+	+	-	+	+	-
nod s12[b]	+	+	-	+	+	-
PSS36[c]	-	-	-	-	-	-
PSS161[c]	+	+	-	+	-	-
PSS189[c]	+	+	+	-	+	-
PSS225[c]	-	-	-	-	-	-
PSS253[c]	-	-	-	-	-	-
PS99[c]	-	-	-	-	-	-
GB03[c]	+	+	+	-	+	-
Ps-Au-11[c]	+	+	-	-	-	-
PsL-11[c]	+	+	+	-	+	-
CLWl579[c]	-	-	-	-	-	-

****: +/-, resistance/susceptivity; [a] Cupriavidus metallidurans; [b] Cupriavidus taiwanensis; [c] Ralstonia solanacearum***
[1] Amp: ampicillin, 50 µg/ml; Chl: chloramphenicol, 20 µg/ml; Kmi: kanamycin, 30 µg/ml; Nal: nalidixic acid, 15 µg/ml; Str: streptomycin, 30 µg/ml; Tet: tetracycline, 12 µg/ml.

The strains rcd 12, 14 and 21 showed cadmium tolerance (6.70 mM) were isolated from cadmium contaminated soil. Strain PSS161 isolated from wilt strawberry stem caused by *Ralstonia solanacearum* had cadmium tolerance (5.81 mM). Strains rcd 12, 14 and 21 collected from cadmium contaminated soil were only resistant to chloramphenicol (Table 5). In addition to chloramphenicol, strains rcd 6, 8 and 19 were found to exhibit resistance to ampicillin and nalidixic acid. Whereas strains nod 1 to nod 5 had the same result as strains rcd 6, 8 and 19. Strains nod s1 to nod s12 had the same antibiotic-resistant results, in addition to strains nod s5, s7 and s8 showed sensitive to nalidixic acid. All strains of *Ralstonia solanacearum* had diverse results, strains PSS189, GB03 and PsL-11 were resistant to four antibiotics, and the remaining strains were resistant to only one of the tested antibiotics.

Among them, PSS36, PS99, PSS225, PSS253 and CLWl579 had no resistant ability to all tested antibiotics. In contrast to strains rcd 12, 14 and 21 from cadmium contaminated soil, the strains of *Ralstonia solanacearum* from fields at different

location had diverse results of antibiotics resistance. However, more informations, eg. soil characteristics and application history of bactericides, are required to confirm the relationship with these findings..

Together with other genes, *czcA* forms the *czc* determinant, which encodes a multi-protein complex associated with a high level resistance to cadmium, cobalt and zinc in bacteria (Nies, 2003). Moreover, this bacterium was resistant to antibiotics (Mowade and Bhattacharyya, 2000), which suggested the existence of multi-resistance mechanisms against drugs and/or the expression of efflux pumps (Pages et al., 2008).

Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment (Andersen et al., 2001; Webber and Piddock, 2003). However, the results of the antibiotic resistance analysis showed that cadmium-resistant strains were not positively correlated to the resistance to antibiotic. It was still needed further study to investigate the relevance between cadmium efflux mechanism and antibiotic efflux system of *Ralstonia* species.

3.3 Cadmium ion uptake and efflux

In the state of 53 ppm cadmium ion, the residual cadmium ion concentration decreased at 20-min incubation by rcd 6 (*C. metallidurans*), rcd 12 and nod s1 (*C. taiwanensis*). At that time, the cadmium ion uptake by tested strains were rcd 12 (2.7 mg/kg), rcd 6 (0.54 mg/kg) and nod s1 (0.53 mg/kg). After that time, the cadmium ion efflux by bacterial cells and the residual cadmium ion concentrations were raised again. On the other hand, the strains nod 1 and nod s7 had not showed a phenomenon of cadmium ion uptake and efflux (Fig. 2).

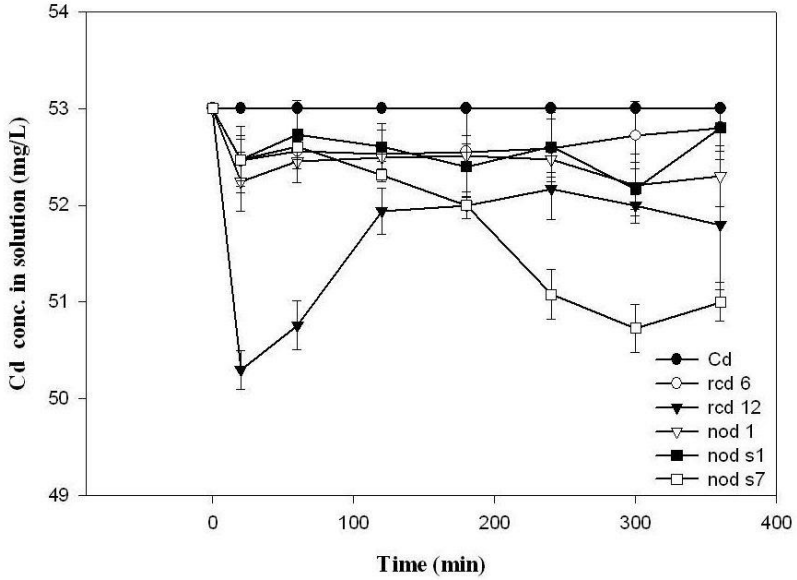

FIG. 2. Uptake of cadmium by strains of rcd 6 (*Cupriavidus metallidurans*), rcd 12, nod 1; nod s1, nod s7 (*C. taiwanensis*) at 30°C. Cd: untreated controls.

Strains PSS36 and PSS161 of *Ralstonia solanacearum* uptake cadmium ion after 20-min incubation (3.11 mg/kg, strain PSS161) and then cadmium ions were effluxed. Strain PSS225 uptaked cadmium ion after 20 to 60 minutes and then cadmium ions efflux occurred. Strain PSS189 uptake cadmium ion after 20 to 360 minutes and had no efflux trend. No cadmium ion uptake and efflux phenomenon were found for strain Ps-Au-11 (Fig. 3).

Thus, at lower cadmium ion concentration (53 ppm), it was found that the most tolerant strain (rcd12, 6.70 mM/1500 ppm) appeared to uptake and efflux cadmium ion at an earlier stage of incubation. Other than strain PSS189, whereas strains with a cadmium tolerance above 1.34 mM (300 ppm), PSS161 (5.81 mM/1300 ppm), rcd 6 and nod s1 (3.13 mM/700 ppm), Ps-Au-11 and PSS36 (2.68 mM/600 ppm), nod 1, nod s7 and PSS225 (1.34 mM/300 ppm), were also showing the phenomenon. On the other hand, when at higher cadmium ion concentration (390 ppm) state, the strains rcd 6, rcd 12 and nod s1 had the same uptake phenomenon after 20-min incubation and the bacterial cell cadmium concentrations were 30.56 mg/kg, 18.48 mg/kg and 10.67 mg/kg, respectively, and then cadmium efflux occurred (Fig. 4). The bacterial cell cadmium concentration of strain PSS161 was 33.71 mg/kg at 20 min incubation and then cadmium efflux occurred (Fig. 5).

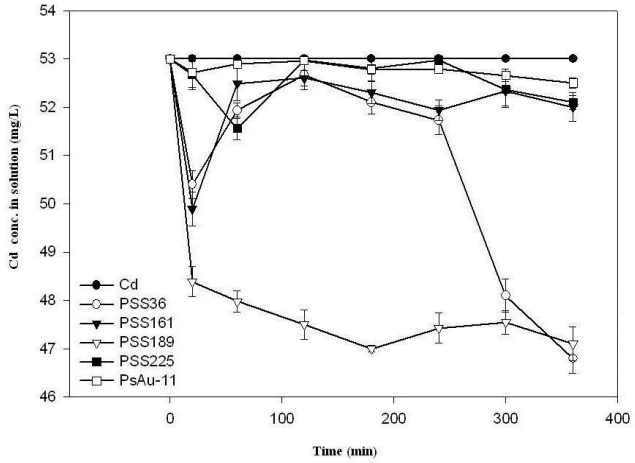

FIG. 3. Uptake of cadmium by strains PSS36, PSS161, PSS189, PSS225, Ps-Au-11 (*Ralstonia solanacearum*) at 30°C. Cd: untreated controls.

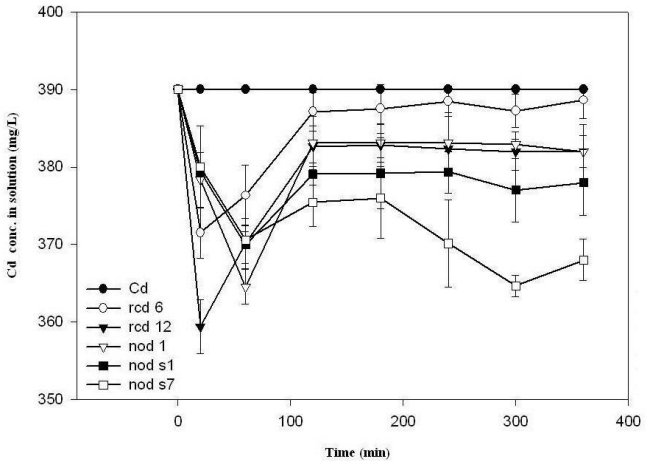

FIG. 4. Uptake of cadmium by strains of rcd 6 (*Cupriavidus metallidurans*), rcd 12, nod 1; nod s1, nod s7 (*C. taiwanensis*) at 30°C. Cd: untreated controls.

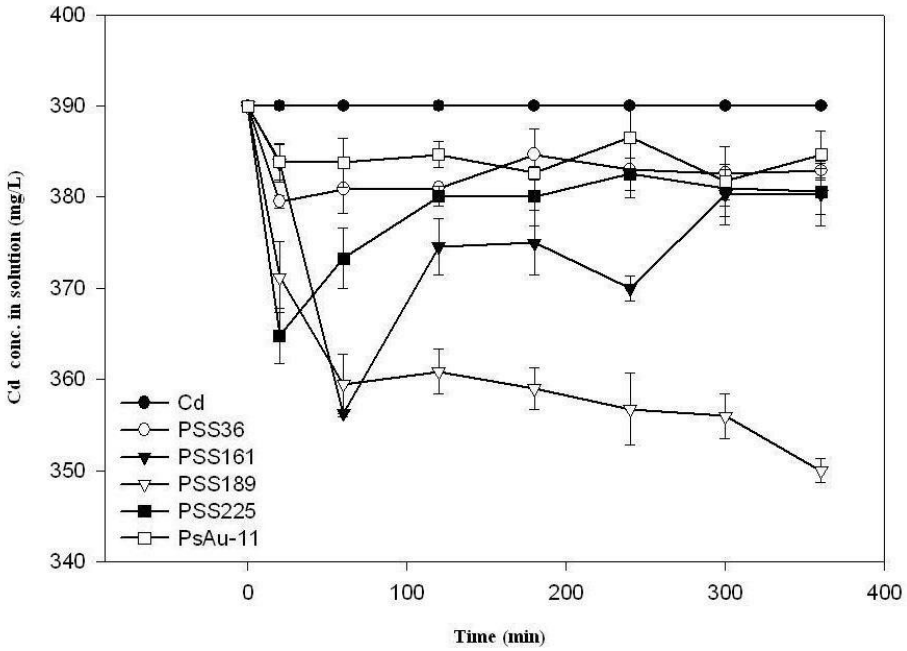

FIG. 5. Uptake of cadmium by strains PSS36, PSS161, PSS189, PSS225, Ps-Au-11 (*Ralstonia solanacearum*) at 30°C. Cd: untreated control.

3.4 Cadmium-resistant genes

It is known that bacteria are able to survive in environment with high concentrations of heavy metal due to their specific mechanisms, including the transport of heavy metal ions out of the cells. Many kinds of membrane-bound-proteins are known to participate in this transport. They are CBA efflux pumps driven by proteins of the resistance–nodulation–cell division superfamily, P-type export ATPases, CDF (cation diffusion facilitators) proteins and chromate proteins, NreB- and CnrT-like resistance factors. Heavy metal resistance is the result of multiple layers of resistance systems with overlapping substrate specificities, but unique functions (Nies, 2003). The best characterized CBA transporter is the CzcCBA complex from *Cupriavidus metallidurans* CH34. The *czc* determinant encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+} by metal-dependent efflux driven by the proton motive force (Nies, 1995; Nies et al., 1987; Nies and Silver, 1989). Chen et al. (2005) reported that cadmium-resistant *C. metallidurans* (=*Ralstonia eutropha*) and *C. taiwanensis* (=*R. taiwanensis*) strains use *czc* operon, which is located on plasmid or chromosome, to release cadmium off the organism.

TABLE 6
CHARACTERISTICS OF CD-RESISTANT MECHANISM OF TESTED STRAINS OF *CUPRIAVIDUS* AND *RALSTONIA* SPP.

Strains[1]	Cadmium (mM)	Cd-resistant genes												
		phsC	*smtA*	*cadA*	*zntA*	*czc* efflux system								
						czcN	*czcI*	*czcC*	*czcB*	*czcA*	*czcD*	*czcR*	*czcS*	*czcE*
rcd 12, 14, 21	6.70	-	-	+	-	+	+	+	+	+	+	+	+	+
PSS161	5.81	-	-	-	-	-	-	+	-	-	-	-	-	-
rcd 6, 8, 19; nod s1 to nod s6, s9, s11	3.13	-	-	-	-	-	-	+	-	-	-	-	-	-
PS99, Ps-Au-11, CLWl579	3.13	-	-	-	-	-	-	-	-	-	-	-	-	-
PSS36, PSS253	2.68	-	-	-	-	-	-	-	-	-	-	-	-	-
nod 1 to nod 5; nod s7, s8, s10, s12; PSS225	1.34	-	-	-	-	-	-	-	-	-	-	-	-	-
PSS189, GB03, PsL-11	0.67	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] ***Cupriavidus metallidurans: rcd 6, 8, 19; C. taiwanensis: rcd 12, 14, 21; nod 1 to nod 5; nod s1 to nod s12; Ralstonia solanacearum: PSS36, PS99, PSS161, PSS189, PSS225, PSS253, GB03, Ps-Au-11, PsL-11, CLWl579***

The *czc* operon located on plasmid or chromosome of Cadmium-resistant *Cupriavidus metallidurans* and *C. taiwanensis* strains is resopsible for releasing cadmium out of these microorganisms (Chen et al., 2005). Cadmium and zinc are removed from cells of *Ralstonia metallidurans* by the CzcCBA efflux pump and by two soft-metal-transporting P-type ATPases, CadA and ZntA. Resistance-Nodulation-Cell division (RND)-type cation efflux systems of the Czc type comprising of an inner membrane pump CzcA associated with two membrane bound factors, CzcB and CzcC (Legatzki et al., 2003). In order to determine the genes responsible cadmium resistance, *phsC*, *smtA*, *cadA*, *zntA* and nine components of efflux pump, *czc* operon were targeted by PCR, all the tested strains consisted of this operon with some variations. Higher cadmium-resistant strains rcd 12, 14, 21 (*C. taiwanensis*) consisted of *cadA* and nine components of *czc* efflux system, whereas strain PSS161 (*Ralstonia solanacearum*) only consisted of *czcC*. Strains rcd 6, 8, 19 (*C. metallidurans*) and nod s1 to nod s6, s9, s11 (*C. taiwanensis*) could grow on moderate cadmium levels (2.1 to 4.9 mM) only consisted of *czcC* (Table 6).

However, no PCR products of *phsC*, *smtA*, *cadA*, *zntA* and nine components of efflux pump, *czc* operon were amplified from lower cadmium-resistant strains PS99, Ps-Au-11, CLWl579, PSS36, PSS253, PSS225, PSS189, GB03, PsL-11 (*R. solanacearum*) and strains nod 1 to nod 5; nod s7, s8, s10, s12 (*C. taiwanensis*). Sequence analysis of these PCR products of *C. taiwanensis* strain rcd 12 showed nucleotide and amino acid sequence differences were 1 to 3% and 0 to 2%, respectively, as compared with the published data from NCBI (Table 7).

The *czcC* nucleotide and amino acid sequences among four tested strains rcd 6 (*C. metallidurans*), rcd 12 (*C. taiwanensis*), nod s1 (*C. taiwanensis*), and PSS161 (*R. solanacearum*) showed a 1 to 25% nucleotide sequence difference and 1 to 2% amino acid sequence difference compared with the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI) (Table 8).

In *Cupriavidus metallidurans* CH34, the regulatory genes of *czcCBA* are arranged in an upstream region consisting of *czcN* and *czcI*, and a downstream region consisting of *czcD*, *czcR*, *czcS* and *czcE*. CzcRS and a periplasmic copper-binding protein designated CzcE, exert metal-dependent control of *czcNICBA* expression via regulation of *czcNp* activity (Van der Lelie et al., 1997; Grosse et al., 2004; Petit-Haertlen et al., 2010). Phosphorylated CzcR activates the expression of *czcCBA* operon encoding an efflux pump specific for zinc, cadmium, and cobalt. Liu et al. (2015) reported that *czcRS*s regulated the expression of *czcCBA* and a cross-link existed between different czcRSs in the heavy metal resistance of *Pseudomonas putida* X4.

TABLE 7
IDENTITY OF CADMIUM-RESISTANT RELATED GENES NUCLEOTIDE AND AMINO ACID SEQUENCES BETWEEN STRAIN RCD 12 OF *CUPRIAVIDUS TAIWANENSIS* AND PUBLISHED DATA FROM NCBI GENBANK

Gene	Strain	NCBI GenBank (CP000354)	Nucleotide sequences identity (%)[1]	Amino acid sequences identity (%)[1]	Suppositional function
cadA	rcd 12	*R. metallidurans* CH34	98	98	Heavy metal translocating P-type ATPase
czcN	rcd 12	*R. metallidurans* CH34	98	98	Cobalt-zinc-cadmium resistance protein CzcN
czcI	rcd 12	*R. metallidurans* CH34	97	98	Cobalt-zinc-cadmium resistance protein CzcI
czcC	rcd 12	*R. metallidurans* CH34	99	99	Outer membrane efflux protein
czcB	rcd 12	*R. metallidurans* CH34	99	99	Cobalt-zinc-cadmium resistance protein CzcB, Membrane Fusion Protein cluster 2
czcA	rcd 12	*R. metallidurans* CH34	99	99	Heavy metal efflux pump CzcA, Cation efflux system protein CzcA
czcD	rcd 12	*R. metallidurans* CH34	99	100	Cobalt-zinc-cadmium resistance protein, CzcD
czcR	rcd 12	*R. metallidurans* CH34	98	98	Two component heavy metal response transcriptional regulator, winged helix family
czcS	rcd 12	*R. metallidurans* CH34	98	98	Heavy metal sensor signal transduction histidine kinase
czcE	rcd 12	*R. metallidurans* CH34	98	99	ORF131 protein

[1] Identity (%) of nucleotide acid sequences was compared using the BLASTn program and amino acid sequences were compared using the BLASTx program of NCBI.

TABLE 8
IDENTITY OF *CZCC* NUCLEOTIDE AND AMINO ACID SEQUENCES BETWEEN TESTED STRAINS OF CUPRIAVIDUS AND RALSTONIA SPP. AND PUBLISHED DATA FROM NCBI GENBANK

Strains	Species	NCBI GenBank (CP000354)	Nucleotide sequences identity (%)[1]	Amino acid sequences identity (%)[1]	Suppositional function
rcd 6	*C. metallidurans*	*C. metallidurans* CH34	75	88	Outer membrane efflux protein
rcd 12	*C. taiwanensis*	*C. metallidurans* CH34	99	99	Outer membrane efflux protein
nod s1	*C. taiwanensis*	*C. metallidurans* CH34	75	88	Outer membrane efflux protein
PSS161	*R. solanacearum*	*C. metallidurans* CH34	75	88	Outer membrane efflux protein

[1] Identity (%) of nucleotide acid sequences was compared using the BLASTn program and amino acid sequences were compared using the BLASTx program of NCBI.

CzcA is one of the primary proteins in cadmium, cobalt and zinc resistance in several microorganisms, including the tolerant bacterium *Cupriavidus metallidurans* CH34 (Nies, 2003), *Caulobacter crescentus* CB15N (Hu et al., 2005), *Pseudomonas putida* CD2 (Hu and Zhao, 2007), *Sinorhizobium meliloti* 1021 (Rossbach et al., 2008) and *Gluconacetobacter diazotrophicus* PAl 5 (Intorne et al., 2012).

When *czcA* used as probe in a Southern blot analysis, the results revealed that this operon was located on plasmid and chromosome as well (Chen et al., 2005). In order to further confirm the results of PCR amplification, the *czcA* and *czcC* probes were prepared for Southern blot hybridization. The results showed only the strain rcd 12 and rcd 21 (*Cupriavidus taiwanensis*) have the hybridization signals on the position of DNA digestion fragment (>10 kb). However, no hybridization results were found on other tested strains rcd 6, rcd 8 (*C. metallidurans*), nod 1, nod s1, nod s2, nod s7 (*C. taiwanensis*), PSS36, PS99, PSS161 and PSS189 (*R. solanacearum*) (Fig. 6 and Fig. 7).

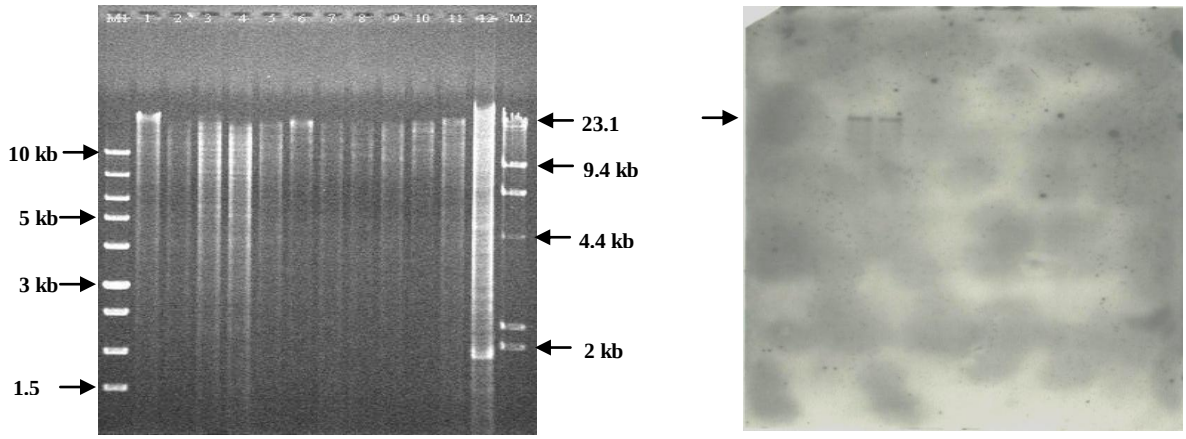

FIG. 6. Bacterial genomic DNA digested with the restriction enzyme *Bam*HI and separated by gel electrophoresis (Left). The detection of specific DNA fragment *czcC* in lanes 3 and 4 by Southern blot hybridization (Right).

M1: 1 kb DNA Ladder Marker; Lanes 1-2: strains rcd 6 and 8 (*Cupriavidus metallidurans*), Lanes 3-4: rcd 12 and 21 (*C. taiwanensis*); Lanes 5-8: strains nod 1, nod s1, s2 and s7 (*C. taiwanensis*); Lanes 9-12: strains PSS36, PS99, PSS161, and PSS189 (*Ralstonia solanacearum*); M2: Lambda DNA/*Hin*dIII Marker.

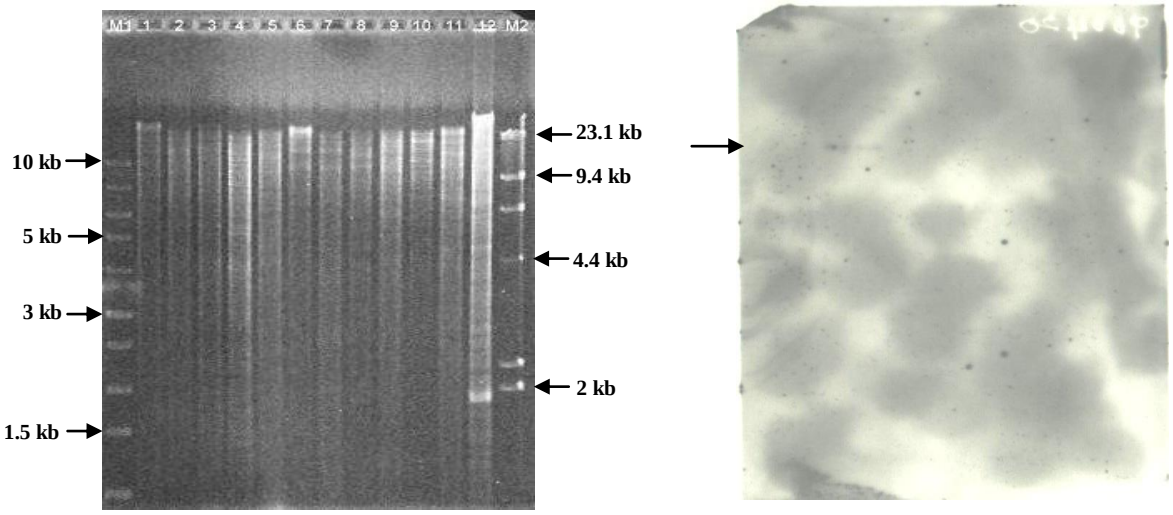

FIG. 7. Bacterial genomic DNA digested with the restriction enzyme *Bam*HI and separated by gel electrophoresis (Left). The detection of specific DNA fragment *czcA* in lanes 3 and 4 by Southern blot hybridization (Right).

M1: 1 kb DNA Ladder Marker; Lanes 1-2: strains rcd 6 and 8 (*Cupriavidus metallidurans*), Lanes 3-4: rcd 12 and 21 (*C. taiwanensis*); Lanes 5-8: strains nod 1, nod s1, s2 and s7 (*C. taiwanensis*); Lanes 9-12: strains PSS36, PS99, PSS161, and PSS189 (*Ralstonia solanacearum*); M2: Lambda DNA/*Hin*dIII Marker.

As concluding remarks, our findings have demonstrated that the higher cadmium resistant capability of strains rcd 12, 14, 21 (*Cupriavidus taiwanensis*) was related to the presence of *cadA* and *czc* efflux system (*czcN*, *czcI*, czcC, *czcB*, *czcA*, *czcD*, *czcR*, *czcS* and *czcE*). In addition to this, some strains of *Cupriavidus* species and *Ralstonia solanacearum*, rcd 6, 8, 19 (*C. metallidurans*), nod s1 to nod s6, s9, s11 (*C. taiwanensis*) and PSS161 (*R. solanacearum*), exhibit moderately elevated cadmium resistance. Our results revealed that cadmium resistant capability in these *Cupriavidus* and *Ralstonia* strains was related to the presence of *czcC*, thought to encode the outer membrane factor (OMF) of *czc* efflux system.

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