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Enterobacter nickelidurans sp. nov., a novel nickel tolerant enterobacteria isolated from Torsa river water of India

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Abstract
A Gram-negative, rod-shaped, nickel-resistant bacterium, designated as strain NiVas 114^T, was isolated from waters of Torsa River in Hasimara, West Bengal, India. The strain NiVas114^T possessing inducible nickel resistance can tolerate maximally 10mM nickel chloride. Southern blot assays of genomic DNA of NiVas 114^T using probe(s) generated from known nickel resistance determinants (*cnr/ ncc/ nre/ ncr/ nir*), under conditions of low stringency, produced no detectable signal except for *cnrA* gene of *Ralstonia metallidurans* CH34 (formerly *Alcaligenes eutropha* CH34) in which weak hybridization signal occurred. Based on 16S rRNA gene sequence similarity, strain NiVas 114^T was identified as a member of *γ-Proteobacteria*, and the nearest phylogenetic relatives are *Enterobacter hormaechei* (98.6-98.5%) and *Enterobacter cloacae* (97.5-98.2 %). In the phylogenetic trees constructed with nucleotide sequence of 16S rRNA gene, hsp60 gene and rpoB gene, strain NiVas 114^T clustered with the subspecies of *Enterobacter hormaechei*. The strain showed DNA-DNA relatedness of 60 -63% with *Enterobacter hormaechei* subsp. *hormaechei*, *Enterobacter hormaechei* subsp. *oharae*, *Enterobacter hormaechei* subsp. *steigerwaltii*, *Enterobacter cloacae* subsp. *cloacae* and *Enterobacter cloacae* subsp. *dissolvens*. The isolate NiVas 114^T differed from the nearest phylogenetic relatives in terms of number of phenotypic characteristics. The G+C content of the genomic DNA of the isolate was 59.5 ±0.4 mol %. The predominant cellular fatty acids of the isolate are $C_{16:0}$, summed feature 3 (comprises $C_{16:1\omega7c/15\ iso\ 2OH}$) and $C_{18:1\omega7c}$; hydroxy fatty acids are found in minor quantities. Thus, on the basis of biochemical characteristics, fatty acid profiles, DNA-DNA relatedness and phylogenetic analysis, the isolate was recognized as a novel species of *Enterobacter*, for which the name *Enterobacter nickellidurans* sp. nov. is proposed with the type strain NiVas 114^T (= LMG 23000^T = CCUG50594^T = JCM13045^T).

Keywords: Inducible nickel resistance, Torsa River, Ni Resistance, *Enterobacter*, *Enterobacter nickellidurans*

Nickel is an essential microelement required for the growth of bacteria. It is used as co-factor for several bacterial enzymes such as urease, CO-dehydrogenase and hydrogenase (Hausinger, 1987). However, at higher concentration nickel is toxic and detrimental to bacterial growth. Some bacteria by virtue of possessing specific efflux system are able to reduce intracellular concentration of nickel ions thus out-competing others to flourish in a nickel polluted environment. The diversity of nickel resistance genetic system(s) in bacteria has been explored to some detail. Two similar genetic loci responsible for nickel resistance, *ncr* and *nir* loci, have been reported from two members of *Enterobacteriaceae*, located in the plasmid pGOE51 of *Hafnia alvei* 5-5 and chromosome of *Klebsiella oxytoca* CCUG 15788 respectively (Park *et al.*, 2003; Stoppel and Schlegel, 1995; Stoppel *et al.*, 1995). Beside these two, three other genetic loci conferring nickel resistance, *cnr*, *ncc*, and *nre*, have been characterized from other bacteria, *Alcaligenes eutropha* CH34 (currently known as *Ralstonia metallidurans* CH34), *Alcaligenes xylosoxidans* 31A (currently known as *Achromobacter xylosoxidans* 31A), and *Achromobacter xylosoxidans* 31A respectively (Grass et al., 2001; Liesegang *et al.*, 1993; Schmidt and Schlegel, 1994).

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The strain, NiVas 114^T was isolated from Torsa River of Hasimara of the state of West Bengal, India, on nutrient agar supplemented with nickel.The maximum tolerable concentration (MTC) of nickel showed by the isolate is 10 mM. Southern hybridization was used to detect homologous sequence, if any, of the known nickel resistance gene(s) in genomic DNA from NiVas 114^T. The inducible nature of nickel resistance in NiVas 114^T was studied by comparing growth curve(s) of cells pre-exposed to low metal ion concentration (inducible concentration) with cells unexposed to inducible concentration of metal(s), in liquid media containing higher concentration of nickel (Grass et al., 2001; Liesegang et al., 1993; Park et al., 2003; Schmidt and Schlegel, 1994).

Detailed polyphasic taxonomic study was done to determine the systematic position of the strain. Almost

Table 1. List of primers used for PCR amplification of nickel resistance genetic locus

Sl.No	Name of the primers	Sequence	Positive control	Amplicon size (expected from +ve coll.)
1	nir 1 nir 2	5' GCTGTCTCTGTCACGTCTG 3' 5' CTGCCTGTCCTATTCGGCT 3'	*Klebsiella oxytoca* CCUG 15788	1000 bp (from both *nir* and *ncr* locus)
2	bbrc 1 bbrc 2	5' TTCGATGGTGACCGCCGCT 3' 5' GCAATCGCCATCGGCACG 3'	*Ralstonia metallidurans* CH34 & *Achromobacter xylosoxidans* 31A	700 bp (from both *cnr* and *ncc* locus)
3	nrc 1	5'GGGCTACGATCACATCCTGT3'	*Achromobacter xylosoxidans* 31A	200 bp (from *nir* locus only)

complete 16S rRNA gene of the isolate was amplified and sequenced, and the sequence showed 97.5-98.6% similarities with the subspecies of *Enterobacter hormaechei*, and *Enterobacter cloacae*. The phylogenetic analysis using *rpoB* and *hsp60* gene sequence(s) further identified *Enterobacter hormaechei* subsp. *hormaechei*, *Enterobacter hormaechei* subsp. *oharae*, *Enterobacter hormaechei* subsp. *steigerwaltii*, *Enterobacter cloacae* subsp. *cloacae* and *Enterobacter cloacae* subsp. *dissolvens* as the nearest phylogenetic relatives of the isolate. Because of DNA-DNA relatedness and differences in phenotypic characteristics with its nearest phylogenetic relatives, the strain $NiVas114^T$ is designated as a novel member of *Enterobacteriaceae*, for which the name *Enterobacter nickelidurans* sp. nov. is proposed.

Material and methods

Reagents and Chemicals

De-ionized double distilled water and analytical grades of metal salts ($NiCl_2 \cdot 6H_2O$; $CoCl_2 \cdot 6H_2O$; $ZnSO_4 \cdot 6H_2O$; $CuSO_4 \cdot 7H_2O$; $CdCl_2 \cdot 2H_2O$ and $CH_3COOPb \cdot 2H_2O$) were used to prepare 1M or 0.1M stock solutions, which were filter-sterilized using filter paper (pore size 0.2µm marketed by Sartorius Ltd., Bangalore, India) before use. The nutrient broth (NB) (containing g l^{-1}, peptone, 10.0; Beef extract, 10.0; NaCl, 5.0; pH 6.8) and nutrient agar (NA) media used in the experiments were purchased from HiMEDIA Chemicals, India. For isolation and growth experiments, required amount of metal salt solution was added singly to sterile medium before inoculation.

Sampling and isolation

Water samples were collected from sampling site Hasimara of District Jalpaiguri, state- West Bengal, India, in January 2002. About 100 ml water samples were collected in sterile glass bottles following standard sampling technique stated in 'Standard Methods' (APHA, 1985). Serial dilutions of each water sample were made with sterile distilled water, which served as the diluents of known volume. Total culturable copiotrophic bacterial populations and their fraction(s) resistant to nickel were enumerated on NA plates containing 3mM $NiCl_2$, after incubation at 36°C for 18-20 hours. The bacterial colonies appeared were purified on the 3mM Ni^{2+} plates, and the maximum tolerable concentration (MTC) towards Ni^{2+} was detected by streaking the purified isolates on NA plates containing 3 -12 mM Ni^{2+} containing plates followed by incubation at 36°C for 48h.

Growth in liquid media to understand the inducible nature of nickel resistance determinant

The inducible nature of nickel resistance in the isolate was studied by comparing growth curve(s) of induced (cells pre-exposed overnight to 100µM metal ion containing media) and uninduced (cells unexposed to inducible concentration of metal) cells in nutrient broth (NB) containing 3mM Ni^{2+} following methods described by earlier authors (Mergeay *et al.*, 1985; Park *et al.*, 2003; Stoppel and Schlegel, 1995; Tibazarwa *et al.*, 2000). Batch cultures were grown in NB at 36°C and 1.0 ml of such culture was centrifuged and washed in sterile distilled water containing 0.5% (w/v) NaCl. The washed cells were incubated overnight in 0.5 ml 'basal medium' [$(NH_4)_2SO_4$ 0.2%, $MgSO_4$ 0.05%, $KHPO_4$ 0.05%, KCl 0.01%; w/v] containing 1% glucose (w/v) and 0.1% NB (v/v), either in presence or in absence of added 100µM Ni^{2+} /Co^{2+}/ Zn^{2+} ion(s). Culture incubated, in the presence of 100µM Ni^{2+} /Co^{2+}/ Zn^{2+} was considered as "induced pre-culture" and, in absence of any added metal ion was considered as 'un-induced pre-culture'. Aliquots of 0.1 ml of both induced and un-induced pre-cultures were then separately inoculated into 10 ml nutrient broth media (in a 100 ml conical flask) containing the Ni^{2+} concentration of 3mM. Flasks were incubated at 36°C with shaking (130 rpm). Optical densities of the cultures were determined every two hours using a spectrophotometer (SIMADJU, Japan) at 560 nm. NB culture of one nickel sensitive (sensitive = growth significantly affected at 0.5mM metal ion concentration) isolate, *Acinetobacter* sp. BB1C, was used as inocula in NB containing nickel and incubated side-by-side with the test flasks to serve as the negative control. Standard growth curve of $NiVas114^T$ in NB at 35°C was also determined to compare with growth of the isolate in presence of nickel.

Isolation of total genomic DNA

The total genomic DNA of the isolate was extracted, for southern hybridization, PCR-amplification, DNA-DNA hybridization and base composition analysis, by the method described by Ezaki *et al.*, (1989) with minor modifications. Lysozyme was not added for lysis, instead the same was achieved by freeze-thaw treatment followed by addition of 1.0 ml of 20% (w/v) SDS solution and 0.02% (w/v) proteinase K. DNA was precipitated with ethanol and suspended in DNase free water. The DNA was quantified by recording absorption at 260nm in a UV-vis Spectrophotometer (Shimadzu, Japan).

Table 2. Phenotypic properties of *Enterobacter nickellidurans* NiVas 114^T sp. nov. (ENI), *Enterobacter hormaechei* subsp. *hormaechei* CCUG 27126^T (EHHO), *Enterobacter hormaechei* subsp. *steigerwaltii* CIP 108489^T (EHST), *Enterobacter hormaechei* subsp. *oharae* CIP108490^T (EHOH), *Enterobacter cloacae* subsp. *cloacae* MTCC 509^T (ECCL), *Enterobacter cloacae* subsp. *dissolvens* JCM 6049^T (ECDI) and *Enterobacter kobei* JCM 8580^T (EKO).

All strains are negative in indole production, lysine decarboxylase, H_2S production and gelatinase, but positive in Voges-Proskauer, β-galactosidase, arginine dihydrolyse, ornithine decarboxylase and Simmon's citrate test. Produced acid from D-glucose, D-galactose, lactose, L-rhamnose, glycerol and α-methyl-D-glucoside. Utilized D-glucose, D-fructose, *cis*-aconitate, D-ribose, D-cellobiose, L-arabinose, sucrose, D-galactose, D-mannose, trehalose, D-xylose, maltose, D-saccharate, D-glucosamine, D-mannitol, glycerol, fumarate, D-galacturonate, succinate, phenylacetate, L-alanine, L-leucine, L-threonine and L-tryptophan as sole source of carbone; but erythritol, 5-keto-D-gluconate L-tartrate, caprate, caprylate, L-aspartic acid and L-asparagine were not utilized.

Tests	ENI	EHHO	EHST	EHOH	ECCL	ECDI	EKO
Urease (Christensen's agar)	–	+	+	+	–	–	–
Esculine Hydrolysis	+	–	–	–	–	+	–
Growth on substrates:							
Adonitol	+	–	+	–	–	–	–
Dulcitol	–	+	–	–	–	–	–
D–Arabitol	–	–	+	–	–	–	–
D–Melibiose	+	–	+	+	+	+	+
L–Fucose	–	+	+	+	–	–	–
3–Methyl–D–glucopyranose	–	+	+	–	–	–	–
1–0–Methyl–α–glucopyranose	+	–	+	+	+	+	+
1–0–Methyl-α-galactopyranose	+	–	+	+	+	+	+
D–Raffinose	–	–	+	+	+	+	+
D–Arabitol	–	–	+	–	–	–	–
D–Sorbitol	+	–	+	+	+	+	+
D–Lactose	+	nd	+	+	–	+	nd
3–Hydroxybutyrate	+	–	–	–	+	+	+
α–Ketoglutarate	+	–	–	–	+	w	–
D–Malate	+	–	–	–	–	+	–
Malonate	+	–	–	–	–	+	–
Putrescine	–	–	–	–	+	+	+
Xylitol	+	–	+	–	w	+	w
L–Proline	+	–	–	–	+	w	nd
L–Serine	+	–	–	–	–	+	–
L–Histidine	+	–	w	–	+	+	+
L–Glutamic acid	+	+	+	+	–	+	–
Mol% G+C content	59.3 ±0.4	58.0 ±0.3	57.7 ±0.4	57.5 ±0.3	53.5 ±0.5	54.8 ±0.4	53.2±0.6

+=positive; –=negative; w=weak positive; nd=not done

Preparation of cnr, ncc, nir, ncr and nre specific probes

Probe(s) for Southern hybridization was prepared from 200-1000 bp amplicon generated using *cnr, ncc, nir, ncr* and *nre* specific primers. The primers used in PCR amplification and their respective positive controls are listed in table 1. Amplifications were performed in 50µl reaction volumes, using 'PCR amplification kit' (GENEI, Bangalore, India), for 30 cycles using *Taq* DNA polymerase (GENEI, Bangalore, India) in a Ampli -Kit thermal cycler (Applied Biosystems). After initial denaturation at 95°C for 2 min, each cycle consisted of, denaturation (95°C, 30 sec), annealing (53°C, 30 sec) and extension (72°C, 60 sec). To rule out primer dimer from the actual amplicon(s), all reaction sets included a negative control where the target DNA was replaced by sterile water. The PCR products were purified using QiaGen gel extraction kit (QiaGen, Germany) and 0.6 µg of purified PCR product were labelled with DIG-labeling and detection kit (Roche, Germany) following manufacturers instructions.

Restriction digestion of the genomic DNA and Southern hybridization

The genomic DNA preparation of isolate NiVas 114^T was diluted (50ng/ µl) and to the 87 µl of diluted DNA 10µl of 10X buffer solution and 3 µl (20,000 U/ml) of *Eco* RI restriction enzyme (GENEI, Bangalore, India) was added and mixed by vortexing. The mix was kept for 24 h at 36°C, and then extracted using phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1) and finally centrifuged at 12000 rpm for 20 min at 4°C after addition of 2.5 volume of dehydrated ethanol. The resultant pellet was washed twice by 70% ethanol, dried in vacuum and suspended in sterile distilled water. The probe DNA (~25 ng), and *Eco* RI restricted genomic DNA of NiVas 114^T (~1.0 µg) and positive control [plasmid DNA bearing nickel resistant determinant (~0.5 µg)] were electrophoresed in an agarose gel (0.8%) without ethidium bromide and transferred to the positively charged nylon membrane (BioDyne) by capillary transfer method, after depurination and denaturation, as described in Sambrook *et al.* (1989). Hybridization was carried out at low stringency (48°C) for 15-18 h (Sambrook *et al.*, 1989) and post

Table 3. Key tests for phenotypic differentiation of *Enterobacter nickellidurans* NiVas 114^{T} sp.nov. and its nearest phylogenetic relatives. Data other than ENI are from Hoffmann *et al.* (2005)

	Biochemical test result^{a}												
	D-Sorbitol	L-Fucose	D-Melibiose	1-0-Methyl-α-galacto-pyranose	Esculin	Lactose	D-Arabitol	Dulcitol	D-Raffinose	Adonitol	3-Methyl-D-gluco-pyranose	3-Hydroxy butyrate	Putrescine
ENI	+	-	+	+	+	+	-	-	-	+	-	+	-
EHOH	+	v	+	+	v	$+^{-}$	-	-	+	$-^{+}$	-	-	-
EHHO	$-^{+}$	+	$-^{+}$	-	-	v	$-^{+}$	+	v	$-^{+}$	v	-	-
EHST	+	+	+	+	-	v	+	-	+	+	+	-	-
ECCL	+	-	+	+	-	v	-	$-^{+}$	+	-	-	+	+
ECDI	+	v	+	+	+	+	-	-	+	v	-	+	+
EKO	+	-	+	+	-	$+^{-}$	-	v	+	-	-	$+^{-}$	+

Abbreviations: *Enterobacter nickellidurans* NiVas 114^{T} sp. nov. (ENI), *Enterobacter hormaechei* subsp. *oharae* CIP108490^{T} (EHOH), *Enterobacter hormaechei* subsp. *hormaechei* CCUG 27126^{T} (EHHO), *Enterobacter hormaechei* subsp. *steigerwaltii* CIP 108489^{T} (EHST), *Enterobacter cloacae* subsp. *cloacae* MTCC 509^{T} (ECCL), *Enterobacter cloacae* subsp. *dissolvens* JCM 6049^{T} (ECDI) and *Enterobacter kobei* JCM 8580^{T} (EKO).

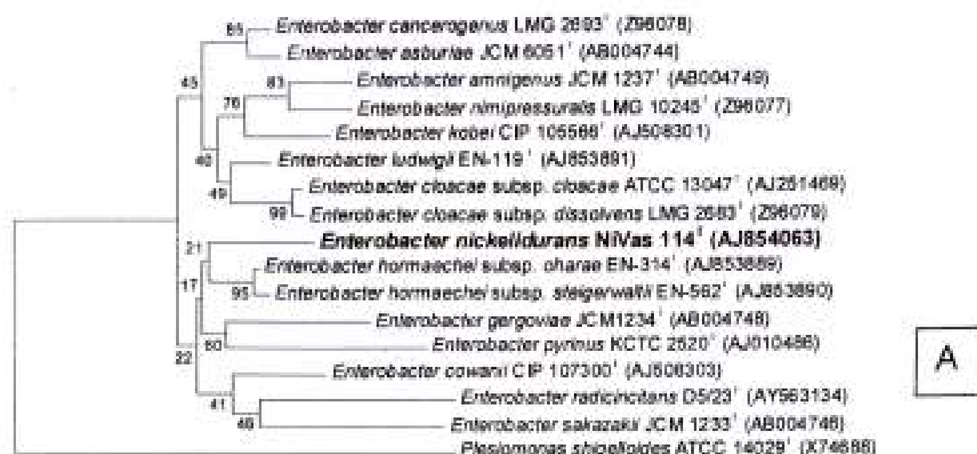
^{a}Incubation was done at 36°C. symbols: -, 0 –10%; $-^{+}$, 10-20%; v, 20-80%; $+^{-}$, 80-90%; +, 90-100%.

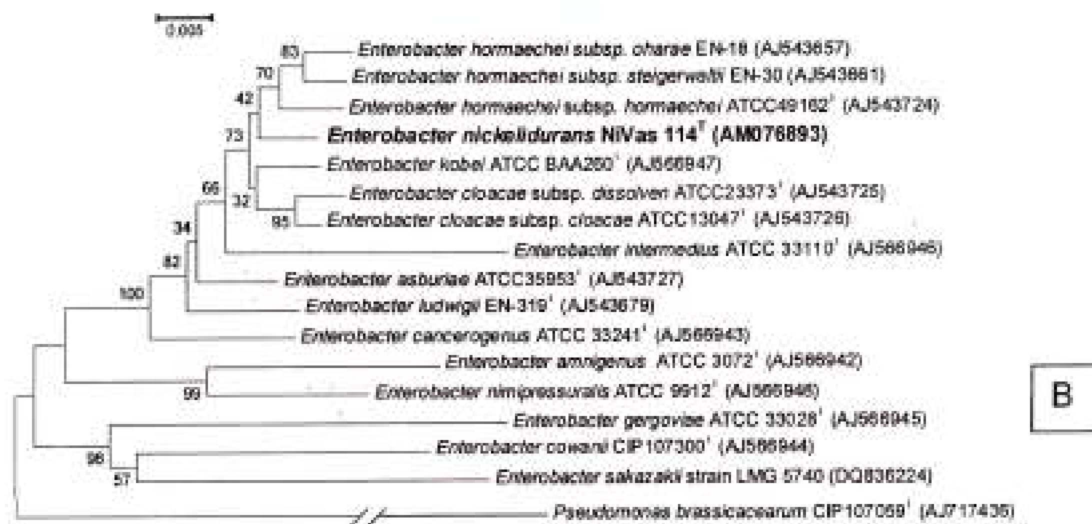
Figure 1. Induction of nickel resistance in NiVas114^{T} in nutrient broth containing 3mM $NiCl_2$. o, Growth curve of un-induced cells; ▲, growth curve of 100 µM Co^{2+} induced cells: ■, growth curve of cells induced by 100 µM Ni^{2+} and, ● growth curve of cells induced by 100 µM Zn^{2+}; □, growth curve of the nickel sensitive strain *Acinetobacter* sp. BB1C. *, Actual OD_{560} multiplied by 1000 has been converted to log value.

hybridization wash was done at 48°C with 0.5X SSC buffer. Immunological detection of the hybridization signal with antiDIG-antibody was done following manufacturers instructions provided with the kit.

Cell morphology and motility were examined by phase-contrast microscope (Olympus, model M021) at 1000X with the cells grown at 36°C for 16h. Gram staining was performed following the method of Gerhardt *et al.* (1994) and colony morphology was studied by using a stereomicroscope (Olympus model SZ11). Nutrient agar medium was used for maintaining NiVas 114^{T} and for determination of growth at various temperatures (5, 10, 22, 28, 36, 40 and 50°C), at different pH (4, 6, 7, 8, 9, 10, and 11) and in the presence of various concentrations of NaCl (5%, 7%, 10%, 12% and 15%) (Shivaji *et al.*, 2005). Indole, methyl red, Voges-Prousker, nitrate reduction, phenylalanine deamination and acid production tests were conducted using Enterobacteriaceae identification kit (HiMEDIA, Mumbai, India). Other physiological tests were done following standard protocols stated earlier (Bhadra *et al.*, 2005; Bhadra *et al.*, 2006) at 36°C and results were recorded after 7 days. *Enterobacter hormaechei* subsp. *hormaechei* CCUG 27126^{T}, *Enterobacter hormaechei* subsp. *oharae* CIP 10849^{T}, *Enterobacter hormaechei* subsp. *steigerwaltii* CIP 108489^{T}, *Enterobacter cloacae* subsp. *cloacae* MTCC 509^{T}, *Enterobacter cloacae* subsp. *dissolvens* JCM 6049^{T} and *Enterobacter kobei* JCM 8580^{T} were used as reference strains for the study. The biochemical characteristics of the isolate were confirmed by MTCC, Chandigarh, India.

Estimation of G+C content





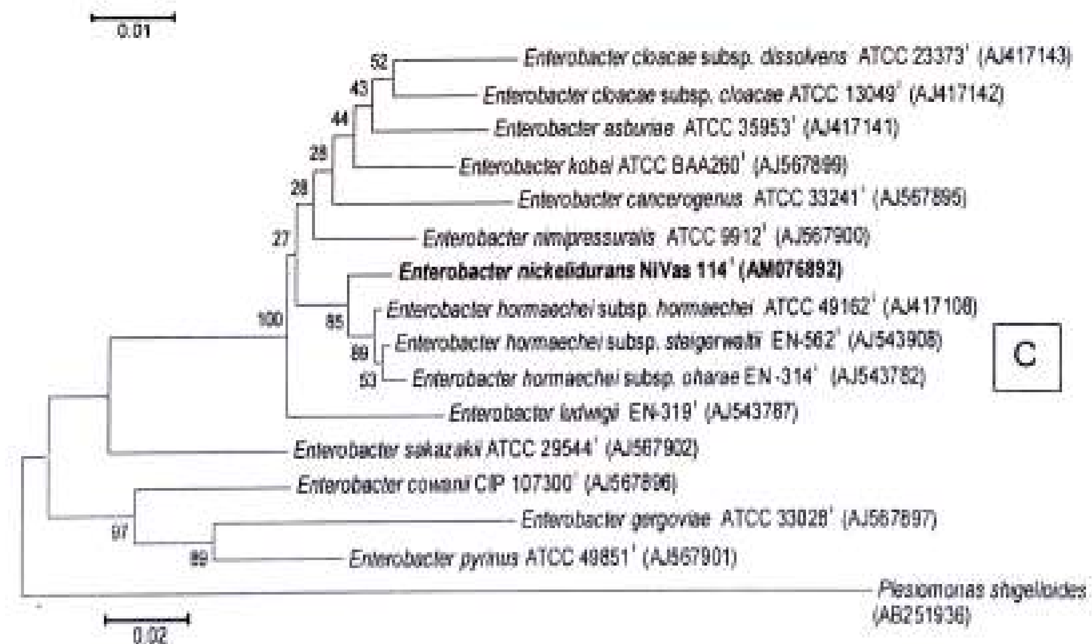


Figure. 2 Neighbour-joining tree based on 16S rRNA gene sequences (A), *rpoB* gene sequence (B) and *hsp60* gene sequence (C) showing the phylogenetic relationship between *Enterobacter nickellidurans* sp. nov. and other closely related species of *Enterobacter*. Bootstrap values, expressed as percentages of 1000 replications, are given at the branching points. The bars represent substitution rate and the accession numbers are given in the parenthesis.

Mol% G+C content of the isolate was determined using thermal denaturation technique using UV-vis spectophotometre (Lambda 2, Parkin Elmer, USA) equipped with TempLab 2 software package (Parkin Elmer, USA) following protocol stated by Shivaji *et al.* (2005, 2006). The mol% G+C content of the isolate was confirmed from BCCM LMG Bacterial Collection, Gent, Belgium.

Fatty acid composition

Fatty acid methyl esters were extracted and prepared from cells grown on Tryptic-soy agar at 28°C following standard protocol of the Microbial Identification System (MIDI: Microbial ID, Inc., Newark, Del.). The cellular fatty acid profile of the strain NiVas 114^T was provided by DSMZ, Germany.

Phylogenetic analyses

The 16S rRNA gene of isolate NiVas 114^T was amplified using universal primers [8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-AGAAAGGAGGTGATCCAGGC-3') corresponding to the *E. coli* numbering system (Brosius *et al.*, 1978; Kumar *et al.*, 2004) and sequenced (Bhadra et al, 2005; 2006). The *rpoB* and *hsp60* gene were amplified and sequenced using primers described by Hoffmann and Roggenkamp (Hoffmann and Roggenkamp, 2003). The sequences of 16S rRNA gene, *rpoB* gene and *hsp60* gene were manually corrected from the chromatogram and subjected to similarity search study using BLAST program (Altschul *et al.*, 1997). The highly similar sequences were retrieved from GenBank and aligned by CLUSTAL_X (Thompson *et al.*, 1997). A neighbour-joining phylogenetic tree (Saitou and Nei, 1987) was constructed using MEGA 3.1 (Kumar *et al.*, 2004) based on evolutionary distance data that was determined with Kimura's two-parameter model (Kimura, 1980). Bootstrap analyses (Felsenstein, 1985) were performed for 1000 replications and are indicated in the branch nodes. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

DNA-DNA relatedness

The DNA-DNA relatedness of the isolate with the nearest phylogenetic relatives was conducted using Dot-Blot hybridization method with DIG-DNA labeling and detection kit (Roche Diagnostics) following protocols described earlier (Bhadra et al., 2005; 2006). The percentage hybridization was calculated using Molecular Analyst software (BioRad Inc.). Cross-hybridization was conducted with the probe prepared from the genomic DNA of NiVas 114^T, *Enterobacter hormaechei* subsp. *hormaechei*, *Enterobacter hormaechei* subsp. *oharae*, *Enterobacter hormaechei* subsp. *steigerwaltii*, *Enterobacter cloacae* subsp. *cloacae* and *Enterobacter cloacae* subsp. *dissolvens*. Hybridization was performed at 55°C and membranes were washed at 53°C with 0.5X SSC following manufacturer's instruction.

Results and discussion:

Torsa is an international river traversing three countries, Tibet, Bhutan and India, before entering Bangladesh. Water quality analysis of the river did not show any alarming level of heavy metal toxicity (Bhadra *et al.*, 2007). The heavy metal ion content of the river was found below the maximum permissible limit for human consumption as set forth by WHO [2], but we have observed and reported sewage pollution of river water due to disposal of waste water from adjoining townships Bhadra et al., 2003; 2005).

Isolation and cultural characteristics

The copiotrophic (or eutrophic) bacterial count of the water samples collected from the sampling station Hasimara, of the state of West Bengal, India, in January 2002 ranged between 5.15-4.11 X 10^3 CFU/ml. When 0.1 ml water sample of a sampling was plated on 3mM nickel containing plate, 30 nickel-tolerant colonies had appeared. Only one isolate, NiVas 114^T, showed confluent growths even at 10mM nickel containing plate, whereas, other isolates could not grow over 5mM Ni^{2+}. In NB, the isolate formed motile rods of 0.8-1.1μm in length and 0.6-0.7μm in width at 36°C after 16h of incubation. In NB, the strain grew between 20-40°C but not at 5, 10 and 50°C, optimally at 36°C. The cells could grow at pH range of 6-8 and tolerate up to 10% NaCl. On NA the isolate formed pale-white colored colony with a diameter of 1-2 mm with entire margin.

Biochemical characteristics

Strain NiVas 114^T produced pale-white colored colonies (2-3 mm) on nutrient agar after 24 h at 36°C. The optimum temperature and pH for growth of the isolate are 36°C and 7.2 respectively, and was found to grow upto 7% NaCl containing media. NiVas 114^T differ from *Enterobacter hormaechei* subsp. *hormaechei*, *Enterobacter hormaechei* subsp. *oharae*, *Enterobacter hormaechei* subsp. *steigerwaltii*, the closest phylogenetic relatives, in terms of number of phenotypic characteristics such as esculine and urea hydrolysis, and utilization of α-ketoglutarate, 3-hydroxybutyrate, D-malate, malonate, L-proline, L-serine and L-histidine (Table 2). The key biochemical tests that differentiate NiVas 114^T from its close phylogenetic neighbours are listed in Table 3.

Induction of Nickel resistance

Growth curve of induced cells of NiVas 114^T showed a reduction of 10h in the duration of lag phase compared to the duration of the same growth phase of uninduced cells in presence of 3mM Ni^{2+} (Fig. 1). Similar comparison between the growth curves of induced and uninduced cells of *Klebsiella oxytoca* CCUG 15788 (which harbor *nir* determinant to confer inducible 10mM Ni^{2+} resistance) demonstrating difference in duration of lag phase has been reported by Stoppel and Schlegel (1995). The cells of *Hafnia alvei* 5-5 (having *ncr* determinant to confer 30mM Ni^{2+} resistance), when induced (exposed overnight to 0.5mM Ni^{2+}) and challenged against 5mM Ni^{2+} , showed reduction in duration of lag phase in comparison to uninduced cells (Park et al., 2003). Similar were the observations in growth studies demonstrating inducible nature of nickel resistance in *Ralstonia metallidurans* CH34 (possessing *cnr* locus) and *Achromobacter xylosoxidans* 31A

(having *ncc* and *nre* locus) (Mergeay *et al*., 1985; Nies *et al*., 1987; Tibazarwa *et al*., 2000). When cells of NiVas 114^T were pre-exposed to 100µm of Ni^{2+}/ Zn^{2+} for overnight, they entered exponential phase after a lag phase of 1-2h in contrast to a prolonged lag phase of 12h shown by uninduced cells, in 3mM Ni^{2+} containing media. The results, therefore, showed the presence of an inducible nickel resistance genetic system in NiVas 114^T.

Southern hybridization

Southern hybridization was carried out on genomic DNA of NiVas 114^T using *ncc*/ *cnr*/ *nre*/ *nir*/ *ncr*/ as a probe, to identify the homologous DNA sequence. At high stringency (60°C) no hybridization signal was detected with any of the probe used. Southern blot analyses clearly suggested that the nickel resistant strain NiVas 114^T did not contain DNA sequence(s) homologous to the known nickel resistance genetic systems except a weak homology with *cnr* system. Using cnrA gene (of R. metallidurans CH34) probe, under conditions of low stringency (hybridization temperature at 48°C), weak hybridization signal could be detected while other gene (*ncc*, *nre*, *ncr*, and *nir*) probes failed to produce any detectable signal.

Cellular fatty acid composition

Strain NiVas 114^T exhibited a fatty acid pattern characterized by high levels of the acid $C_{16:0}$ (32.2%), $C_{18:1\ w7c}$ (22.24%) and Summed feature 3 ($C_{16:1\ w7c}$ and $C_{15:0\ iso\ 2OH}$, 20.95%). The acids $C_{12:0}$ (1.51%), $C_{14:0}$ (6.59%), $C_{14:0\ 2OH}$ (1.95%), $C_{17:0\ CYCLO}$ (5.53%), Summed feature 2 ($C_{16:1\ iso\ I}$ and $C_{14:0\ 3OH}$, 8.04%) were also detected. The cellular fatty acid profile of NiVas 114^T was compared with the library of the MIDI system and was found similar (but not identical) to *Enterobacter cloacae* subsp. *cloacae*, *Enterobacter cloacae* subsp. *dissolvens*, *Enterobacte hormaechei* subsp. *hormaechei* and *Enterobacte cancerogenus*, i.e., the members of *Enterobacter cloacae* complex. Therefore, the isolate was regarded as a member of *Enterobacter cloacae* complex.

Phylogenetic analysis based on 16S rRNA gene, rpoB gene and hsp60 gene sequence

The 16S rRNA gene sequence of the isolate showed 98.7 and 98.5% similarity to *Enterobacter hormaechei* subsp. *oharae* EN-314^T (AJ853889) and *Enterobacter hormaechei* subsp. *steigerwaltii* EN-562^T (AJ853890). The 16S rRNA gene sequence of *Enterobacter hormaechei* subsp. *hormaechei* ATCC 49162^T (AJ417450) is only 422 nucleotides in length and hence not compared. The strain showed 97.5-98% similarities with the other members of *Enterobacter cloacae* complex (Hoffmann et al., 2005). Rest of the members of *Enterobacter* showed less than 97.5% 16S rRNA gene sequence similarity to the 16S rRNA gene of NiVas 114^T. It is clearly understood from the 16S rRNA gene sequence analysis that NiVas 114^T is a member of *Enterobacter cloacae* complex (Hoffmann et al., 2005) and is close to *Enterobacter hormaechei*. In the phylogenetic tree constructed using the 16S rRNA gene sequence, isolate NiVas 114^T clustered with *Enterobacter hormaechei* subsp. *oharae* EN-314^T (AJ853889) and *Enterobacter hormaechei* subsp. *steigerwaltii* EN-562^T (AJ853890) (Fig. 2A).

Mollet *et al*., (1997) explained the importance of *rpo*B gene sequence for the identification of the *Enterobacteriaceae*. Analysis of *rpo*B gene sequences were used as a criterion to propose a new genus *Raoultella* under *Enterobacteriaceae*, where 6% difference of *rpoB* gene sequence was used as a criterion to delineate a new genus (Drancourt *et al*., 2001). A 1008 bp *rpoB* gene sequence of the strain NiVas 114^T showed 97.9-98.6% similarities with *Enterobacter hormaechei* subsp. *hormaechei* ATCC 49162^T (AJ543724), *Enterobacter hormaechei* subsp. *oharae* EN-18 (AJ543657), *Enterobacter hormaechei* subsp. *steigerwaltii* EN-30 (AJ543661), *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^T (AJ542736), *Enterobacter cloacae* subsp. *dissolvens* ATCC 23343^T (AJ543735) and *Enterobacter kobei* ATCC BAA260^T (AJ566947). In the phylogenetic tree NiVas 114^T clustered with *Enterobacter hormaechei* subsp. *hormaechei* ATCC 49162^T (AJ543724), *Enterobacter hormaechei* subsp. *oharae* EN-18 (AJ543657) and *Enterobacter hormaechei* subsp. *steigerwaltii* EN-30 (AJ543661) (Fig. 2B).

Hoffmann and Roggenkamp (2003) have analysed the nucleotide sequences of four protein coding genes, *hsp60*, *rpoB*, *hemB* and *ampB*, and found that the sequence of *hsp60* gene is one of the strongest tool to classify *Enterobacter*. To confirm the systematic position of NiVas 114^T the *hsp60* gene of the isolate was sequenced and the sequence showed 99% similarity to *Enterobacter hormaechei* subsp. *steigerwaltii* EN-562^T (AJ543908), 98.5% similarity to *Enterobacter hormaechei* subsp. *hormaechei* ATCC 49162^T (AJ417108) and 97.6% similarity to *Enterobacter hormaechei* subsp. *oharae* EN-314^T (AJ543782). In the phylogenetic tree (Fig. 2C) isolate NiVas 114^T clustered with *Enterobacter hormaechei* subsp. *hormaechei* ATCC 49162^T, *Enterobacter hormaechei* subsp. *steigerwaltii* EN-562^T, *Enterobacter hormaechei* subsp. *oharae* EN-314^T with a bootstrap support of 85 (Fig. 2C).

Based on the phylogenetic analysis of 16S rRNA gene, *rpoB* gene and *hsp60* gene, the nearest phylogenetic relatives of isolate NiVas 114^T were identified as *Enterobacter hormaechei* subsp. *hormaechei*, *Enterobacter hormaechei* subsp. *steigerwaltii*, *Enterobacter hormaechei* subsp. *oharae*, *Enterobacter cloacae* subsp. *cloacae* and *Enterobacter cloacae* subsp. *dissolvens*.

DNA-DNA relatedness study

The strain NiVas 114^T showed >70% DNA-DNA similarity to *Enterobacter hormaechei* subsp. *hormaechei* (63%, reciprocal 62%), *Enterobacter hormaechei* subsp. *steigerwaltii* (63%, reciprocal 61%), *Enterobacter hormaechei* subsp. *oharae* (61%, reciprocal 60%), *Enterobacter cloacae* subsp. *cloacae* (61%, reciprocal 62%) and *Enterobacter cloacae* subsp. *dissolvens* (62%, reciprocal 59%). Using the genomic

DNA of NiVas 114^T as probe 36% and 58% DNA-DNA similarities were observed with *Enterobacter kobei* and *Enterobacter cancerogens* respectively. Considering 70% DNA-DNA relatedness as a cutoff value for species delineation (Wayne et al., 1987) NiVas 114^T stood strong to be regarded as a new species of *Enterobacter*.

Thus, based on phenotypic characteristics, phylogenetic analysis, cellular fatty acid profile and DNA-DNA relatedness studies, strain NiVas 114^T was regarded as a novel species of *Enterobacter*, for which the name *Enterobacter nickellidurans* sp. nov. is proposed. The strain demonstrating inducible nickel resistance system is capable of tolerating 10mM of Ni^{2+} in rich media. Southern blot assays showed the possibility of existence of a diverse nickel resistance genetic system in strain NiVas 114^T. Identification and molecular characterization of nickel resistance genetic locus in this isolate may be useful to explore the diversity and evolution of nickel resistance determinants in bacteria.

Description of *Enterobacter nickellidurans* sp. nov. (nic.ke.li.du'rans. N. L. n. nickelum, nickel; L. part. Adj. durans, insensible, resisting; N.L. part. adj. nickelidurans, nickel-resisting).

Cells are aerobic, Gram-negative, and motile and rod shaped (length 1.5-2 mm and width 0.8-0.52 mm). Colonies (2-3 mm) on nutrient agar are rounding, shiny, elevated and pale-white in color after 24 h at 36°C. Strain NiVas 114^T grows between 20 to 40°C and a pH range of 6 to 9. The optimum temperature and pH for growth are 36°C and 7.2 respectively. The isolate could tolerate 7% NaCl and grows also without salt. The strain could grow in presence of 10mM nickel chloride in rich medium (nutrient agar) and bears inducible nickel resistant determinant homologues to *cnrA* locus of *Ralstonia metallidurans* CH34. The strain is positive in Voges-Proskauer, β-galactosidase, arginine dihydrolyse, ornithine decarboxylase, esculine hydrolysis and Simmon's citrate test, but negative in indole production, lysine decarboxylase, H_2S production, urea hydrolysis and gelatin liquefaction. It produced acid from glucose, galactose, lactose, L-rhamnose, glycerol and α-methyl-D -glucoside. Used D-glucose, D-galactose, lactose, D-fructose, L-arabinose, sucrose, D-ribose, D-cellobiose, D-melibiose, D-mannose, trehalose, D-xylose, maltose, D-lactose, D-saccharate, D-glucosamine, 1-0-methyl-α-glucopyranose, 1-0-methyl-α-galactopyranose, D-mannitol, D-sorbitol, xylitol, adonitol, D-sorbitol, glycerol, *cis*-aconitate, fumarate, D-malate, malonate, D -galacturonate, succinate, phenylacetate, α-ketoglutarate, 3-hydroxybutyrate, L-alanine, L-leucine, L-threonine, L-proline, L-serine, L-histidine, L-glutamic acid and L-tryptophan as sole source of carbon; but L-fucose, D-raffinose, D-arabitol, erythritol, dulcitol, 3-methyl-D-glucopyranose, 5-keto-D-gluconate, putrescine, L-tartrate, caprate, caprylate, L-aspartic acid and L-asparagine are not utilized. The strain is resistant to tetracycline (25μg/ml) and ampicillin (50μg/ml), but sensitive to rifampicin (25μg/ml), nalidixic acid (20μg/ml) and chloramphenicol (30μg/ml). The major cellular fatty acids are $C_{16:0}$ (32.2%), $C_{18:1\,\omega7c}$ (22.24%) and Summed feature 3 ($C_{16:1\,\omega7c}$ and $C_{15:0\,iso\,2OH}$, 20.95%). The mol% G+C content of the genomic DNA of the isolate is 59.3.

The type strain is NiVas 114^T (= LMG 23000^T = CCUG50594^T = JCM13045^T), isolated from waters of Torsa River, Hasimara, West Bengal, India.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Research 25: 3389-3402.

APHA (1985) Standard Methods for the Examination of Water and Wastewater, 17th edn. Washington, DC: American Public Health Association.

Bhadra B, Nanda AK and R. Chakraborty R (2006) Inducible Nickel resistance in a river isolate of India phylogenetically ascertained as a novel strain of *Acinetobacter junii*. World. J. Microbiol. Biotech. 22: 225-232.

Bhadra B, Roy P and Chakraborty R (2005) *Serratia ureilytica* sp. nov., a novel urea-utilizing species. Int. J. Syst. Evol. Microbiol. 55: 2155-2158.

Bhadra B, Nanda AK, Chakraborty R (2007) Fluctuation in recoverable nickel and zinc resistant copiotrophic bacteria explained by the varying zinc ion content of Torsa River in different months. Arch. Microbiol. 188: 215-224.

Bhadra B, Das S, Chakraborty R and Nanda AK (2005) Investigation of some basic water quality parameters of the North Bengal terai river Kaljani- a tributary of River Torsa, and comparison thereof with the mainstream. *J. Environ. Biol.* 26: 277-286.

Bhadra B, Mukherjee S, Chakraborty R and Nanda AK (2003) Physicochemical and Bacteriological investigation on the river Torsa of North Bengal. *J. Environ. Biol.* 24: 125-133.

Brosius J, Palmer ML, Kennedy PJ and Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal rRNA gene from Escherichia coli. Proc. Nat. Acad. Sci. USA 75: 4801–4805.

Drancourt M, Bollet C, Carta A and Rousselier P (2001). Phylogenetic analysis of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov., and *Raoultella planticola* comb. nov., Int. J. Syst

Evol. Microbiol. 51: 925-932.

Ezaki B, Ogura T, Mori H, Niki H and Hiraga S (1989) Involvement of DnaK protein in mini-F plasmid replication: temperature-sensitive seg mutations are located in the dnaK gene. Mol. Gen Genet. 218: 183-189.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution 39: 783-791.

Gerhardt P, Murray RGE, Wood WA and Krieg NR (editors) (1994)Methods for General and Molecular Bacteriology. Washington, DC: American Society for Microbiology (1994).

Grass G, Fan B, Rosen BP, Lemke K, Schlegel HG and Rensing C (2001). NreB from *Achromobacter xylosoxidans* 31A is a nickel induced transporter conferring nickel resistance. J. Bacteriol. 183: 2803-2807.

Hausinger RP (1987) Nickel utilization by microorganism, Microbiol. Rev. 51: 22–42.

Hoffmann H and Roggenkamp A (2003) Population genetics of the nomenspecies Enterobacter cloacae, Appl. Environ. Microbiol. 69: 5306-5318.

Hoffmann H, Stindle S, Ludwig W, Stumpf A, Mehlen A, Monget D, Pierard D, Ziesing S, Heesemann J, Roggenkamp A and Schleifer KH (2005), *Enterobacter hormaechei* subsp. *oharae* subsp. nov., *E. hormaechei* subsp. *hormaecei* comb. Nov., and *E. hormaechei* subsp. *steigerwaltii* subsp. nov, three new subspecies of clinical importance. J. Clin. Microbiol. 43: 3297-3303.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.

Kumar S, Tamura K and Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinf. 5: 150-163.

Liesegang H, Lemke K, Siddiqui RA and H. G. Schlegel HG (1993) Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL 28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. 175: 767-778.

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P and Van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162: 328-34.

Mollet C, Drancourt M and Raoult R (1997) *rpoB* sequence analysis as a novel basis for bacterial identification, Mol. Microbiol. 26: 1005-1011.

Nies D, Mergeay M, Friedrich B and Schlegel HG (1987). Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. 169: 4865–4868.

Park EP, Young KE, Schlegel HG, Rhie HG and Lee HS (2003), Conjugative plasmid mediated inducible nickel resistance in *Hafnia alvei* 5-5. Int. Microbiol. 6: 57-64.

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: a Laboratory Mannual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmidt T, Schlegel HG (1994). Combined nickel-cobalt-cadmium resistance in *Alcaligenes xylosoxidans* 31A. *J Bacteriol* 176: 7045-7054.

Shivaji S, Chaturvedi P, Suresh K, Reddy GSN, Dutt CB, Wainwright M, Narlikar JV and Bhargava PM (2006). *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. Int. J. Syst. Evol. Micorbiol. 56: 1465-1473.

Shivaji S, Gupta P, Chaturvedi P, Suresh K, Dellile D (2005). *Marinobacter maritimus* sp. nov., a psychrotolerant strain isolated from sea water off the subantarctic Kerguelen islands. Int. J. Syst. Evol. Microbiol. 55: 1453-1456.

Stoppel RD and Schlegel HG (1995). Nickel resistant bacteria from anthropogenically nickel-polluted and naturally nickel-percolated ecosystems. App. Enviro. Microbiol. 61: 2276-2285.

Stoppel RD, Mayer M, Schlegel HG (1995) Nickel resistant determinant cloned from the enterobacterium *Klebsiella oxytoca*: conjugal transfer, expression, regulation and DNA homologies to various nickel resistant bacteria. Biometals 8: 70-79.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1997), The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acid. Res. 25: 4876-4882.

Tibazarwa C, Wuertz S, Mergeay M, Wyns L and van Der Lelie D (2000). Regulation of the cnr cobalt and nickel resistance determinant of Ralstonia eutropha (Alcaligenes eutrophus) CH34, J. Bacteriol. 182: 1399-1409.

Wayne LG, Brenner DJ, Colwell RR & nine other authors (1987) International committee on Systematic Bacteriology. Report of the ad-hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37: 463-464.