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Isolation and Characterization of Nickel Uptake by Nickel Resistant Bacterial Isolate (NiRBI)

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Objective Bioremediation technology has gained importance because microbes could be the convenient source of bio-absorption/bioaccumulation of metals from effluent streams. **Methods** The nickel-resistant bacterial isolates (NiRBI) were selected from various bacterial isolates from industrial effluent and grown in nutrient broth containing different concentrations of nickel sulfate (0.3-3.0 mmol/L) and their capability of accumulating metal from the medium. **Results** Well-defined growth of NiRBI was observed in the medium containing up to 2.5 mmol/L of nickel. The isolate was identified using 16S rRNA and closely related to *Pseudomonas fragi.* Maximum accumulation of nickel (0.59 mg/g dry weight of bacterial cells) was observed when NiRBI was grown in media containing 2 mmol/L of nickel. The protein profile of the NiRBI cellular extract by SDS-PAGE showed two metal stress-induced proteins of molecular weight 48 KD and 18 KD with a simultaneous down regulation of four proteins of 46.7 KD, 42.2 KD, 19.7 KD, and 4.0 KD. **Conclusion** 48 KD and 18 KD proteins play a role in metal resistance mechanism by NiRBI.

Key words: Nickel; Nickel-resistant bacterial isolates; Induction; Repression; Proteins

INTRODUCTION

Today indiscriminate and uncontrolled discharge of metal-contaminated industrial effluent in the environment has become an issue of major concern. Heavy metals are the major toxicants found in industrial waste water. Nickel toxicity is comparable to cobalt but its toxic effect on humans is better documented, up to 20% of the populations in industrially developed countries have positive results in epidermal testing[1]. Many industries such as electroplating, paint, pigments, batteries, and gas turbines, discharge aqueous effluents containing relatively high levels of nickel. Trace elements such as chromium, lead, and nickel, have been detected from industrial effluents collected in and around industrial areas[2].

Bioremediation can be used to effectively reduce contaminant toxicity, mobility or volume to levels that are innocuous to human health and ecosystem[3]. Microorganisms possess mechanisms that regulate metal ion accumulation to avoid heavy metal toxicity and there are many reports about microbial resistance to heavy metals. Various microorganisms such as bacteria[4], yeast[5], fungi[6], algae[7-8], and plants[9] have been reported to tolerate and remove heavy metals from aqueous solutions. The advantages of using microbes for bio-remediation include natural occurrence, cheap production, easy availability to treat large volumes of wastewater due to rapid kinetics and high selectivity in terms of removal and recovery of specific metals[10]. Levinson *et al.*[11] have reported that lead-resistant strain of *Staphylococcus aureus* tolerates approximately 600 times higher lead nitrate concentration than lead-sensitive strains[11]. The potential of several bacterial strains to detoxify chromate has been described with a view to developing process for microbiological detoxification of polluted water[12-13].

The study of nickel-resistant microorganisms that are capable of resisting and surviving in these polluted environments provides the knowledge for nickel bioremediation.

MATERIALS AND METHODS

Isolation of Bacteria

The effluent samples were collected under aseptic condition in sterilized bottles from Marigold

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Paint Pvt. Ltd., G. I. D. C. Estate, Vithal Udyognagar, Dist, Anand, Gujarat, and used for isolation of nickel-resistant bacterial isolates (NiRBI) using Bromfield[14] medium containing 0.16 mmol/L nickel ($NiSO_4.6H_2O$). Plates were incubated at 30℃ for 72 h and colonies were randomly picked, isolated, and purified. The well-defined isolated colonies were picked up on the basis of colony morphological characteristics and transferred to nutrient agar and preserved at 4℃ in a refrigerator.

Screening, Identification, and Growth Kinetics of Nickel-resistant Bacterial Isolates (NiRBI)

A number of purified nickel-resistant bacterial isolates were grown on sterilized nutrient agar plates containing different concentrations of 0.0 mmol/L, 0.3 mmol/L, 0.5 mmol/L, 0.7 mmol/L, 1.0 mmol/L, 1.5 mmol/L, 2.0 mmol/L, 3.0 mmol/L, 4.0 mmol/L, and 5.0 mmol/L of nickel ($NiSo_4.H_2O$) at 37℃ for 24 h to 120 h to find out their resistance towards nickel.

Bioaccumulation of Nickel

One strain of NiRBI was selected based on its maximum metal tolerance limit and identified on the basis of morphological and phenotypic criteria. Identification of the strain was done by amplification of the partial 16S rRNA gene using 8F (5′AGAGTTTGATCCCTGGCTCAG3′) and 519R (5′G(AT) ATTACCGC GGCC(GT)GCTG 3′). The sequencing was performed at Stopford Sequencing Facility, Department of Biological Sciences, University of Manchester, UK. Preliminary identification of the strain was obtained using BLAST programme. The 16S rRNA sequences were aligned by using Clustal W (1.8), a pair-wise progressive-alignment algorithm was used to align the sequences. The method of Jukes and Cantor[15] was used to compute the evolutionary distances, and the final tree was assembled with Tree View (Page, R. D. M. 1996). The strain NiRBI grown in a 250 mL flask containing nutrient broth with different concentrations of nickel ($NiSO_4.H_2O$) on a rotary shaker (37℃ temperature and 200 rpm) to study its growth kinetics.

NiRBI grown in nickel free media (control) and in different concentration of nickel (experimental) were harvested during stationary phase by high-speed centrifugation (10 000 g for 30 min). The cell pellets were washed 2-3 times with normal saline, dried to constant weight, digested with concentrated acid mixture (nitric acid: sulfuric acid: perchloric acid-6:3:3)[16] and used for nickel estimation by inductivity coupled plasma spectrophotometer (Perkin Elmer 3300RL). The results were expressed as mean of triplicate observations and the data were compared with those of cells grown in nickel free medium.

SDS-Polyacrylamide Gel Electrophoresis of Total Cell Proteins

The cell pellets of NiRBI grown with 2.5 mmol/L nickel and in metal free medium were used to study their protein profile. The cells were harvested during stationary phase and suspended in SDS sample buffer (62 mmol/L Tris HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated for 5 minutes at 100˚C immediately before electrophoresis[17]. Chromosomal DNA was sheared by a sonicator with an immiscible tip. Protein was separated using cell extract of NiRBI grown with 2.5 mmol/L of nickel and nickel-free medium by vertical gel electrophoresis using denaturing 12% SDS polyacrylamide gel and stained with Coomassie brilliant blue R 250.

RESULTS

Table 1 suggests that NiRBI grown without or with different concentrations of nickel (0.0 mmol/L to 3.0 mmol/L) showed a growth up to 2.5 mmol/L nickel concentration in the medium. The well-defined colonies of NiRBI were observed after 24 h of incubation in the medium up to 1.0 mmol/L concentration of nickel, and further increase in nickel concentration showed a depression in the growth of NiRBI.

TABLE 1

Capacity and Resistance of NiRBI to Growth on Nickel at Different Concentrations

Concentration (mmol/L)	Colony Observation				
	24 h	48 h	72 h	96 h	120 h
0.0	+++++	+++++	+++++	+++++	+++++
0.3	+++++	+++++	+++++	+++++	+++++
0.5	+++++	+++++	+++++	+++++	+++++
0.7	+++++	+++++	+++++	+++++	+++++
1.0	+++++	+++++	+++++	+++++	+++++
1.5	+++	++++	+++++	+++++	+++++
2.0	++	+++	++++	++++	++++
2.5	+	++	+++	+++	+++
3.0	–	–	–	–	–

Fig. 1 depicts the phylogenetic tree derived from 16S rRNA sequence data of a strain of NiRBI and other related species. Phylogenetic analysis of the 16S rRNA sequence of the strain of NiRBI placed it within the Gram-negative phylum of the bacteria. The nucleotide sequence of a 496bp 16S rRNA fragment

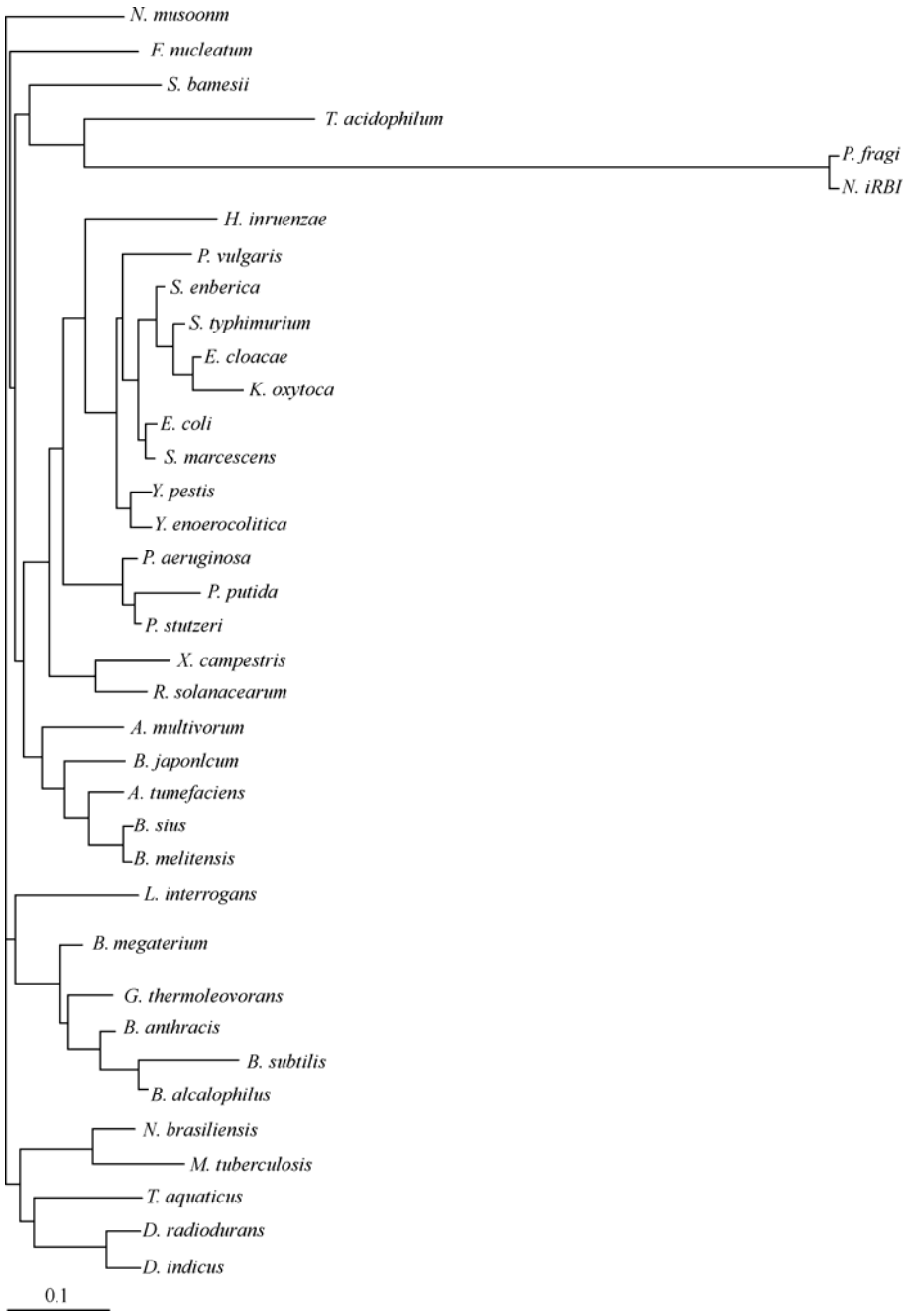

FIG. 1. Phylogenetic tree derived from the 16S rRNA sequence data of a strain of NiRBI and other related species. The scale bar corresponds to 1% nucleotide sequence difference.
Fusobacterium; Proteus; Salmonella; Enterobacter; Klebsiella; Escherichia; Yersinia; Xanthomonas; Brucella; Leptospira; Bacillus; Bacillus; Nocardia; Mycobaterium; Thermus

was obtained, which aligned closely with the sequence of *Pseudomonas fragi* (position 1-496, EMBL accession No. AF467304) with two mismatches (G instead of C at position 326 and A instead of G in 412 position in the NiRBI sequence).

The results of growth kinetics studies of NiRBI have shown that lag phase increases as the concentration of nickel is increased in the medium (Fig. 2). NiRBI grown at 2 mmol/L-2.5 mmol/L nickel containing medium showed a four-fold increase in lag phase compared with the control. Cellular growth of NiRBI at the concentration of 0.3 mmol/L to 1.0 mmol/L nickel was better than that in metal-free medium, whereas 1.5 mmol/L to 2.5

mmol/L concentration of nickel in the medium decreased the cellular growth of NiRBI as compared with control cells.

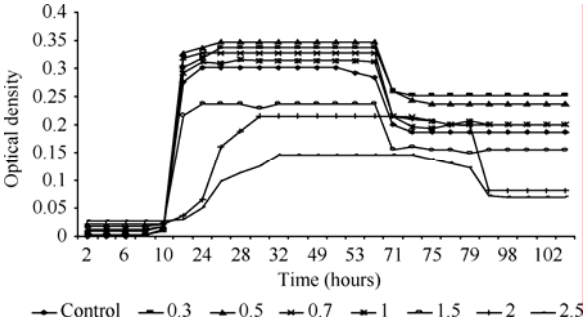

FIG. 2. Growth kinetic of NiRBI grown in the absence and presence of different concentrations (0.3-2.5 mmol/L) of Nickel.

The cellular uptake of nickel by NiRBI was found to depend on the concentration of metals in the medium and was increased with increase in nickel concentration in the liquid medium up to 2.0 mmol/L (Fig. 3). Maximum accumulation of nickel was observed at 2.0 mmol/L nickel concentration, which remained at the same level till 2.5 mmol/L nickel concentration.

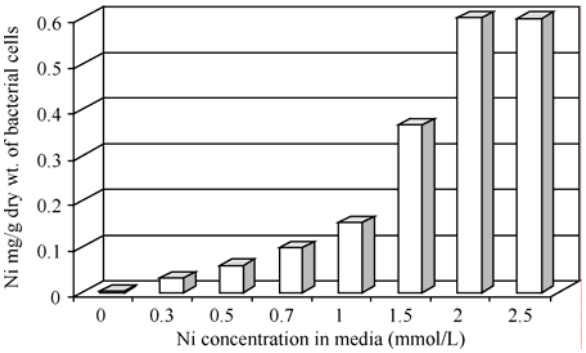

FIG. 3. Nickel accumulation at different concentrations (0.3-2.5 mmol/L) of NiRBI.

SDS-polyacrylamide gel electrophoresis of the whole cell extract of NiRBI grown in the medium containing 2.0 mmol/L nickel and metal-free medium is shown in Fig 4. A number of proteins were seen in control and cell growth in nickel containing medium was down-regulated by NiRBI. The four proteins with molecular weights of 46.7 KD, 42.2 KD, 19.7 KD, and 4.0 KD, respectively, were repressed in the cells grown with nickel, whereas two proteins with molecular weights of 48.0 KD and 18 KD, respectively, were not observed in the control cells but observed in NiRBI grown at the optimal nickel concentration.

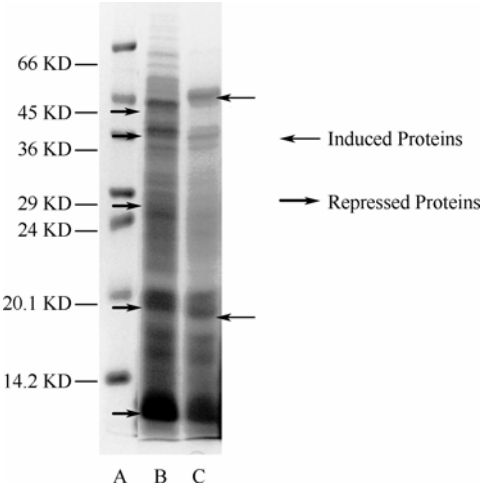

FIG. 4. Separation of total cellular protein from NiRBI grown with and without nickel by polyacrlamide gel eletrophoresis.

DISCUSSION

During the present investigation efforts were made to isolate new strains of bacteria which tolerate and accumulate nickel. Morphological characterization of NiRBI showed NiRBI as Gram-negative species. At higher concentrations metal ions formed unspecific complex compounds in the cells, which may lead to toxic effects. The excess zinc ions showed toxic effects on the cells of isolated cultures and considerable inhibition of growth as compared with metal-free controls[18]. The phylogenetic analysis of 16S rRNA sequence of the strain of NiRBI revealed the sequence of two mismatches. Thus the partial sequence was not submitted to the nucleotide database as an independent sequence, based on the consistent phylogenic placement of the strain of NiRBI within the *Pseudomonas* group of Gram-negative phylum regardless of the algorithm used to infer the tree[19] . The findings are in accordance with the results of other researches on a variety of heavy metals with different microorganisms[20].

The possible reason for the maximum accumulation of nickel at 2.0 and 2.5 mmol/L nickel concentration could be due to fact that the binding sites might be getting saturated at 2.0 mmol/L external nickel concentration. The similar concentration-dependent accumulation of metals was also shown in a number of other metals. The amount of zinc accumulation in *Pseudomonas putida* strain S4 increased as the zinc concentration was increased in the medium but was stabilized at 2.0 mmol/L[20]. Lee *et. al.*[21] reported that *P. putida* strain 06909 tolerates 11.5 mmol/L zinc and 1.0 mmol/L nickel.

SEM and EDEX studies have shown the concentration-dependent accumulation of manganese in the cell membrane of *Pseudomonas aurogenosa*[22].

Protein plays an important role in heavy metal resistance[23]. The two proteins observed in the cells extracts of NiRBI, grown at optimal nickel concentration suggest that these proteins are modified or induced protein, which may have some role in transport and/or storage of nickel by bacterial isolates. Darke[24] also reported that several nickel-containing proteins exist in *Clostridium pasturianum.* Liesegang *et al.*[25] have characterized the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. An ABC-transporter and a periplasmic nickel binding protein are shown to uptake nickel by *Escherichia coli*[26-27]. The *ncc* of *Alcaligenes xylosoxidans* causes a high level of combined nickel, cobalt and cadmium resistance[28]. The metal stress-induced proteins in NiRBI grown with nickel may be involved in metal resistance, transport and/or sequestration of nickel, which needs further investigation.

The present results thus provide some interesting observations about the new strains of bacteria, which tolerate and accumulate nickel. However the practical application of this organism to the treatment of nickel-containing wastewater needs further studies.

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