Polish Journal of Microbiology
2016, Vol. 65, No 1, 115–118

SHORT COMMUNICATION

Enterobacter asburiae KUNi5, a Nickel Resistant Bacterium for Possible Bioremediation of Nickel Contaminated Sites

ANIRUDHA PAUL and SAMIR KUMAR MUKHERJEE*

Department of Microbiology, University of Kalyani, Kalyani, India

Submitted 6 February 2015, revised 25 May 2015, accepted 31 May 2015

Abstract

Nickel resistant bacterial strain *Enterobacter asburiae* KUNi5 was isolated and showed resistance up to 15 mM and could remove Ni optimally better at 37°C and pH 7. Maximum removal was found at initial concentration of 0.5 to 2 mM, however, growth and Ni removal were affected by other heavy metals. Major amount of the metal was accumulated in the membrane fractions and certain negatively charged groups were found responsible for Ni binding. KUNi5 could also produce 1-aminocyclopropane-1-carboxylate deaminase, indole-acetic acid and siderophore. It seems that KUNi5 could be a possible candidate for Ni detoxification and plant growth promotion in Ni-contaminated field.

Key words: *Enterobacter* sp., bioremediation, nickel

Nickel (Ni) contamination of the environment from industrial sources has been reported to affect living organisms and is being considered as a global concern (Khodadoust *et al*., 2004; Cecchie and Zanchi, 2005). Irrigation of agricultural fields with Ni containing wastewater is affecting crop yield and also creating potential risk of biomagnification by entering into the food chain (Sanders *et al*., 1987; Singh *et al*., 2012).

Different mechanisms of Ni resistance have earlier been documented (Gadd, 1988; Nies, 1999; Sar *et al*., 2001; Salvador *et al*., 2007; Desale *et al*., 2014), of which certain mechanisms could be exploited for successful remediation of sites contaminated with such hazardous metal. Screening of Ni resistant microbes from contaminated sites with potential Ni immobilizing ability could provide a significant impact on environmental management. Moreover, such bacterial inoculants having metal immobilizing ability with plant growth promoting (PGP) features have opened other eco-friendly measures for sustainable agriculture (Burd *et al*., 2000; Faisal and Hasnain, 2006; Denton, 2007; Rajkumar and Freitas, 2008). Considering this background, the objectives of this study were to isolate and characterize a Ni resistant bacterial candidate from Ni contaminated soil and to examine its remediating ability with PGP features under experimental conditions.

Ni resistant bacterial strain was isolated from industrial waste contaminated soil (pH 7.4, Ni content – 2.9 mg/Kg of soil) near Durgapur, West Bengal, India. Dilution plate technique was followed to isolate the Ni resistant strain. The bacterial strain that showed maximum tolerance to Ni (as $NiCl_2$, $6H_2O$) on nutrient agar (NA) medium (HiMedia, India) was selected and designated KUNi5. The colony was further transferred to modified Tris minimal agar (TMA) medium (Sar *et al*., 1998) for culture purification and was maintained. The maximum tolerance levels of KUNi5 to other heavy metals were also assessed in modified Tris minimal broth (TMB). The isolate KUNi5 was identified on the basis of its morphological and biochemical features (Vos *et al*., 2009) and 16S rDNA sequence analysis. The base sequence was aligned using BLAST function (Altschul *et al*., 1990) at the National Center for Biotechnology Information (NCBI) database for its identification.

To determine the effect of Ni on bacterial growth under aerobic culture conditions, equal volume of young cell suspensions were inoculated (~4 log CFU/mL) into modified TMB with different concentrations of Ni (0.5, 1.0, 2.0, 3.0 and 4.0 mM) as $NiCl_2$, $6H_2O$ separately and each set including the control set was incubated at 37°C on a rotary shaker. The growth of KUNi5 was measured by colony count method at certain time intervals on modified TMA plates. Ni removal by KUNi5 at each treatment regime was also determined by quantifying the residual amount of Ni present in the medium. Supernatant was harvested by centrifugation and was acid-digested; Ni contents in the supernatants were measured by Atomic Absorption Spectrophotometer

* Corresponding author: S.K. Mukherjee, Department of Microbiology, University of Kalyani, Kalyani, India; **e-mail: dr.samirmukherjee@gmail.com**

(iCE 3000 AAS, Thermo Scientific, Austria). An uninoculated set for each treatment regime was also maintained and processed similarly to monitor the artifacts that might arise owing to metal chelation on the surface of the container. The percentage of Ni removal was calculated following the equation: $R\ (\%) = (C_o - C_e) \times \times 100/C_o$ (C_o = Initial Ni concentration; C_e = Equilibrium Ni concentration).

To study the effect of initial pH on Ni removal under culture conditions, modified TMB supplemented with 2 mM Ni with pH values ranging from 5 to 8 was inoculated with equal volume of bacterial inoculum and incubated at 37°C. The growth patterns of KUNi5 and Ni removal at respective treatment regimes were also measured. The effect of temperature on Ni removal and growth under aerobic growth conditions was determined in TMB (pH 7) having 2 mM of Ni at different temperatures (30°C, 37°C, and 40°C).

The effects of other heavy metals separately (0.1 mM Cu as $CuSO_4$, $5H_2O$ or 0.1 mM Co as $CoCl_2$, $6H_2O$ or 0.1 mM Zn as $ZnCl_2$ or 0.1 mM Cd as $CdCl_2$, $5H_2O$) on Ni removal were studied. Equal volume of cell suspension was inoculated in modified TMB (pH 7, 2 mM Ni) to each treatment regime and incubated for 48 h at 37°C. Culture set without having any selected heavy metals other than Ni was considered as control. After 48 h the amount of Ni removal was measured as mentioned earlier.

Amount of Ni in different cell fractions were measured by growing the cells in modified TMB supplemented with 2 mM Ni for 48 h at 37°C. Cellular fractions were harvested and prepared following Sar *et al.* (2001). The amount of Ni in different fractions was measured after acid digestion as mentioned before. Fourier transform infra red (FTIR) spectroscopy was performed to visualize the shifts in the absorption spectrum of the treated cell fractions in comparison to the control one. Cells from both control (Ni free) and treated (2 mM Ni) sets were harvested after 48 h of growth and washed with HEPES-NaOH buffer (100 mM, pH 7.2), and were then lyophilized. Infra red spectra were obtained by using Spectrum One FT-IR Spectrometer (Perkin Elmer, USA).

For determining possible PGP features of KUNi5, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-acetic acid (IAA) and siderophore producing abilities were considered. ACC-deaminase activity of KUNi5 was qualitatively assayed following Dworken and Foster (1958). Siderophore and IAA production by KUNi5 were qualitatively determined following respective standard methods (Schwyn and Neilands, 1987; Sosa-Morales *et al*., 1997).

KUNi5 cells were found to be Gram-negative and rod shaped. Based on the analysis of 16S rDNA sequence (NCBI GenBank Accession No. KM277458), KUNi5 has been identified as a strain of *Enterobacter asburiae*. It could tolerate 15 mM Ni in nutrient broth and 10 mM Ni in modified TMB. The difference in tolerance limit in two different media might be due to the complexation of Ni with undefined ingredients in the nutrient broth thus lowered toxicity (Sau *et al*., 2008). KUNi5 showed varied degree of tolerance to heavy metals in the order of Ni>Zn>Cu>Co>Cd (10, 7, 5, 2 and 1 mM respectively) in TMB. Tolerance to different toxic metals might be due to the selection pressure of contaminant metals at the sampling site posed on the KUNi5 population. The strain showed ability to produce ACC deaminase, IAA and siderophore, which might be of important agronomic value for using as PGP candidate. PGP features of *E. asburiae* and its suitability of using in sustainable agriculture were also documented earlier (Ahemad and Khan, 2010; Zhao *et al*., 2011).

Growth retardation and extended lag phase of KUNi5 were found with increasing Ni concentration in the medium (Fig. 1a). Irrespective to the Ni concentration, highest cell mass was obtained after 48 h of growth. The extended lag phase might be due to the requirement of time for buffering the metal stress (Patel *et al*., 2006; Das *et al*., 2014). KUNi5 grew optimally better at pH 7 and 37°C under Ni stress (2 mM) conditions (Fig. 1b, c). Irrespective of the initial Ni concentration, pH of the medium and incubation temperature, highest Ni removal was found after 48 h and thereafter remained same (Fig. 1d, e, f). When initial Ni concentration was considered as the factor, maximum removal was found at an initial concentration ranging from 0.5 m to 2 mM of Ni in the medium. Incubation temperature of 37°C and pH 7 were found to be conducive for Ni removal. From the experimental analysis it seems that active biomass might play a crucial role on Ni removal. Other heavy metals were found to antagonize growth and Ni removal (Fig. 2). Cadmium was found to be most antagonistic than Co, Zn and Cu. Similar types of results were also observed with other bacteria (Fu and Maier, 1991; Hussein *et al*., 2004; Das *et al*., 2014). Decrease in Ni removal might be due to the growth inhibitory or competitive effect of other metals on Ni binding (Kaltwasser and Frings, 1980; Fu and Mier, 1991).

From the cellular fractionation study it was observed that most of the metals were accumulated (nmol of Ni/mg dry weight) in the membrane fractions (~53%) followed by periplasmic space (~34%) and cytosol (~13%). Such pattern of accumulation and sequestration of toxic cations was also reported earlier (Cha and Cooksey, 1991; Sar *et al*., 2001). In order to ascertain the role of different functional groups involved in Ni binding, FTIR analysis were done for both lyophilized control and treated (2 mM Ni) biomass. A large shift was found due to Ni exposure in the intensity of absorption band peak for OH stretch from 3300.61 cm^{-1} to 3344.68 cm^{-1} (Fig. 3). Small shifts occurred in the intensity of the bands 1539.03 cm^{-1} to 1546.21 cm^{-1}

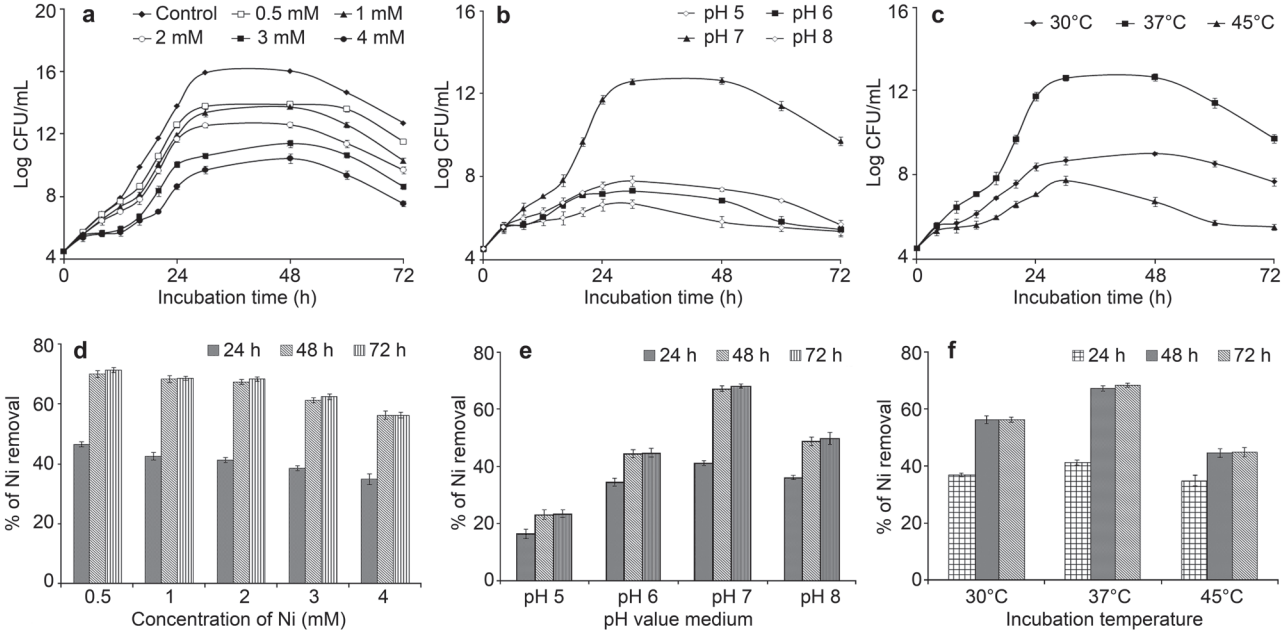

Fig. 1. Growth response of KUNi5 in TMB with different concentration of Ni and without Ni as control (a), having 2 mM Ni at different pH (b), and having 2 mM Ni at different temperatures (c). Ni removal ability of KUNi5 grown in TMB at different time intervals with increasing Ni concentrations (d), at different pH values (e), and at different temperatures (f). Data are the mean of three replications with ± SE ($p < 0.05$)

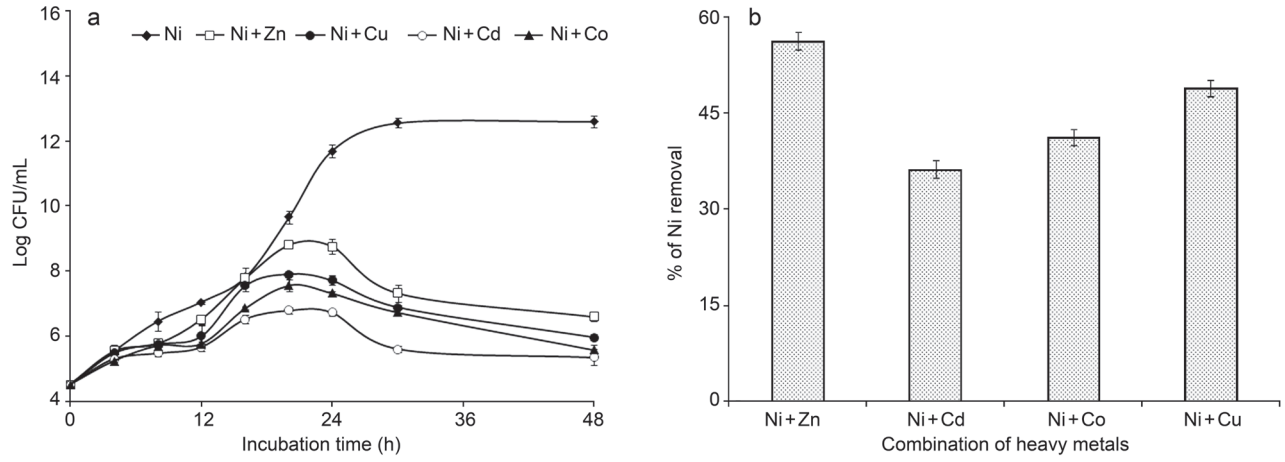

Fig. 2. Growth response (a) and Ni removal ability (b) of KUNi5 grown in TMB having 2 mM Ni after 48 h in presence of other heavy metals (0.1 mM). Data are the mean of three replications with ± SE ($p < 0.05$).

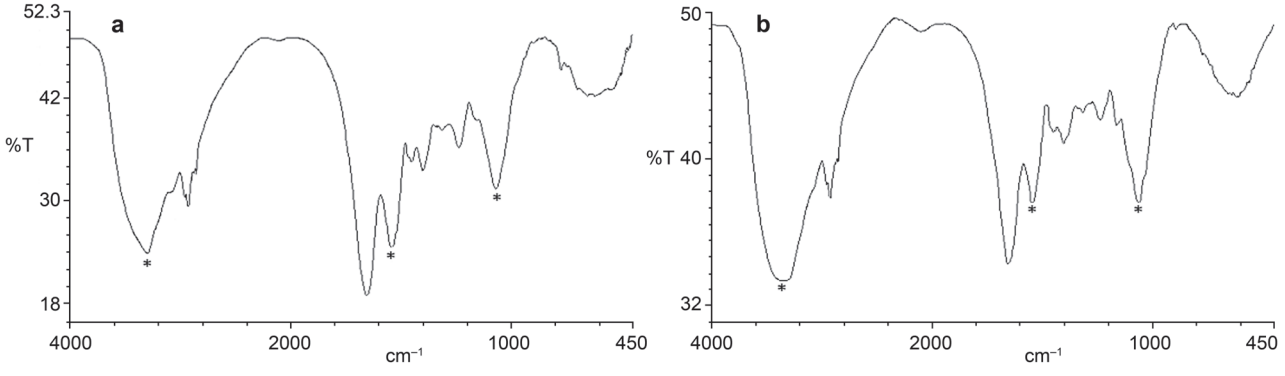

Fig. 3. FTIR spectra of untreated (a) and 2 mM Ni treated (b) cell biomass of KUNi5. Major shifting in the intensity of absorption band peaks are indicated by [*].

indicating N-H bending and C-H stretching in amide II, and 1068.86 cm^{-1} to 1063.29 cm^{-1} suggesting asymmetric and symmetric stretching of PO^{2-} and $P(OH)_2$ or C-OH bonds in alcohols or polysaccharide of bacterial biomass (Jiang *et al*., 2004). It seems that positively charged metal ions bind to such negatively charged functional groups present on the bacterial cell fractions (Anand *et al*., 2006; Desale *et al*., 2014).

Thus, the strain KUNi5 showed its promise for bioremediation of Ni-contaminated crop field and also as a plant growth promoting agent, although the extent of Ni immobilization and plant growth promotion under actual metal-polluted field conditions warrants further investigation.

Acknowledgments

Authors acknowledge the support received from University of Kalyani, Kalyani, India and Department of Science and Technology under the PURSE program, Government of India, New Delhi, India.

Literature

Ahemad M. and M.S. Khan. 2010. Plant growth promoting activities of phosphate solubilizing *Enterobacter asburiae* as influenced by fungicides. *Eurasia. J. Bio. Sci.* 4: 88–95.

Altschul S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 219: 403–410.

Anand P., J. Isar, S. Saran and R.K. Saxena. 2006. Bioaccumulation of copper by *Trichoderma viride. Bioresour. Technol*. 91: 1018–1025.

Burd G.I., G.D. Dixon and B.R. Glick. 2000. Plant growth promoting bacteria that decrease heavy metal toxicity in plants. *Can. J. Microbiol.* 46: 237–245.

Cecchie C.G.S. and C. Zanchi. 2005. Phytoremediation of soil polluted by nickel using agricultural crops. *Environ. Manage.* 36: 675–681.

Cha J.S. and D.A. Cooksey. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc. Natl. Aca. Sci. USA* 88: 8915–8919.

Das P., S. Sinha and S.K. Mukherjee. 2014. Nickel bioremidiation potential of *Bacillus thuringiensis* KUNi1 and some environmental factors in nickel removal. *Bioremed. J.* 18(2): 169–177.

Denton B. 2007. Advances in phytoremediation of heavy metals using plant growth promoting bacteria and fungi. MMG 445. *Basic. Biotechnol. J.* 3: 1–5.

Desale P., D. Kashyap, N. Nawani, N. Nahar, A. Rahman, B. Kapadnis and A. Mandal. 2014. Biosorption of nickel by *Lysinibacillus sp.* BA2 native to bauxite mine**.** *Ecotoxicol. Environ. Saf.* 107: 260–268.

Dworken M. and J. Foster. 1958. Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* 75: 592–601.

Faisal M. and S. Hasnain. 2006. Plant growth promotion by *Brevibacterium* under chromium stress. *Res. J. Bot.* 1: 24–29.

Fu C. and R.J. Maier. 1991. Competitive inhibition of an energy-dependent nickel transport system by divalent cations in *Bradyrhizobium japonicum* JH. *Appl. Environ. Microbiol.* 57: 3511–3516.

Gadd G.M. 1988. Accumulation of metals by microbes and algae. *Biotechnology* 60: 401–430.

Hussein H., S.F. Ibrahim, K. Kandeel and H. Moawad. 2004. Biosorption of heavy metals from waste water using *Pseudomonas* sp. *eJ. Biotechnol.* 7(1). doi: 10.2225/vol7-issue1-fulltext-2.

Jiang W., A. Saxena, B. Song, B.B. Ward, T.J. Beveridge and S.C.B. Myneni. 2004. Elucidation of functional groups on gram positive and gram negative bacterial surfaces using infrared spectroscopy. *Langmuir* 20: 11433–11442.

Kaltwasser H. and W. Frings. 1980. Transport and metabolism of nickel in microorganisms, pp. 463–491. In: Nriagu J.O. (ed). *Nickel in the environment.* John Wiley & Sons, New York.

Khodadoust A.P., K.R. Reddy and K. Maturi. 2004. Removal of nickel and phenanthrene from kaolin soil using different extractants. *Environ. Eng. Sci.* 21: 691–704.

Nies D.H. 1999. Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.* 51: 730–750.

Patel J.S., C.P. Patel and K. Kalia. 2006. Isolation and characterization of nickel uptake by nickel resistant bacterial isolate (NiRBI). *Biomed. Environ. Sci.* 19: 297–301.

Rajkumar M. and H. Freitas. 2008. Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. *Chemosphere* 71: 834–842.

Salvador M., G. Carolina and E. Jose. 2007. Novel nickel resistance genes from the rhizosphere metagenome of plants adapted to acid mine drainage. *Appl. Environ. Microbiol.* 73(19): 6001–6011.

Sanders J.R., S.P. Mc Grath and T. Adams. 1987. Zn, Cu, and Ni concentration in soil extracts and crops grown on four soils treated with metal loaded sewage sludges. *Environ. Pollut.* 44: 193–210.

Sar P., S.K. Kazy and S.P. Singh. 2001. Intracellular nickel accumulation by *Pseudomonas aeruginosa* and its chemical nature. *Lett. Appl. Microbiol.* 32: 257–261.

Sar P., S.K. Kazy, R.K. Asthana and S.P. Singh. 1998. Nickel uptake by *Pseudomonas aeruginosa*: role of modifying factors. *Current. Microbiol.* 37: 306–311.

Sau G.B., S. Chatterjee and S.K. Mukherjee. 2008. Isolation and characterization of a Cr(VI) reducing *Bacillus firmus* strain from industrial effluents. *Pol. J. Microbiol.* 57: 327–332.

Schwyn B. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical. Biochem.* 160: 47–56.

Singh S., M. Zacharias, S. Kalpana and S. Mishra. 2012. Heavy metal accumulation and distribution pattern in different vegetable crops. *J. Environ. Chem. Ecotoxicol.* 4(10): 170–177.

Sosa-Morales M.E., F. Guevara-Lara, V.M. Martinez-Juarez and O. Paredes-Lopez. 1997. Production of indole-3-acetic acid by mutant strains of *Ustilago maydis* (maize smut/huitlacoche). *Appl. Microbiol. Biotechnol.* 48: 726–729.

Vos P., G. Garrity, D. Jones, N.R. Krieg,W. Ludwig, F.A. Rainey, K.H. Schleifer and W. Whitman. 2009. *Bergey's Manual of Systematic Bacteriology*. 2nd ed. Vol. 3., Springer, New York, USA.

Zhao H., H. Yan, S. Zhou, Y. Xue, C. Zhang, Lihuozhang, X. Dong, Q. Cui, Y. Zhang, B. Zhang and Z. Zhang. 2011. The growth promotion of mung bean (*Phaseolus radiatus*) by *Enterobacter asburiae* HPP16 in acidic soils. *Afr. J. Biotechnol.* 10(63): 13802–13814.