Summary

Because all of biology is connected, one can often make a breakthrough with an organism that exaggerates a particular phenomenon, and later explore the generality

The genus *Deinococcus* is represented by members that are extremely resistant to ionising as well as non-ionising radiation, desiccation, H_2O_2 and several mutagenic agents as mitomycin C and hence are aptly described as "polyextremophiles". The isolation of these organisms often employs ionising radiation as selective pressure that eliminates the radiation sensitive population, obliterating any information regarding the comparative distribution of this group in natural habitats.

A molecular method based on 16S rRNA gene was developed for easy detection and understanding the deinocccocal diversity from different environmental samples. An oligonucleotide sequence corresponding to positions 202-222 (according to the numbering in *D.indicus* 16SrRNA gene), is present in > 70% of the total deinococcal spesies listed at the RDP and has negligible possibility of being found in other taxonomic groups. Employing PCR primer corresponding to this sequence, a two step hemi–nested PCR protocol for the detection of deinococci from mixed microbial communities was developed. Under optimised conditions, this procedure was highly specific and had sensitivity to detect 1pg of the deinococcal 16S rDNA obtained from the first round of PCR while 16SrDNA of none of the other bacteria tested was detected. Using this method, the presence of deinococci was detected in 10 different soil samples from diverse ecological settings in India. To comprehend the deinococcal diversity of the environmental samples, a group specific 16S rDNA clone library was prepared from representative samples. Clones from the CRC sample showed maximum identity to *D.radiopuganans* while clones from GRK represented phylotypes showing maximum homology to *D. murrayi* and *D. hopeinsis*. The hemi – nested PCR approach was extended to develop a three step PCR-DGGE protocol for obtaining fingerprints of the deinococcal community in the environmental samples. A rich diversity of deinococci was detected in the samples, which was greater than that revealed by the clone library. Sequence analysis of the DGGE bands demonstrated the dominance of *D. radiopuganas* obtained from different environments. *D. radiodurans*, *D. deserti* , *D. proteolyticus*, and *D. murrayi* were also represented amongst the other DGGE band sequences. Culturing of radiation resistant bacteria from these samples lead to isolation of only a few deinococcal pure cultures indicating thst the clonal diversity was from unculturable deinococci. Three novel radiation resistant isolates were obtained from GRK samples and were ascertained to be deinococci with hemi-nested PCR approach. 16S rRNA gene sequence analysis of the isolates revealed Grk2 was similar to *D. proteolyticus* while Grk4 and Grk5

showed maximum similarity to *D. ficus*. The newly isolated strains were catabolically more versatile than *D. radidurans* R1 (DR1) and showed broader spectrum of carbohydrate utilisation as compared to the type strain DR1 indicative of robust physiology. Some possessed better resistive properties than DR1. Considering the potential use of radiation resistant bacteria for bioremediation of the nuclear waste sites, tolerance of the new the isolates to heavy metals commonly found at the radioactive waste sites, was also analysed and some isolates showed signifi cantly better metal tolerance than DR1.

Most bacteria are reported to be more resistant to external stress during the stationary phase. On the contrary we observed that DR1 was more sensitive to Cd^{2+} during the stationary phase and the log phase cells of DR1 exhibited a D_{50} three times as much as the stationary phase culture. The sensitivity of the stationary phase culture to Cd^{2+} was rescued by the addition of 100μM Mn^{2+}, which then exhibited a D_{50} comparable to the log phase culture. Cd^{2+} induced stasis was observed for the stationary phase culture for 48h. During recovery from Cd^{2+} induced stasis several morphological changes were observed, most profound changes being, after 3h and 6h post recovery.

The reactive oxygen species (ROS) induced by Cd^{2+} was demonstrated to produce in DR1. Carbonylated proteins which are indicators of the oxidative damage were found to increase in a dose dependent manner in DR1 exposed to Cd^{2+}. Lipid damage was in coherence with the ROS produced. Both superoxide dismutase (SOD) and catalase enzymes, which are regarded as first line of defence against the oxidative stress, were inhibited in the presence of Cd^{2+}. It can be assumed the loss of catalase activity could be because of the Fe displacement by Cd^{2+} by from the active as catalytic site of catalases from DR1 *kat* A, known to be heme catalase. *sod* A^- mutant of DR1, defective in SOD, was 5 times as sensitive to Cd^{2+}as compared to the wild type. In contrast to the wild type, Mn^{2+} aggravated Cd^{2+} toxicity to the *sod* A^-.

Pre-exposure to sub-lethal concentration of H_2O_2 provided cross-resistance Cd^{2+}. Conversely, DR1 cells grown in presence of Cd^{2+} decimate the catalase activity and reduce the ability of the culture to withstand H_2O_2. Similarly, prior exposure to UV sensitises DR1 culture for better recovery in presence of Cd^{2+} indicating a possible role of the UV repair pathway in tolerance to Cd^{2+} in DR1. The *rec* A^- mutant of DR1 was found to be 4 fold sensitive to Cd^{2+} as compared to the wild type though the

addition of Mn^{2+} didn't alter the tolerance to Cd^{2+} for the *rec* A⁻ mutant. The *lac* Z reporter gene under *rec*A promoter exhibited a dose dependent increase in beta-galactosidase activity with Cd^{2+} exposure, indicating a possible role of *rec* A in Cd^{2+} tolerance in DR1. PprI, known to regulate both SOD and *rec* A in DR1, when mutated showed sensitivity to Cd^{2+} exhibiting a D_{50} less than either rec A⁻ or sod A⁻.

Proteomic analysis of the DR1 grown under log phase, stationary phase and log phase amended with Cd^{2+} or Mn^{2+} indicate that there exists an overall large amount of similarity amongst the proteins expressed during stationary phase, Mn^{2+} induced and Cd^{2+} induced cultures of DR1, therefore it can be concluded that exogenous Mn^{2+} can also exert stressful conditions in DR1. No proteins were detected exclusively for both stationary phase cultures and Cd^{2+} affected cells indicating that DR1 may have more generalised response to combat the Cd^{2+} stress and no unique mechanism to combat Cd^{2+}. Metalloproteome analysis of the DR1 grown under log phase, stationary phase and log phase amended with Cd^{2+} or Mn^{2+} was done using immobilised metal affinity chromatography. Mn^{2+} and Cd^{2+} binding proteome in DR1 under all conditions bear a significant resemblance. Although there were significant differences obtained for Mn^{2+} binding proteome, but the Cd^{2+} binding proteome was similar under all conditions examined.

Construction of recombinant strains of DR1 that can survive high metal concentrations at the radioactive waste sites and subsequent co-transformation of such strains with metabolic genes for mineralization of toxic hydrocarbon and metal can enhance the applicability of DR1 for bioremediation at the nuclear waste sites. Cloning and expression of natural metallothionein gene, *smt* A and synthetic phytochelatin gene, *ec* 20 was carried out in DR1. The synthetic phytochelatin was synthesised by overlap extension PCR using synthetic oligonucleotides. *ec* 20 and *smt* A were expressed individually in DR1 under the effect of groE promoter in the shuttle vector, pRADZ3 cloned in place of the *lac* Z fragment. The transformant DR1 (pRAD-EC) exhibited, 1.5 fold higher tolerance to Cd^{2+} than the control and accumulated 1.21 fold greater Cd^{2+} as opposed to the control. Heterologous expression of natural metallothionein gene, *smt* A, in DR1 imparted the transformant, superior tolerance to Cd^{2+} wherein DR1 (pRAD-*smt*A) amassed 2.5 fold greater Cd^{2+} than DR1-EC. Addition of cysteine enhanced the growth of the DR1 strains harbouring the metallotheionin however it did not translate into efficient metal accumulation.

Briefly, the highlights/achievements of the present work can be summarised as follows

- A deinococcus specific primer was demonstrated to be useful for group specific PCR and studying deinococcal diversity by different culture-independent approaches
- Three new deinococcal isolates have been obtained from Great Rann of Kutch, an Indian salt desert. These are catabolically more versatile and more robust in terms of their resistive properties than the most popular strain, *D. radiodurans* R1.
- Deinococcal diversity, in terms of their DGGE fingerprints, has been reported for the first time. Distinct communities have been observed in different Indian ecological settings and preponderance of specific species observed.
- Several new deinococcal 16S rRNA gene sequences from uncultured as well as the three new isolates are reported from different geographic regions of India, some locations for the first time. Presence of deinococci in contaminated environments (Cr contaminated and petrol contaminated samples) has been detected, however their isolation was not possible.
- A detailed study of the effects of Cd, a toxic metal, in DR1 has been undertaken, particularly to undersand growth phase dependent sensitivity as well as Mn-Cd interactions in this strain. The studies have provided new insights about the oxidative stress imposed by Cd^{2+} in DR1 and some aspects of its dependence on other factors both genetic and physiological
- The expression of synthetic phytochelatin and a natural heterologous metallotionein gene in DR1 is reported to bring about modest increase in Cd^{2+} tolerance.