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Micro Diversity in Individuals of Same Species Residing in Restricted Ecotypes

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A combination of cultivation-based methods with a molecular biological approach was used to investigate whether isolated bacteria with identical 16S rRNA gene sequences can represent distinct eco – and – genotypes. A set of 8 strains of *Pseudomonas putida* and *Actinetobacter calcoaceticus* were isolated from mushroom casing compost community by conventional plating or by using a liquid most probable number (MPN) dilution series. All the 8 strains showed genetic diversity but each strain utilized a specific combination of 31 carbon substrates, and the niche overlap indices were low, suggesting that each strain occupied a different ecological niche. Our results concluded that the extent of physiological diversity masked by identical partial 16S rRNA sequences is much large and that this so-called micro diversity has ecological relevance.

Key words: 16S rRNA sequence, Microdiversity, Sole source carbon utilization, Morphotypical, Phylogenetical.

Analysis of 16S rRNA (ribosomal RNA) gene sequences has become the primary approach for studying the naturally occurred distribution of bacteria in a culture independent manner (Amann *et al.,* 1995).

The vertical seasonal distributions of distinct 16S rRNA gene sequences (phylotypes) within one ecosystem have been used to infer the ecological niches of bacteria (Pernthaler et al., 1998; Ward *et al.,* 1998). This approach is especially valuable if the physiology of bacteria that have not been cultured yet is to be elucidated.

In many cases phylogenetically closely related bacteria (whose 16S rRNA sequences differ by between 2.7 & 0.3%) have been detected in the same fresh water, marine, or soil habitat (Postine *et al.,* 1999; Ward *et al*., 1998). According to macroecological principles of competitive exclusion, physiologically similar micro organisms should not co-occur in nutrient poor systems which are dominated by physical & chemical fluctuations (Pernthaler *et al*., 1998).

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Accordingly, phylogenetically closely related bacteria coexisting in the same habitat occupy distinct ecological niches (Gray *et al*., 2001; Moore *et al.,* 1998; Postius *et al*., 999; Ward *et al*., 1998).

For pathogenic bacteria it is well established that even phylogenetically identical strains or species can exhibit distinct ecophysiological properties. Certain serovars of *Mycobacterium intracellulare* (Boddinghaus *et al*., 1990), serovars of *Ochrobactrum anthropi* (Lebuhn *et al.,* 2000), *strains of Yersinia pestis, Yerinia pseudo tuberculosis* (Trebesins *et al.,* 1998), or strains of *Bacillus anthracis, Bacillus cereus* (Ash *et al*., 1991) contain identical 16S rRNA gene sequences. These phylogenetically identical organisms are also genetically highly similar based on DNA-DNA hybridization data but clearly represent different ecotypes based on their virulence properties or host ranges. Often, phenotypic differences can be traced back to the presence of plasmids, as in *B. anthracis*, in which the major virulence determinants are encoded by the 181-kb plasmid pX01 and the 95-kb plasmid pX02 not present in *B. cereus* (Read *et al*., 2003).

However, the genomes of certain phylogenetically identical strains exhibit profound differences. *Escherichia coli* K-12 & 0157: H7 differ not only in genome size (by 0.89 Mb) but also in a considerable number of chromosomal genes. Twenty-five percent of the genes present in the enterohemorrhagic organism *E.coli* 0157: H7 are not found in the nonpathogenic organism *E. coli* K-12, whereas 12% of the genes in the latter organism are absent in the former organism (Blattner *et al*., 1997; Perna *et al*., 2001). Nevertheless, some of the 16S rRNA gene sequences (eg, the two rrsEgenes) are identical in the two organisms. Similarly, genomic fingerprinting (Sass *et al*., 1998; Sikorski *et al.*, 2002; Wieringa *et al.*, 2000) and analysis of fosmid libraries of DNA fragments from marine samples (Beja *et al*., 2002) have indicated that nonpathogenic bacteria with identical 16S rRNA gene sequences but distinctly different genomes coexist in natural ecosystems (Rossello-Mora *et al*., 2001). The term micro diversity has been used to describe the phenomenon of phylogenetically closely related but physiologically distinct bacterial populations (Moore *et al*., 1998). In order to assess the extent of microdiversity present in a natural habitat, the niche separation between the different genotypes with identical 16S-rRNA genes, and finally the potential limitations of 16S-rRNA-based methods, more information about the genetic and ecophysiological differences of such bacteria is required.

Inspite of having limited information on microdiversity of bacterial fours of mushroom casing niche the main objective of present study was to assess the ecophysiological differences between bacterial isolates of restricted ecotypes based on sole source carbon utilization (SSCU).

MATERIAL AND METHODS

Isolates

Total 8 bacterial strains were brought for physiological characterization by kind permission of Head, Department of Microbiology, CBSH, G.B. Pant University of Agriculture Technology, Pantnagar – 263145 (Uttaranchal), India, out of 8 bacterial strains 3 were *Pseudomonas putida*, isolated from mycelium impregnated stage (MIS) of casing farm yard manure + vermicompost (3:1) (FYM + VC, 3:1) and farm yard manure + spent compost (3:1) (FYM + SC, 3:1). Rest 5 isolates were belonged to *Acinetobacter calcoaceticus* isolated from MIS of FYM + VC and FYM + SC. All the 8 strains were characterized by employing partial 16S rRNA sequencing.

Nucleotide sequence accession numbers

The following strains were studied for the physiological characterization.

UVC2 (AY 961043), CVC 2 (AY 967724), USC 31 (DQ 074752), UVC 4 (AY 961045), UVC 3 (AY 961044), UVC 8 (AY 961047), USC 29 (AY 961061) and USC 30 (DQ 074751)

Carbon Source

A total of 31 C-sources were selected which were relevant to mushroom compost ecosystem. The sugars and sugar derivatives used were D-Sorbitol, D-Mannitol, Dextrose monohydrate, Lactose monohydrate, sucrose, D(+) Maltose monohydrate, B-Cyclodextrin, D-Fructose, D(+) Galactose, L(+) Rhamnose monohydrate, D (+) Mannose, D(+) Arabitol, L(+) Arabinose, D(+) Cellobiose and D(+) Trehalose dihydrate. The organic acids were DL-Malic acid, Citric acid anhydrous, Malonic acid and Succinic

acid. In addition the amino acids DL-Methionine, L-Histidine, L-Alanine, L-Leucine, L-Asparagine, DL-Aspartic acid, DL-Alanine, L-Valine, L-Threonine, DL-Serine, L-Arginine and L-Histidine hydrochloride were tested. All C-sources were tested at 1% level.

Preparation of bacterial suspension

The bacterial cultures were washed to remove the residual substrates. Pure single colony were inoculated in 50 ml of King's B broth and were incubated at 28 +_ 2 ° C for 2 days untill log phase growth took place. Centrifugation was done to obtain pellet at 10,000 rpm for 10 minutes. Supernatant was discarded and remaining pellet was washed with 85% NaCl. Again centrifuged at 10,000 rpm for 10 minutes. Above steps was repated twice. O.D. was taken at 465 nm (0.12 for $10^4 - 10^6$ $cfmt^{-1}$) (Jaspers and Overmam, 2004).

Preparation of micro titer plate

For growth test, each microtiter well received 50 ml of bacterial suspension, 50 ml of triphenyl tetrazoluim chloride (TTC) (0.5%), 50 ml of C-source (1%) and 50 ml of M9 medium devoid of glucose. The plates were incubated for 5 days at 30 ± 2^0C.

Niche overlap index (NOI)

The niche overlap index (NOI) between two strains A and B, was calculated by determining the ratio of the number of substrates utilized by both strains ($N_{A \cap B}$) to the total number of substrates utilized by either of the two (N tot) (Wilson and Lindow, 1994).

$$NOI = N_{A \cap B} / N \text{ tot}$$

Compositional Similarity

The physiological similarity of the 8 bacterial strains was determined by cluster analysis. Cluster analysis was performed with the SAHN program of the NTSYS – by employing the unweighted pair group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

Colour formation in microtiter plates

Differences in sole source carbon utilization have been used to distinguish among different bacterial types over 50 years. Development of redox sensitive dyes such as TTC and incorporation of these dyes into microtiter plates has allowed for rapid profiling of Sole Source Carbon Utilization by bacterial isolates [Bachner & Savagrave, 1997, Bochher 1989 (a) 1989 (b)].

As the bacterial cultures respired the oxidation of substrate takes place by reduction of dye into the product formazen which gives red colour in the well. No colour development was observed in the control well (Fig. 1 & Fig. 2).

Niche differentiation among strains with partially identical 16S rRNA gene sequences

Strains were morphotypically quite similar (based on Gram Staining) and they were belonged to *Pseudomonas putida* and *Acinetobacter calcoaceticus* species based on partial 16S rRNA gene sequencing. Consequently, their potential ecological niches were assessed based on their carbon substrate utilization patterns.

Each strain used a unique combination of the 31 carbon substrates (Table 1). The lowest metabolic diversity was observed for strain USC 31 (*P. putida*) isolated from FYM + SC (3:1) and grew only on L (+) Arabinose, L (-) Asparagine, Dl- Aspartic acid DL(-) Alanine, l (-) Threonine and L(-) Histidine hydrochloride whereas other two strains of *P.putida* viz, UVC 2 and CVC 2 utilized 23 and 21 different C-substrates respectively. The NOI calculated for all pairs of *P. Putida* strains indicated that they occupy different ecological niches i.e. cannot coexist. Strains UVC 2 and CVC 2 had NOI value 1.91 i.e. no stable coexistance. The strains i.e. USC 31 was not able to coexist with UVC 2 and CVC 2. Similarly, NOI for strain UVC 3 was 1.8 with UVC 4 and 1.7 with UVC 8 which was quite high i.e. they cannot exist similarly strain UVC 4 had NOI value 1.7 with UVC 7 which was considerable high i.e. they occupied different ecological niches. Likewise, strain USC 29 had NOI value 1.6 with USC 30 i.e. high NOI values do not allow stable coexistence.

Wilson and Lindow (1994) calculated niche overlap index for Ice (Sup+) *P.syringae* strain with respect to Ice (Sup-) *P.syringae* TLP2 del. It was uniformly high indicated that they were ecologically similar but had low level of coexistence. They found that in the phyllosphere resource partitioning among different bacterial

species with NOI values of 0.25 to 0.59 allowed stable coexistence (Wilson and Lindow, 1994), whereas catabolically identical strains (NOI 1.0), even if they belong to different species cannot coexist (Wilson and Lindow, 1994).

Intresting observation was found in microtiter plate i.e. no one strain utilized carboxylic acids viz, DL- Malic acid, Citric acid, Malonic acid and Succinic acid.

Compositional Similarity

Measure of niche overlap index (NOI) does not provide information about the types of substrates that are utilized by the bacterial strains. Four strains showed identical NOI but still catabolized different substrates. Beside NOI, a consistent relationship among strains was made by cluster analysis, based on the presence or absence of utilized (Fig. 3.) Two strains UVC 2 and UVC 8 showed 78% similarity and they had 65% similarity with CVC 2 whereas USC 29 and USC 30 showed 65% similarity with each other. The least relationship was observed for UVC 4,

Table 1. Substrate utilization pattern of the 8 strains belonged to *Pseudomonas putida* and *Acinetobacter calcoaceticus*

Substrate	*Pseudomonas putida*			*Acinetobacter calcoaceticus*				
	UVC2	CVC2	USC31	UVC4	UVC3	UVC8	USC29	USC30
1. Sugar and derivatives								
Sorbitol	+	-	-	+	+	+	-	-
Mannitol	-	+	-	+	-	+	+	+
Dextrose monohydrate	+	-	-	-	-	+	-	+
Lactose monohydrate	+	+	-	-	-	+	+	+
Sucrose	+	+	-	+	-	+	-	-
D(+) Maltose monohydrate	-	+	-	-	-	+	-	+
B-cyclodestrin	+	+	-	-	-	+	-	-
D-F ructose	s+	+	-	-	-	+	+	+
D(+) Galactose	+	+	-	+	-	+	+	+
L(+) Rhamnose monohydrate	+	-	-	-	-	+	-	-
D(+) Mannose	+	-	-	-	-	+	-	-
D(+) Arabitol	-	+	-	+	+	+	-	+
L(+) Arabinose	+	+	+	+	+	+	+	+
D(+) Cellobiose	+	+	-	+	+	+	+	-
D(+) Trehalose dihydrate	+	-	-	-	+	+	-	+
2. Organic acids								
DL-malic acid	-	-	-	-	-	-	-	-
Citrc acid anhydrous	-	-	-	-	-	-	-	-
Malonic acid	-	-	-	-	-	-	-	-
Succinic acid	-	-	-	-	-	-	-	-
3. Amino Acids								
DL Methionine	+	+	-	-	-	+	-	-
L-Histidine hydrochloride	+	+	+	+	-	+	-	+
L-Alanine	+	+	-	+	+	+	-	+
L-Leucine	+	+	-	+	+	+	+	-
L-Asparagine monohydrate	+	+	+	+	-	+	-	-
DL-Aspartic acid	+	-	+	+	+	+	-	+
DL-Alanine	+	+	+	-	+	+	-	+
L-Valine	-	+	-	-	-	+	+	-
L-Theronine	+	+	+	+	+	+	-	+
DL-Serine	+	+	-	+	+	+	+	+
L-Agrinine hydrochloride	+	+	-	-	-	+	-	+
L-Histidine	+	+	+	+	+	+	+	-
Control	-	-	-	-	-	-	-	-

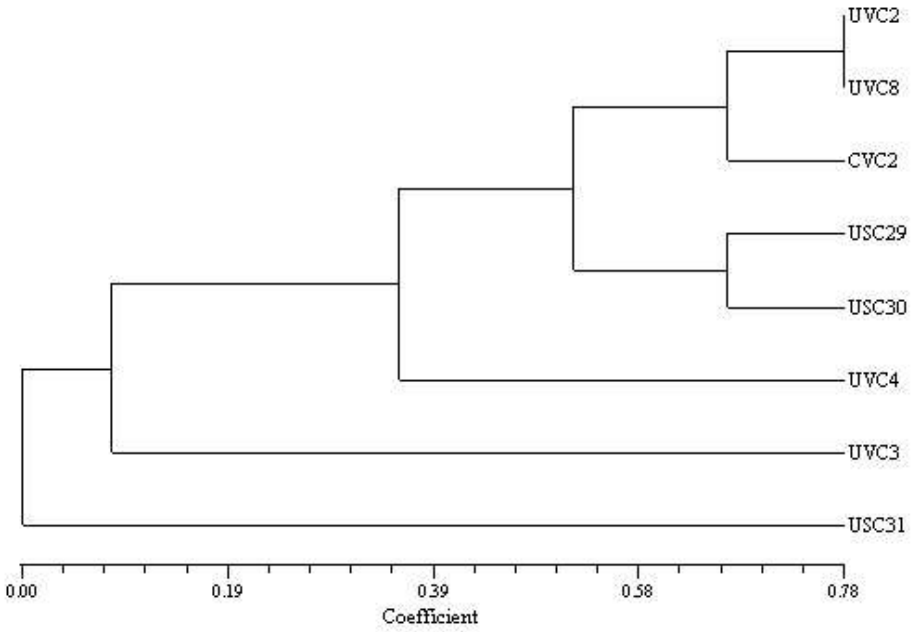

Fig. 3. Compositional similarity among bacterial strains based on physiological characterization by employing UPGMA

They isolated II strains of *Brevun dimonas alba* from a freshwater community by employing conventional plating and MPN dilution series. All the II strains had identical 16S rRNA gene sequences and each strain utilized a specific combination of 59 C-substrates and the NOI were low suggested that each strain occupied in different ecological niche.

CONCLUSION

The 8 bacterial strains investigated in the present study were retrieved from the two types of mushroom casing niche viz, FYM+SC (3:1) and FYM+VC (3:1). All 8 strains were belonging to *P.putida* and *A.calcoaceticus*. Two strains of *P.putida* belonged to FYM+VC and third one isolated from FYM+SC, whereas three strains of *A.calcoaceticus* isolated from FYM+VC and other two were belong to FYM+SC. Therefore a hitheto to unknown multitude of ecotypes must thrive in the some habitat. Based on above results it has to be concluded that the extent of physiological diversity masked by identical partial 16S rRNA sequences is much large and that this so-called microdiversity has ecological relevance.

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