INT. J. BIOL. BIOTECH., 3 (4): 733-738, 2006.

PHENOTYPIC DIVERSITY AMONG INDIGENOUS SOIL BACTERIAL STRAINS FROM DIFFERENT GEOGRAPHICAL REGIONS OF KARACHI

Muhammadi and Nuzhat Ahmed

Centre for Molecular Genetics, University of Karachi, Karachi-75270, Pakistan

ABSTRACT

In order to study the phenotypic response of indigenous soil bacterial community to soil diversity, fifty bacterial strains were isolated from four geographically different sites of Karachi. Among the soil bacterial strains, mostly were found to be gram-negative, facultative and lactose non-fomenter rods. Diversity in colonial morphology and antimicrobial tolerance phenotype was markedly found. Fourteen percent strains showed phenotypic characteristics specific for exopolysaccharides and cellulose production. Twenty four percent bacterial strains showed tolerance to different antibiotics, heavy metals and NaCl. These characters have indicated pollution in the sampling sites.

Key words: Soil bacterial stains, antibiotics, cellulose, exopolysaccharide, heavy metal tolerance.

INTRODUCTION

Soil is a natural habitat for large range of organisms among which bacterial flora counts the largest portion of life. Their metabolic and genetic diversity speaks of the roles of these species in soil and of the variability of the soil environment (Andrew, 1998). Further, bacteria in these diverse environments are also diverse in their biological activities and characteristic features. It is well recognized that the diversity of bacterial communities remain largely uncharacterized and less than 1% of soil bacteria are cultivable by standard methods. The widespread pollutants such as heavy metals and antibiotics have resulted in the contamination of surface soil layer of vast areas and have established serious ecological risks (Singh and Steinnes, 1994) which may produce hazardous effects on plants, animals and human beings (Anne and Alm, 2003). Bacteria in such ecological niches have evolved several mechanisms to tolerate toxic agents. When they are exposed to antimicrobial agents they may acquire additional tolerance genes through horizontal gene transfer or gene mutations and the resident bacteria may be the reservoir or source of widespread tolerant organisms found in many environments. Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial tolerance is observed (Levy, 1997). Tsutomu *et al*., (1997) studied that cultivation of isolated bacteria supplies us with a great amount of information useful for both the taxonomy and the ecology. Therefore, testing of tolerance phenotypes in bacterial strains isolated from contaminated sites could be used as witness to the presence of these contaminants in the environment. Another common feature of bacteria is the production of exopolysaccharides which is logical response to selection pressures in environment (Whitfield, 1988). These bacterial polymers offer a number of novel material properties and commercial opportunities. The versatility exhibited by bacteria in synthesizing ionic and neutral polysaccharides with a high structural regularity, and greatly varying composition and properties does not find its counterpart in the vegetable world and can not be imitated by synthetic chemistry. The aim of present study was to study the phenotypic diversity among soil bacterial strains from different sites of Karachi.

MATERIALS AND METHODS

Soil Sample Collection

Soil samples from following four sites were collected from 0-15cm layer in sterile universal sampling vials; 1) garden soil from Safari Park labeled as GSSP, 2) contaminated soil from SITE Karachi labeled as CSSK, 3) dry and aggregated soil of Karachi University labeled as DSKU, and 4) Bean and okra fields at Malir Halt Karachi and labeled as BOFM. pH and temperature of each soil sample were measured at collection sites.

Isolation and Purification of Bacterial Strains

By using standard method pure bacterial strains were isolated and were coded as CMG 1401 to1450. The over night (ON) cultures of purified bacterial strains in nutrient broth (NB) were preserved in 20 % Glycerol at –20^{o}C (Miller, 1972).

Phenotypic Characterization of Bacterial Strains

Mode of bacterial growth (aerobic, anaerobic and facultative) was studied by growing the strains in 20ml test tubes with half-filled NB for over night. Colony morphology was studied after 1 to 2 days growth on nutrient agar (NA) plates. To study the motility 30µl ON culture was dropped to a clean glass cavity slide and observed under light microscope. Cellular morphology was studied by using gram-staining technique. Lactose fermentation was studied on MacConkey's (Merck) agar plates.

Screening of Bacterial Strains for Production of Exopolysaccharide and Cellulose

Bacterial strains with phenotypically mucoid, slimy colonies and with alcoholic precipitation of their culture supernatant were chosen as exopolysaccharide (EPS) producers (Fusconi *et al*., 2002). Bacterial strains forming milk-white, swollen colonies on NA plates and pellicle in liquid NB were selected for cellulose production (Hiroshi et al., 1995).

Extraction of EPS and Cellulose

5 days old culture was centrifuged (12000 rpm) at 4^{o}C in refrigerating centrifuge to sediment the cells. Viscous supernatant was added to two volumes of absolute ethanol and precipitates were collected, washed three times with distilled water and freeze dried. Cellulose was extracted by Dudman method.

Chemical Analysis of EPS and Cellulose

Chemical composition of EPS and cellulose was determined in terms of total contents of the following; uronic acid was determined by carbazol reaction (Bitter & Muir, 1962) with D-mannuronic acid standard, total protein content was estimated by Bradford method with bovine serum albumin standard (Bradford, 1978), total neutral sugar was determined by phenol-sulphuric acid method with D-glucose standard (Kochert, 1978) and total lipid content was estimated by the gravimetric method of Salton (1953).

Estimation of Maximum Tolerable Concentrations of Heavy Metal, Antibiotics and NaCl

Maximum tolerable concentrations (MTC) of selected heavy metals, antibiotics and NaCl were evaluated on agar plates (Miller, 1972). For heavy metal tolerable concentration, cells were grown on tris-gluconate agar plates supplemented with varying concentrations (mM/ml) of heavy metal salts such as $CdCl_2$, $CrCl_3$, $CuSO_4$, $NiCl_2$ and $ZnSO_4$. For antibiotics and NaCl tolerable concentrations, bacterial strains were grown on NA plates supplemented with varying concentrations of antibiotics (µg/ml) (ampicillin, chloramphenicol, kanamycin, neomycin, streptomycin, and tetracycline) or NaCl (1-12%). The selective agar plates were incubated at 37°C for 24-72hrs. MTC of each tested selection pressure was estimated in terms of observable growth.

RESULTS AND DISCUSSION

From four indigenous soil samplesof Karachi, fifty bacterial strains were isolated (Table 1). 90% of these were gram negative and only 10% strains were gram positive. This was further confirmed when the growth of the gram positive strains was inhibited on MacConkey's agar (data not shown). On MacConkey's agar all gram negative bacterial strains produced colorless colonies except CMG1419 which appeared as pink to red (data not shown). All bacterial strains showed a facultative growth except two gram positive strains i.e. CMG1407 and 1428 which grew aerobically suggesting its ubiquitousness. As far as cellular morphology is concerned, 38% strains were cocci and 62% bacilli. Substantial diversity was observed in colonial morphology among the bacterial strains (Table. 1). 10% strains showed mucoid colony phenotype and formed precipitates when their culture supernatants were added to ethanol (Table.1). This phenotypic characteristic was correlated to EPS production in the medium (Govan *et al*., 1992; Robertson and Firestone, 1992). 4% strains were found to grow as milk-white swollen colonies and formed pellicle on surface of liquid medium (Table. 1). Such strains have been reported as cellulose producing bacteria (Hiroshi *et al*., 1995). Further chemical composition substantiated the production of EPS and cellulose by these strains (Table. 2). Since cellulose is resistant to hydrolysis therefore, it could not be hydrolyzed completely. EPS extracted from CMG 1418 and 1421 consisted of acidic sugar and those from rest of producers contained mostly neutral sugar (table 2). All soil bacterial strains in aqueous medium exhibited observable motility under microscope except CMG1407, 1422, 1440 and 1446 (data not shown). Bacterial motility is apparently an intelligent behavior achieved towards favorable (nutrients) and away from unfavorable (temperature, light, gravity, etc) stimulus (Alfred, 1999). MTC data related to only tolerant strains were tabulated in this study and those of sensitive strains have been omitted due to their large volume. Among these bacterial strains, only 24% were found to tolerate NaCl concentrations ranging from 4-11% as shown in table 3. These NaCl tolerant strains give an estimated measurement

of NaCl concentration or salinity in sampling sites. All bacterial strains were found to be sensitive to tetracycline, kanamycin, chloramphenicol and neomycin except CM1407 which showed tolerance for chloramphenicol and CMG1405 which showed mild tolerance for chloramphenicol (25µg/ml) and neomycin (25µg/ml). The results (table 4) have shown that among bacteria strains only 18% showed tolerance against ampicillin and streptomycin while rest were found to be sensitive (Table 4).

Table 1. Characterization of soil bacterial strains.

Soil sample	CMG strain	Gram	Cellular morphology	A/An/F	Colonial morphology	Ethanolic ppt	Pellicle
GSSP	1401	-	small bacilli	F	round, opaque	No	No
	1402	-	small bacilli	F	thick, circular	No	No
	1403	-	cocco bacilli	F	mucoid, light brown	**Yes**	No
	1404	-	bacilli	F	spherical, swelling	No	No
	1405	-	bacilli	F	round to irregular	No	No
	1406	-	cocci	F	regular, dull white	No	No
	1407	+	large bacilli	A	dry and irregular	No	No
	1408	-	small cocci	F	viscous, thick	No	No
CSSK	1409	-	small cocci	F	thick, circular	No	No
	1410	-	large bacilli	F	dull brownish, spherical	No	No
	1411	-	cocco bacilli	F	swelling, regular, round	No	No
	1412	-	small cocci	F	thick, circular	No	No
	1413	-	cocco bacilli	F	irregular, off white	No	No
	1414	-	bacilli	F	round, regular, swelling	No	No
	1415	+	large bacilli	F	round, regular, swelling	No	No
	1416	-	cocco bacilli	F	soft, round, regular	No	No
	1417	+	cocci	F	soft, milky-white, swollen	No	**Yes**
	1418	-	cocco bacilli	F	mucoid, light yellow	**Yes**	No
	1419	-	small bacilli	F	round, milky, viscous	No	No
	1420	-	cocco bacilli	F	spherical, swelling	No	No
DSKU	1421	-	cocco bacilli	F	mucoid, florescent green	**Yes**	No
	1422	-	small cocci	F	regular, off white	No	No
	1423	-	small cocci	F	deep globular, soft	No	No
	1424	-	small cocci	F	round and viscous	No	No
	1425	-	small cocci	F	round, regular, swelling	No	No
	1426	-	cocco bacilli	F	soft, round, regular	No	No
	1427	-	small bacilli	F	thick, circular	No	No
	1428	+	large bacilli	A	hard, irregular	No	No
	1429	-	cocco bacilli	F	soft, milky-white, swollen	No	**Yes**
	1430	-	cocci	F	soft, round, regular	No	No
	1431	-	cocco bacilli	F	irregular, off white	No	No
	1432	-	small bacilli	F	oval, soft	No	No
	1433	-	cocco bacilli	F	viscous, milky	No	No
	1434	+	bacilli	F	round, soft, regular	No	No
	1435	-	small cocci	F	circular, greenish	No	No
	1436	-	small cocci	F	mucoid, light-pinkish	**Yes**	No

Table contd…

BOFM	1437	-	cocco bacilli	F	circular, opaque	No	No
	1438	-	bacilli	F	spherical, swelling	No	No
	1439	-	small bacilli	F	irregular, grayish	No	No
	1440	-	cocci	F	hard, dry, regular	No	No
	1441	-	small cocci	F	viscous, light-greenish	No	No
	1442	-	cocci	F	circular, grayish-white	No	No
	1443	-	small cocci	F	granular, brownish	No	No
	1444	-	small cocci	F	circular, bronish	No	No
	1445	-	cocco bacilli	F	regular, off white	No	No
	1446	-	cocci	F	milky, vawey	No	No
	1447	-	cocco bacilli	F	mucoid, lihgt-brownish	**Yes**	No
	1448	-	small bacilli	F	oval, soft	No	No
	1449	-	cocci	F	round, lihgt brownish	No	No
	1450	-	small bacilli	F	circular, opaque	No	No

- : negative**, +**: positive, **A, An, F:** aerobic, anaerobic and facultative respectively.

Table 2. Distribution of EPS and cellulose chemical composition.

Exopolymer	CMG	Contents expressed as g % w/w			
	strain	Neutral sugar	Uronic acid	Protein	Lipid
EPS	1403	80.12	3.63	3.677	-
	1418	22.552	77.333	-	-
	1421	2.457	96.106	-	-
	1436	91.31	2.52	1.8	-
	1447	88.45	4.11	4.72	-
Cellulose	1417	71.232	2.342	-	-
	1429	74.345	-	2.546	-

Table3. Estimation of maximum tolerable concentration of NaCl.

CMG	Bacterial growth at NaCl Concentration(%)											
strain	1	2	3	4	5	6	7	8	9	10	11	12
1403	++	++	++	+	+	+	+	+	+	+	+	-
1405	++	++	++	+	+	-						
1407	++	++	++	+	+	+	+	+	+	+	+	-
1411	++	++	++	+	+	+	+	+	+	+	+	-
1414	++	++	++	+	+	-						
1415	++	++	++	+	-							
1417	++	++	++	+	+	-						
1418	++	++	++	+	+	-						
1421	++	++	++	+	+	-						
1429	++	++	++	+	+	+	+	+	+	+	+	-
1436	++	++	++	+	+	+	+	+	+	+	+	-
1447	++	++	++	+	+	+	+	+	+	+	+	-

+: Growth appeared, -: No growth.

Table 4. Estimation of maximum tolerable concentration of heavy metal and antibiotics

CMG strain	MTC of antimicrobial agents exhibited by CMG strains										
	Antibiotics (µg/ml)						Heavy metal (mM/ml)				
	AMP	CHL	KAN	NEO	STR	TET	Cd	Cr	Cu	Ni	Zn
1403	-	-	-	-	50	-	0.5	2	-	-	-
1405	250	25	-	25	75	-	3.5	2	-	-	-
1407	250	50	-	-	250	-	3	2	-	-	1
1411	25	-	-	-	150	-	-	1	-	-	-
1414	-	-	-	-	-	50	3.5	2	-	-	-
1415	250	-	-	-	250	-	1	2	-	1.5	-
1417	-	-	-	-	75	-	1	2	-	-	1
1418	250	-	-	-	250	-	3.5	2	-	-	1
1421	250	-	-	-	25	-	3.5	2	-	-	-
1429	100	-	-	-	-	25	-	-	-	-	-
1436	250	-	-	-	100	-	3.5	-	-	-	-
1447	200	-	-	-	-	-	-	-	-	-	-

AMP: ampicillin, STR: streptomycin, KAN: kanamycin, TET: tetracycline, NEO: Neomycin and CHL: chloramphenicol

It was noticed that CMG 1407, 1415 and 1418 showed equal maximum tolerance (250µg/ml) for ampicillin and streptomycin while CMG 1405, 1421 and 1436 could also tolerate the same concentration of ampicillin only. Among tested heavy metals, Cu and Ni could not be tolerated by any strain except CMG1415 showing tolerance for only Ni. Cd and Cr were tolerated by only 18% strains. Only 6% bacterial strains i.e. CMG1407, 1417 and 1418 tolerated the equal concentration of Zn. Similarly MTC among Cr tolerant strains was the same except CMG1411. Among the bacterial strains only 10% exhibited the maximum equal tolerance for only Cd among all tested heavy metals (table 4). These tolerance capabilities exhibited by bacterial strains against certain antibiotics, heavy metals and NaCl, suggested the presence of these contaminants in soil environmental or their previous exposure. Unlike many organisms, bacteria share their DNA with one another by horizontal transfer of tolerant genes that allow them to tolerate toxins in natural environment (Gitte *et al*., 2003). Montserrat and Erland, (1996) studied that prolong exposure of soil bacteria to toxic materials present in soil resulted in the development of toxin-tolerant bacterial communities. According to Hughes and Datta, (1983) antibiotic tolerance has been shown to have occurred rarely in bacteria collected before the antibiotic era. But extensive use and misuse of antibiotics for therapeutic and prophylactic purposes and for growth promotion in animal husbandry have exerted a selective pressure on the microbial community in such a way that it has resulted in the evolution of tolerant species. It was observed that most of the antibiotic tolerant bacterial strains showed heavy metal tolerance too (Table. 4). The logical reason is that a correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that tolerance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together (Anne and Alm, 2003). It is concluded that indigenous soil had EPS and cellulose producing bacteria that may have economic value in different industries. Further, based on the published studies, it has been suggested that the expression of tolerance phenotype in response to different antibiotics, heavy metals and NaCl is an estimation of these contaminants in the soil. So, these bacterial strains which address hazardous compounds in soil are useful and can be used as biological tool for environmental monitoring.

REFERENCES

Alfred, M.S. (1999). Gliding motility in bacteria: Insights from studies of *Myxococcus xanthus. Microbiol Mol Biol Rev*., 63: 621–641.

Andrew, O. (1998). Teaching soil bacterial diversity from a phylogenetic perspective: A term project utilizing the ribosomal database project. *J. Nat. Resour. Life Sci. Ed*., 27: 93-96.

Anne, S and E. Alm (2003). Implications of microbial heavy metal tolerance in the environment. *Rev. in Undergraduate Res.,* 2: 1-6.

Bitter, T and H.M. Muir (1962). A modified uronic acid carbazol reaction. *Anal. Biochem.* 4: 330-334.

Bradford, M.M. (1978). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *J. Anal. Biochem*., 72: 248-254.

Fusconi, R and M.J.L. Godinho (2002). Screening for exopolysaccharide producing bacteria from sub-tropical polluted groundwater. *Braz. J. Biol.*, 62: 363-369.

Gitte, S., A. Yvonne, H. Bent, B.B. Suraj, S.A. Jens and L.B. Jensen (2003). Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ. Int.,* 28: 587– 595.

Govan, J. R. W., P. Sarasola, D. J. Taylor, P.J. Tatnell and N.J. Russell (1992). Isolation of a mucoid alginate producing *Pseudomonas aeruginosa* strain from equine guttural pouch. *J. Clin. Microbiol*. 30: 595-599.

Hiroshi, T., N. Takaaki, A. Seto, M. Matsuoka, T. Tsuchida and F. Yoshinaga (1995). Screening of bacterial cellulose producing *Acetobactor* strains suitable for agitated culture. *Biosci. Biotech. Biochem.,* 59: 1498-1502.

Hughes, V.M and N. Datta (1983). Conjugative plasmids in bacteria of the dpre-antibioticT era. *Nature*, 302: 725–726.

Kochert, G. (1978). *Carbohydrate by phenol-sulphuric acid method*. (J.A. Hellebust and J.S. Cagie eds.). Physiological and chemical methods. Cambridge University Press, Cambridge, pp. 96-97.

Levy, S.B. (1997). *Antibiotic resistance: an ecological imbalance*. (D.J. Chadwick and J. Good eds.). Antibiotic Resistance. Origins, Evolution, Selection and Spread. John Wiley & Sons, Chichester, pp. 1– 14.

Miller, J.H. (1972). *Experiments in molecular genetics.* Cold spring Harbor Lab publication New-York, pp. 27-29.

Montserrat, D.A.A, and B. Erland (1996). Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. *Appl. Environ. Microbiol*., 62: 2970–2977.

Robertson, E. B, and M. K. Firestone (1992). Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas sp*. *App and Environ, Microbiol.*, 58: 1284-1291.

Salton, M. R. J. (1953). Studies of bacterial cell wall. IV. The composition of cell walls of some gram-positive and gram-negative bacteria. *Biochem. Biophysics. Acta*., 10: 512-523.

Singh, B.R and E. Steinnes (1994). *Soil and water contamination by heavy metals*. (R. Lal and B.A. Stewart eds). Soil Processes and Water Quality. CRC Press Inc., Boca Raton, pp. 233–271.

Tsutomu, H., M. Hisayuki, H. Hideki, W. Norio, S. Shuichi, G. Krystyna, K. Yasuhiro, A. El-Beltagy and H. Reiko (1997). Advances in soil microbial ecology and the biodiversity. *Antonie van Leeuwenhoek*. 72: 21–28.

Whitfield, C. (1988). Bacterial extracellular polysaccharides. *Can J. Microbiol.*, 34: 415-420.

(*Accepted for publication September 2006*)