

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

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### 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.

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### 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°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°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<sub>2</sub>, CrCl<sub>3</sub>, CuSO<sub>4</sub>, NiCl<sub>2</sub> and ZnSO<sub>4</sub>. 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 samples of 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        | Ethanol 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        | <b>Yes</b>  | 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          | <b>Yes</b> |
|             | 1418       | -    | cocco bacilli       | F      | mucoid, light yellow       | <b>Yes</b>  | 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   | <b>Yes</b>  | 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          | <b>Yes</b> |
|             | 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      | <b>Yes</b>  | 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 | mucoïd, lihgt-brownish  | <b>Yes</b> | 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 strain | Contents expressed as g % w/w |             |         |       |
|------------|------------|-------------------------------|-------------|---------|-------|
|            |            | 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 strain | Bacterial growth at NaCl Concentration(%) |    |    |   |   |   |   |   |   |    |    |    |
|------------|-------------------------------------------|----|----|---|---|---|---|---|---|----|----|----|
|            | 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    | MTC of antimicrobial agents exhibited by CMG strains |     |     |     |     |     |                     |    |    |     |    |
|--------|------------------------------------------------------|-----|-----|-----|-----|-----|---------------------|----|----|-----|----|
| strain | 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 ( $250\mu\text{g/ml}$ ) 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.

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