Identification of Oligotrophic Bacteria bearing Class 1 integron

[A]: Identification of isolates using basic approaches

3A.1. Background

Scientific naming of organisms dates back to 18th century. A famous Swedish botanist, Carolus Linnaeus, also called as "Father of taxonomy", proposed binomial nomenclature, the system that is still used today to name all living things. In addition to binomial system of nomenclature, Linnaeus also propounded a hierarchy of taxonomic ranks: species, genus, family, order, class, phylum or division, and kingdom. An order of taxonomic hierarchy from larger to smaller taxon is shown in Fig. 3.1. The overall philosophy of Linnaeus is also adapted in classifying bacteria as well. The Bacterial taxonomy or systematics is generally defined as "the branch of bacteriology that deals characterization and naming of organism and organizing them into groups". It can be separated into three branches-

(a) nomenclature: naming of bacteria
(b) classification: grouping of bacteria sharing common properties
(c) identification: to determine bacterial genus followed by species

"Bergey's Manual of Determinative Bacteriology", coming to existence in late 1957, published reports relating to the identification of bacteria largely based on Artificial classification and Numerical taxonomy: Artificial classification is based on the characteristics expressed by bacteria that we observe, and results generated increases the accuracy with which we can detect similarities among them and the organizing of these data in a special manner to conclude how that species are closer to each other in terms of characteristics leads to numerical taxonomy. In numerical taxonomy, each characteristic is allocated a value of '1' if present and '0' if not present, means all characteristics are given equal weight. Characteristics may be genetical, morphological, physiological or biochemical response of bacteria like cell shape, cell margin, colour, reaction to Gram staining, aerobic, anaerobic, capsule present or absent, catalyse production, citrate utilization, properties of nucleic acids and proteins, and the presence or absence of particular enzymes and chemical reactions can be evaluated. Bacteria are then compared, and patterns of similarities and differences are computed and analysed. In this system, each strain is compared with everyone and if two organisms shared a 90% or more of the characteristics studied, they are supposed to be the same species. Though the idea of numerical taxonomy was developed much before the introduction of computers applications in biology, but after the development of user-friendly computer algorithms, a large number of data now can be compared in a fraction of second. Hence computerized numerical taxonomy offers great promise for improving our understanding of relationships among all organisms. A dendorgram can be generated from computed similarity values. Separating isolates according to colour, cell shape, size, flagella,

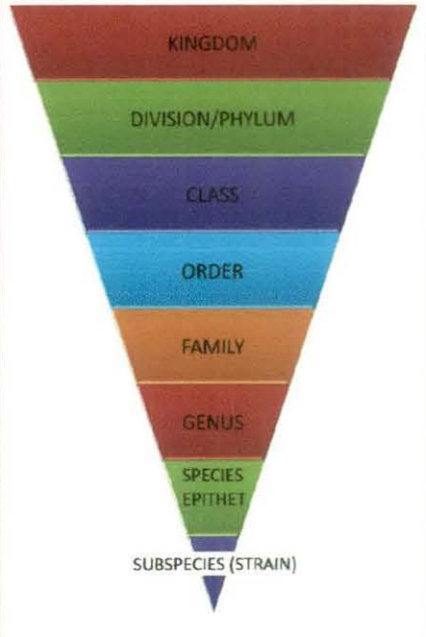

Fig. 3.1: inverted pyramid showing hierarchical relation of different taxa.

endospores, capsules and other physiological characteristics neither produce a very significant data to classify bacteria nor allow identification of particular species. For that reason, other criteria must be considered. However, Gram test is still the first requirement of all bacteriologists among all the known characteristics used to classify bacteria. However, with time and advancement of modern biology, some molecular techniques were perceived by some as useful in standard bacterial classification. Several efforts, though in lesser scale were mounted to improve classification by various molecular approaches. Genetic characterization, deoxyribonucleic acid (DNA) base ratios, nucleic acid hybridization studies, cell wall analyses, and protein sequencing began to reveal phylogenetically valid groupings. "These early molecular approaches, though useful, were not powerful enough to reveal the higher bacterial taxa, and in any case conventional wisdom did not perceive doing so as important" (Woese, 1987, Woese *et al.*, 1990). The sequencing of proteins and nucleic acids further strengthened the methodology to measure evolutionary relationships and a novel way of looking at them, in terms of the "evolutionary clock" (Wilson *et al.*, 1977).

In 1987, Carl Woese's seminal work has added a new dimension to this field. An important observation was that by examining structural feature in the small subunit rRNA, the hairpin loop (a side lump made-up of six nucleotide in all eubacteria protruding from stalk of the structure) found between positions 500 and 545 (*E. coli* numbering), could distinguish eubacteria from archaebacteria and eukaryotes (Woese *et al.*, 1990). In subsequent years, ease of sequencing of rRNA gene sequences revolutionized the molecular phylogeny. Genetic information, i.e., sequence information, was found as superior tool over phenotypic data in two main ways: (a) more readily, reliably, and precisely interpreted and (b) inherently more informative of evolutionary relationships than phenotypic information is. "Unlike three-dimensional phenotypic patterns, a sequence pattern is one dimensional and One-dimensional pattern can be measured in simple ways, in terms of simple relationships" (Woese, 1987). Five important points summarized by Woese *et al.* (1990), as stated below have provided the strongest logic in support of using ribosomal RNA for evolutionary phylogeny.

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I. They possess a high degree of functionally fidelity, which assures relatively good clock like behaviour over other.
II. They found in all organisms, and different positions in their sequences change at very different rates, allowing most phylogenetic relationships (including the most distant) to be measured, which makes their range all-encompassing.
III. Their sizes are large and they consist of many domains.
IV. There are about 50 helical stalks in the 16S rRNA secondary structure and roughly twice that number in the 23S rRNA which makes them accurate chronometers on two counts.
V. The most compelling reason for using rRNAs as chronometers is that they can be sequenced directly."

As multiple copies of 16S rRNA gene(s) possessing highly conserved regions at both upstream and downstream and hypervariable regions providing conserved species-specific signature sequences has advantage of easy PCR based amplification, it has become more relevant for phylogenetic studies.

Later on, bacteriologist realized that there should be a uniform rule for naming. In 1992, bacteriologists at international level agreed to set a rule for naming Bacteria and Archea. These rules are termed as "International Code for the Nomenclature of Bacteria" (ICNB). ICNB stated that each bacterium will be named in the same manner as of plant and animals i.e Linnaeus's binomial system. *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) is the journal devoted to the taxonomy for the publication of newly discovered bacterial species. Hence it has been accepted universally that any claim for a new species requires validation by the authorities of IJSEM. The

recent edition of *"Bergey's Manual of Systematic Bacteriology (2001-2008)"*, published by Springer contains a complete list of prokaryotic species and their classification.

There were many rules formulated for bacterial classification. For example, rule 9 (Lapage *et al.*, 1992) of the *Bacteriological code* (1990 Revision), the name of taxon between genus and class will be figured by the addition of a suitable suffix to the stem of the name of the type genus. Stackebrandt *et al.* (1997) proposed a list of suffixes to denote class and subclass. However, there is no such hard-and-fast official document in prokaryotic taxonomy (Sneath and Brenner, 1992) and still it is a matter of debate. The latest "Taxonomic Outline", now known as "Taxonomic Outline of the Bacteria and Archaea" (TOBA) release 7.7 (Garrity *et al.*, 2007). At present, to define a bacterial species accurately, a set of tests like phenotypic features, 16S rRNA gene based phylogenetic data; results of DNA-DNA hybridization assay, typing of housekeeping genes, DNA base composition, chemical composition of cell, fatty acid methyl ester analysis and some genus or species specific characteristics are required. This collective species identification approach called as polyphasic characterization.

In the first phase, phenotypic characterization along with total cellular protein profiling of the isolates were performed. The results of the first phase enabled to create the dendrogram based on similarity coefficient. In the second phase, representative isolate(s) from each cluster were picked up for determining phylogeny using 16S rRNA gene sequences for identification at least up to the genus level. A polyphasic approach was also utilized to characterize and classify one novel Gram-positive bacterium, *Brevibacterium siliguriense* discovered during the study. This novel species have been validated and published in IJSEM (Kumar *et al.*, 2012).

3A.2. Materials and methods

3A.2.1. Bacterial isolates

All ninety facultatively oligotrophic bacteria which yielded amplicon(s) of the variable region of class 1 integron were characterized up to genus level.

3A.2.2. Morphological and physiological characterization of isolates

Conventional tests for catalase, oxidase, IMViC, casein hydrolysis, citrate utilization, gelatin hydrolysis, amylase production, H2S production, acid production from carbohydrate were performed following methodology described earlier (Cappuccino and Sherman, 1996; Aneja, 2001). Ready to use test kits for biochemical characterization were also used as per manufacturer's instructions (HiMedia, India Ltd). The data generated from these tests were converted into binary numbers and used for numerical taxonomy for categorizing the bacteria.

3A.2.3. Numerical taxonomy followed by total protein profiling

The tentative genus of class 1 integron positive oligotrophic bacteria was ascertained by the application of numerical taxonomy. The phenotypic data generated from physiological and biochemical tests were converted into binary characters (1, for positive character and 0, for negative character) and similarity matrix was generated by using the Sneath and Sokal methodology (Sneath and Sokal, 1973). The Jaccard (Tanimoto) coefficient was computed from the set of variable (similarity: a /a+b, where a, is the homolog character present in two bacterial isolate and b is the number of non-homolog character present in two bacterial isolate). The similarity dendogram was generated using unweighted pair group method with arithmetic means (UPGMA) tree building method with the help of DendroUPGMA tool available at http://genomes.urv.cat/UPGMA/. The obtained output also can be visualized in tree view tool. The isolates clustered together in groups were subjected for total protein profiling. The total protein of individual isolates of each cluster was extracted followed by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer of pH 8.3 [composition (g/L): Glycine, 14.4; Tris-buffer, 3; and SDS, 1) at 100v 30 amps for 3 h

(Sambrook and Russell, 2001). For extracting total proteins, 3-4 colonies were suspended in 200 µL 2X SDS-gel loading buffer [composition: 100mM Tris-cl, pH 6.8; 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol and 200mM DTT or β-mercaptoethanol (β-ME) (should be added at time of experiment)]. Total proteins separated on 12% polyacrylamide gel, according to their molecular size (molecular weight), was stained in 0.25% (w/v) coomassie-blue [staining solution: coomassie blue 0.25% (w/v); 90 mL 50% methanol+ 10 mL absolute glacial acetic acid] for 3h at gel dancing apparatus. Gel was visualized under white light. Pattern were compared and considered identical when all the protein bands (migrated bands) were at same distances.

3A.2.4. Amplification, cloning and sequencing of 16S rRNA gene sequence

One representative culture from each group of bacteria, exhibiting similar phenotype and nearly identical proteins band pattern, was used for amplification of 16S rRNA gene sequence using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) primer pair in DNA engine (BioRAD, USA). For PCR assay total DNA was extracted by boiling lysis method as described in chapter 2. A 100 µL of supernatant from the lysed cell suspension (after centrifugation step) was aspirated and transferred to the fresh micro-centrifuge tube and stored at 4 °C until use. The supernatant was used as template in the PCR-reaction mixture for amplification of 16S rRNA gene sequence (or 16S rDNA). PCR cycling conditions used were: initial denaturation at 94 °C for 3 min; followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and amplification at 72 °C for 1 min and final extension at 72 °C for 7 min. Amplified product was resolved on 0.7% or 1% agarose gel containing 0.3- 0.5 µg/mL ethidium bromide. Amplified product was purified using commercial PCR purification kit [Elute™ PCR Clean-up kit (Sigma-Aldrich, St. Louis, MO)]. If the multiple bands of PCR product appeared on gel then the gel extraction method was followed to get the desire amplicon for cloning and sequencing purpose. Desired band of ~1.5kb was visually identified by comparing with the marker lane. The DNA band was excised with the help of sterile surgical blade and DNA was extracted using gel extraction kit (Genie, India) following manufacturer's instruction. The purified DNA was cloned in to pGEMT easy vector-II (Fig. 3.2) and transformed in to *E. coli* JM109 host cell. The cells bearing recombinant plasmid, were screened by blue-white screening on ampicillin (100 µg/mL; ampicillin is the selective marker of pGEMT easy vector), X-gal (5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside, concentration used: 0.5mM) and IPTG (isopropylthio-β-galactoside) containing Luria agar plate. The white colonies (colony containing recombinant plasmid) along with one blue (for negative control) were picked and purified on ampicllin containing Luria agar plate. The pure white and blue colonies were transferred to the fresh Luria broth containing ampicllin [concentration, 100µg/mL] and incubated at 37°C for overnight. The recombinant and non-recombinant plasmids were isolated from overnight grown culture following alkaline lysis method (Sambrook and Russell, 2001). The extracted plasmids were visualized over UV trans-illuminator (Genie, India) after run on 0.7% agarose gel containing ethidium bromide and compared with control (non-recombinant). An upward shifting in test plasmid in compare to control is the indication of chimeric DNA (clone containing desired insert). Further the cloning was confirmed by restriction digestion of recombinant plasmid DNA using *Eco*RI endonuclease (Genie, India). The clone was sequenced as described in chapter 2.

3A.2.5. Analyses of 16S rRNA gene sequences (derived from the isolates) using tools of Bioinformatics

DNA sequences of cloned 16S rRNA genes were edited and vector sequences were removed from both the ends after examining via vec-Screen tool (www.ncbi.nlm.nih.gov) or manually. Orientation of the gene sequence was examined manually by searching forward primer location on sequenced 16S rRNA gene sequence. Nearest matches were determined in the GenBank database using BlastN (www.ncbi.nlm.nih.gov) program. Best five or more similar 16S rRNA gene sequences were retrieved from databank and compared using Clustal W/or X. A pairwise sequence alignment

(http://www.ebi.ac.uk/Tools/psa/) was conducted to determine the homology percent. The closest known genera with maximum score and percentage homology were considered as nearest probable genus.

3A.2.6. 16S rRNA gene sequence based phylogeny

The 16S rRNA gene sequence (16S rDNA sequence) of representative isolate was used as a query for search of homologous sequence in the nucleotide sequence databases by using BlastN program (Altschul *et al.*, 1997). Sequences showing high similarities with maximum scores were retrieved from the GenBank database and were aligned with 16S rRNA gene sequences of the isolate by using CLUSTAL W software (Thompson, 1994). Distances were calculated according to Jukes & Cantor (1969) one-parameter/ or Kimura two-parameter (1980)/ and or Tamura and Nei (1993) four-parameter methods. Phylogenetic trees were inferred by using the neighbor-joining (Saitou and Nei, 1987)/ or maximum-likelihood (Yang, 1999)/ and or by maximum-parsimony (Eck and Dayhoff, 1966) algorithms. The tree topology was evaluated by the bootstrap analysis based on 1000 re-samplings (Felsenstein, 1985). For tree building, a MEGA 4.0 software package was used (Tamura *et al.*, 2007).

Accession numbers: The 16S rRNA gene sequences of the isolates were deposited in GenBank/EMBL nucleotide database (Table 3.2).

3A.3. Results and Discussions

The biochemical characteristics exhibited by all class-1 integron-positive isolates are shown in Table 3.1. Out of ninety, eighty nine integron positive oligotrophic isolates were Gram-negative and a single isolate was determined to be Gram-positive. Two isolates, one Gram positive, MB18, and one gram negative, MB12, were excluded from the numerical taxonomy analyses because they responded differently and were not amenable for comparison [Isolate, MB18 was excluded because it was the only isolate which was Gram-positive, while MB12 did not respond conclusively to any of the biochemical test performed]. **Extensive characterization of the strain MB18 has been presented separately** (under the subtitle: *Polyphasic taxonomy of Brevibacterium siliguriense, strain MB18^T, a novel Gram positive facultative oligotrophic bacterium isolated from River Mahananda, Silguri, India*). All gram negative facultatively oligotrophic isolates were grouped in two major categories (i) isolate showing positive reaction to oxidase test and (ii) isolates showing negative reaction to oxidase test. All phenotypic characters were converted in to binary numbers and similarity matrix was calculated using DendroUPGMA tool. Similarity matrix of phenotypic data computed with Jaccard (Tanimoto) coefficient is shown in supplementary table (Annexure-I, available in the soft copy of the thesis). An UPGMA dendogram of all the isolates were constructed from binary data generated from phenotypic characters (Fig. 3.3). It was observed that bacteria possessing same phenotype were found to exhibit nearly same protein pattern under the same cultural condition (Fig. 3.3).

The 16S rRNA gene sequencing (molecular approach) and phenotypic data (similarity matrix, a numerical taxonomic approach) of representatives of each cluster revealed that all oxidase positive and oxidase negative isolates fell under two main classes, *Betaproteobacteria* and *Gammaproteobacteria*. Results revealed that *Betaproteobacteria* comprised of only two genera, *Comamonas* and *Acidovorax* of family *Comamonadaceae* while other isolates were identified under super class *Gammaproteobacteria*. The representative genera of class *Gammaproteobacteria* were constituted by families, *Moraxellaceae, Pseudomonadaceae, Aeromonadaceae* and *Enterobacteriaceae*. Similar to previous reports published on presence of integrons in copiotrophic bacteria, majority of the identified integron positive oligotrophic bacteria of super class *Gammaproteobacteria* were detected from the family *Enterobacteriaceae*. (Mukherjee and Chakraborty, 2007; Chen *et al.*, 2011;

Xu *et al.*, 2011; Mokracka *et al.*, 2012). Eight facultative oligotrophic isolates MB25, MB28, MB41, MB44, MB48, MB54, MB81, MB83 and one oligotrophic isolate MB12 could not be assigned a specific genus from the data derived either phenotypically or by 16S rRNA gene sequence (Fig. 3.3 to 3.8). The 16S rRNA gene sequences of these unclassified isolates (the isolates which could not be assigned any genus) shared insignificant homologies with the known genera of different classes. The 16S rRNA phylogeny showed that they were branching with uncultured bacteria. However on comparing the phenotypic data, the group of unknown species was found to cluster with members of family *Enterobacteriaceae* (Fig 3.3). Due to taxonomic uncertainties, these strains were kept under category of unknown spp.

Majority of the integron positive oligotrophic bacteria were detected from the family *Enterobacteriaceae* similar to earlier reports on presence of integrons in copiotrophic bacteria (Mukherjee and Chakraborty, 2006; Chen *et al.*, 2011; Han *et al.*, 2012; Su *et al.*, 2012). The results indicate that the members of this family are more prone to acquire antibiotic-resistance genes to get selective advantages over other. In the present study fifty five integron-positive oligotrophic isolates were identified as the members of the family *Enterobacteriaceae*. Isolate MB05 and MB24 was identified as member of genus *Shigella* (Table 3.2; Fig 3.3 and 3.9). Genus *Kluyvera* was only represented by the single isolate MB66 (Fig. 3.10 and Table 3.2). Eight isolates, MB19, MB26, MB29, MB42, MB45, MB49, MB51 and MB72 were recognized as the member of genus *Klebsiella* (Table 3.2; Fig.3.3, 3.11 and 3.12). Genus *Enterobacter* were represented by six integron positive oligotrophic bacterial isolates named as OD21, SR19, MR01, MB40, MB59, and MB73 (Table 3.2; Fig. 3.13). Four isolates MB30, MB34, MB47 and MB57 were identify as member of genus *Proteus* (Table 3.2; Fig. 3.13). Phenotypic data and 16S rRNA sequence homology revealed that isolates MB20, MB38, MB43, MB64, MB67, MB74, MB75, MB76 and MB82 belongs to the genus *Salmonella* (Table 3.2; Fig. 3.13). Only two isolates MR04 and NV66 represented the genus *Providencia* (Table 3.2; Fig. 3.14). Genus *Serratia* was represented by isolates MB23 and MB53 (Table 3.2; Fig. 3.15). Phenotypic and genotypic study revealed that the isolates MR03, MB35, MB61, OC16, OC24, OD05, OD08, OD10 and OD24 represented the genus *Citrobacter* (Table 3.2; Fig. 3.13 and 3.15). Twelve isolates, OC75, OC78, MB27, MB31, MB32, MB33, MB36, MB37, MB60, MB65, MB68, MB69, and MB79 were tentatively classified under genus *Escherichia* (Table 3.2; Fig. 3.13 and 3.16).

Family *Comamonadaceae* was represented by nine integron positive isolates, MB09, OB05, MR02, MB16, MB50, MB56, MB58, MB70 and MB71 (Fig.3.3 and Table 3.2). Isolate MB09 was identified as the member of genus *Comamonas* while rest eight isolates were tentatively characterized under genus *Acidovorax* (Table 3.2; Fig. 3.17 and 3.18). All known species of *Acidovorax* are aerobic and less is known about them, few of them are found to cause diseases in vegetables. *Acinetobacter* was the only genus identified amongst the isolates that was under the family *Moraxellaceae*. Seven isolates, MB03, MB22, MB46, MB52, MB55, MB63 and MB80 were assigned to the genus *Acinetobacter* (Table 3.2; Fig 3.3 and 3.19). *Acinetobacter* strains are capable of surviving in extremely nutritionally deficient (oligotrophic) abiotic environments of hospitals for a substantial period, for example, up to ten days on dust and dry particles (Webster *et al.*, 2000) or for more than 4 months on both moist and dry surfaces such as polyvinyl chloride, rubber, ceramics, and various types of medical equipments (Wendt *et al.*, 1997) and are an important cause of infection in immunocompromised patients.

Aeromonas was the only genus that was identified for the family *Aeromonadaceae* of super class *Gammaproteobacteria*. On the basis of habitat genus *Aeromonas* divided in two groups-psychrophilic and mesophilic. Psychrophilic are omnipresent aquatic environmental species and are mostly pathogenic to the aquatic (mainly fish) and terrestrial animals while mesophilic *Aeromonas* spp. are grow in temperature ranging 10-42 °C and are important pathogens for humans (Lee *et al.*, 2008). In a study from conducted in Taiwan it was observed that 13.9% of the total were carrying class 1 integron (Lee *et al.*, 2008). A report on *Aeromonas* isolated from aquaculture showed that

~60% members were found to carry gene cassettes in variable region of class 1 integrons (Lukkana *et al.*, 2012; Ol Ndi and Barton, 2011). Four isolates MB21, MB39, MB77 and MB78 were classified under this genus (Table 3.2; Fig 3.3, 3.20, 3.21 and 3.22). Family *Pseudomonadaceae* was covered by a single genus *Pseudomonas*. Four isolates OB12, MB08, MB62, and OC74 were categorized in the genus *Pseudomonas* (Table 3.2; Fig 3.3 and 3.23). Pseudomonads are recognized as opportunistic pathogen of human and other animals (Haenen and Davidse, 2001; Fonseca *et al.*, 2005) and have been recovered from diverse sources even from distilled water (Favero *et al.*, 1971). Reports revealed that member of *Pseudomonas* are potential carrier of antibiotic resistance genes in class 1 integrons (Poirel *et al.*, 2001; Yan *et al.*, 2007).

[B]: Identification of a novel gram positive bacterium using polyphasic approach

3B: Polyphasic taxonomy of *Brevibacterium siliguriense*, strain MB18^T, a novel Gram positive facultative oligotrophic bacterium isolated from River Mahananda, Siliguri, India

The genus *Brevibacterium* was established by Breed (1953) for some non-sporulating, non-branching, Gram-positive rods, which were earlier assigned to genus *'Bacterium'*. While several gram-positive, non-spore-forming, non-branching rods were classified as members of the genus *Brevibacterium*, further chemotaxonomic studies showed that not all of these species are the members of the genus *Brevibacterium*. Eventually, the genus *Brevibacterium* has been described after the type species, *Brevibacterium linens* (Collins *et al.*, 1980). As per available reports, *Brevibacterium* spp. have been isolated from diverse sources such as milk products (Kollöffel-*et al.*, 1999), clinical specimens (Collins *et al.*, 1983; Pascual *et al.*, 1996; Wauters *et al.*, 2001; Wauters *et al.*, 2003; Wauters *et al.*, 2004; Mages *et al.*, 2008; Roux and Raoult, 2009), human body parts (McBride *et al.*, 1993), soil (Gavrish *et al.*, 2004; Tang *et al.*, 2008), sediment (Lee, 2006; Bhadra *et al.*, 2008), brown algae (Ivanova *et al.*, 2004), paintings (Heyrman *et al.*, 2004), poultry (Pascual and Collins, 1999), marine environments (Lee, 2008), fresh water (Manage *et al.*, 2009), insects (Kati *et al.*, 2010), wall colonized by moulds (Kämpfer *et al.*, 2010), salt-lake (Tong-Wei Guan *et al.*, 2010) and recently, *B. daeguense*, isolated from industrial wastewater treatment plant (Cui *et al.*, 2012). So far 46 species were classified in genus *Brevibacterium* (http://www.bacterio.cict.fr/b/brevibacterium.html).

A facultatively oligotrophic *Brevibacterium siliguriense* sp. nov. strain MB18^T, isolated from waters of river Mahananda at Siliguri (Longitude, 88°25'22.89"E; Latitude, 26°44'23.20"N) West Bengal, India, representing a novel member of the genus *Brevibacterium*, has been described in details in this chapter.

Oligotrophic growth of the strain MB18^T was demonstrated. In diluted (10^{-3}) Luria Broth (LB), an increase of approximate 12 times from the initial cell number was noted in span of 4 days incubation, at 28 °C (Fig. 3.24). Oligotrophic growths of the strain were also recorded in other diluted (10^{-3}) media like nutrient broth (NB, HiMedia, India) and tryptone soy broth (TSB, HiMedia, India). However strain MB18 could grow well in the undiluted rich media like LB, NB and TSB. Since the strain was able to grow on both nutrient-rich and nutrient-deficient media, it has been described as a facultative oligotrophic bacterium.

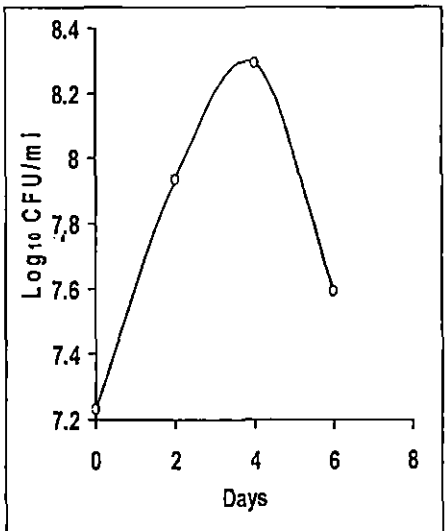


Fig. 3.24: Viability and growth of MB18^T in diluted (10^{-3}) Luria broth

Cell morphology and motility were determined under phase contrast microscope (Olympus, Japan); detail of the cell shape (Fig. 3.25) was ascertained with help of scanning electron microscope (LEO 1430 VP). Result of Gram-staining (Claus, 1992) was confirmed by the KOH lysis method (Murray *et al.*, 1999). Growth of the strain MB18 was tested at 10, 20, 28, 30, 37, 40 and 45 °C (±1). Salt tolerance was tested in peptone-yeast extract (PY) medium (composition g/L; Peptone 10, yeast extract 5) supplemented with following concentrations (%w/v): 0, 2, 4, 6, 8, 10, 15 and 20 of KCl and NaCl. To assess growth at different pH, the pH of the sterile LB medium was adjusted from pH 3.0 to 12.0 by using either 0.1 M HCl or 0.1 M NaOH. Catalase activity was examined by air bubble production after the addition of few drops of 3% (v/v) H_2O_2. Ability to hydrolyze starch was

determined by assessing the development of clear zones around the streaked culture. Lipase production was determined by standard procedure. Hemolytic activity and gelatin hydrolysis were tested according to the method described earlier (Kumar *et al.*, 2010). Pyrazinamidase activity and acid production from 2, 3-butylene glycol were detected as described by Wauters *et al.* (2001). Acid production from ethylene glycol and phenyl acetate; and Alkali production from sodium-formate was detected by methods described earlier (Wauters *et al.*, 1998, 2003). Hydrolysis of casein, tyrosine and xanthine were examined using the method described by Gordon *et al.* (1974). Oxidase, lysine utilization, ornithine utilization, urease activity, deamination of phenyl alanine, reduction of nitrate, H_2S production, citrate utilization, VP test , MR test, malonate utilization and carbon source utilization/fermentation tests were carried out using HiBio-ID/*HiCarbo* system (HiMedia, Mumbai, India) and GP card (VITEK 2 system, BioMérieux) according to manufacturer's instruction. Results were scored after 7 day at 28 °C. The biochemical characteristics of strain MB18^T were also determined using the Biolog GP2 MicroPlate system (BioMérieux). Bacterial suspensions prepared in GP sterile inoculation fluid transferred to GP2 Microplates as described by the manufacturer.The tests were repeated three times. The metabolic fingerprint patterns were noted. Antibiotics susceptibility tests (specific for oligotrophic bacteria) determined according to the method described in chapter 2. Susceptibility to some of the drugs was tested using GP card (VITEK 2 system, BioMérieux) and results were interpreted according to manufacturer's instructions.

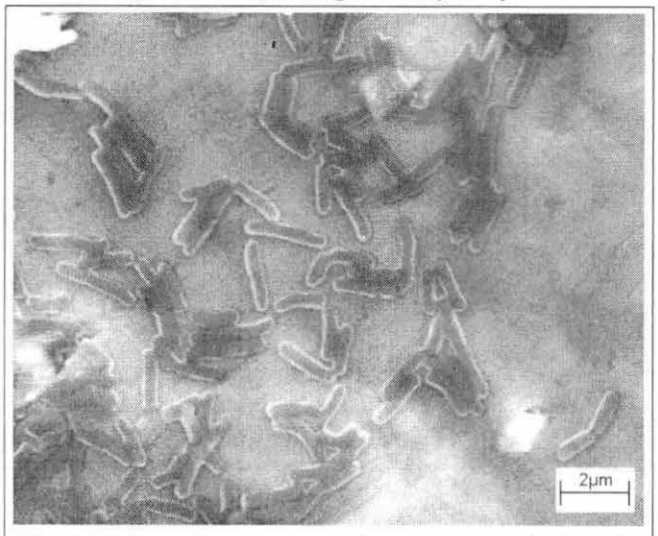


Fig. 3.25: Scanning electron micrograph of 16 h old cells of *Brevibacterium siliguriense* sp. nov. strain MB18^T grown in undiluted Luria broth at 28 °C. Bar, 2 µm.

For amplification of 16S rRNA gene, whole cell DNA was extracted according to the method described in chapter 3. The amplified 16S rRNA gene sequence was purified, cloned and sequenced. Nearly complete 16S rRNA gene sequence comprising 1433 bp was obtained from strain MB18 using 27F and 1492R primers. 16S rRNA gene sequence comparisons with entries in the updated GenBank and EMBL databases were performed with the FASTA and BLAST programs (Pearson, 1990; Altschul *et al.*, 1990, 1997). To determine the phylogenetic affiliation, the 16S,rRNA gene sequence of strain MB18 was aligned with the sequences of species of the genus *Brevibacterium* using Clustal-W (Thomson *et al.*, 1994). Approximately 1360 bp long stretch of 16S rRNA gene sequences present in all strains of *Brevibacterium* between position 13 and 1371 selected from nucleotide database from EMBL European Bioinformatics Institute (http://www.ebi.ac.uk) for further analysis. The rest of the lanking nucleotide sequences were omitted due to alignment ambiguities. Phylogenetic tree(s) [maximum-parsimony (MP) and neighbour-joining (NJ)] were constructed from continuous stretch containing ~1360 bp (Fig. 3.26 and 3.27). Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The fidelity of the tree topologies were evaluated by the bootstrap analysis with 1000 replicates (Felsenstein, 1985). The phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

The phylogenetic tree (Fig. 3.27) showed that strain, MB18 belong to the genus *Brevibacterium*. In the phylogenetic tree, strain formed a cluster comprising five *Brevibacterium* species: *B. epidermidis* NCDO 2286^T, *B. iodinum* DSM 2062^T, *B. permense* VKM Ac-2280^T and *B. linens* DSM 20425^T and *B. oceani* LMG 23457^T. Strain MB18 makes a deep branching with *B. epidermidis* NCDO 2286^T (= DSM 20660 = LMG 21455) with 16S rRNA gene sequence similarity value of 96%.

According to previous studies if any strain, showing 3% or more than 3% 16S rRNA gene sequence dissimilarity with their neighbours, it can be assigned as separate species (Stackebrandt and Goebel, 1994; Stackebrandt *et al.*, 2002; Lee, 2006 and Tindall *et al.*, 2010) without the need of DNA-DNA hybridization. As the level of similarity between 16S rRNA gene sequences of strain MB18 and the closest *Brevibacterium* species, *Brevibacterium epidermidis* NCDO 2286^T was 96%, therefore, this strain, MB18 can be endorsed to a separate genospecies without entering into DNA–DNA hybridization. The differences are also evident between the strains in terms of biochemical and chemotaxonomic characters.

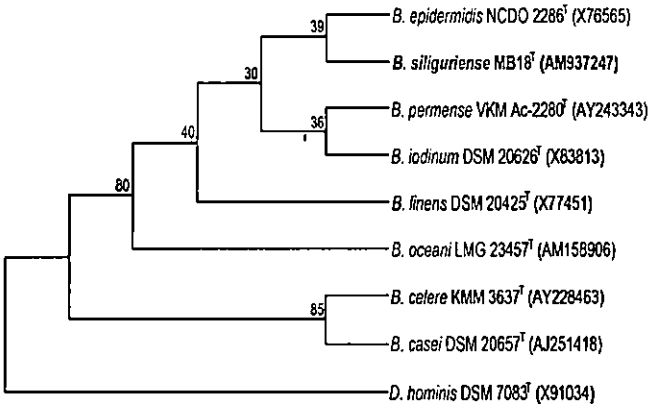


Fig. 3.26: MP tree based on 16S rRNA gene sequences, showing the position of strain MB18^T (Bold face) within the closely related members of the genus *Brevibacterium*. Bootstrap values (expressed as percentages of 1000 replications) are given at the branch nodes. *Dermabacter hominis* DSM 7083^T was used as outgroup.

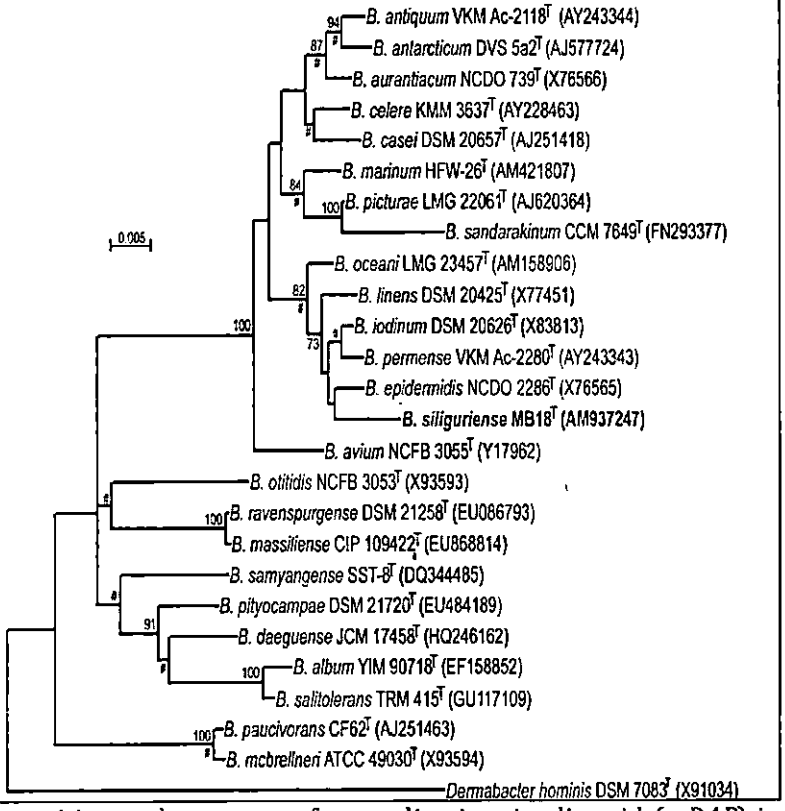


Fig. 3.27: Consensus NJ tree derived from 16S rRNA gene sequences showing the position of strain MB18^T within the members of the genus *Brevibacterium*. The tree was reconstructed using maximum parsimony method and common clusters obtained in both NJ and MP tree are indicated by hash (#). Bootstrap values (>70%) expressed as percentages of 1000 replications are given at each branch point. *Dermabacter hominis* DSM 7083^T was used as out-group. Bar, 5 nucleotide substitutions per 1000 nucleotide.

To determine the sugar composition and presence of *meso*-diaminopimelic acid (*mDAP*) in strain MB18^T, 200 mg (wet weight) cells were scraped from LA plate grown at 28 °C for 3 days. The cell walls were prepared according to the method of Boone and Pine (1968), and sugars and *m*DAP in acid hydrolysates were identified by one-dimensional paper chromatography following the method described earlier (Staneck and Roberts, 1974) with slight modification (Whatman paper No.1 was used instead of cellulose thin- layer chromatographic plate). Using butanolic ninhydrin (0.3% w/v ninhydrin in isobutanol) spray, the appearance of gray-green spot with low Rf value (0.37-0.38) which turned yellow on keeping in dark for more than 24 h, confirmed the characteristic

presence of *m*DAP (Fig. 3.28). *m*DAP standard was kindly provided by Dr. B. Bhadra (CCMB Hyderabad, now at dupont, Hyderabad).

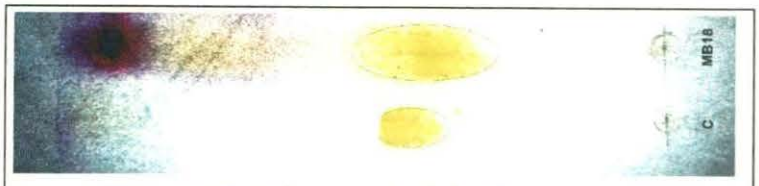


Fig. 3.28: mDAP (yellow spot) of *Brevibacterium siliguriense* sp.nov. strain MB18^T. C, control

Sugars present in cell wall acid hydrolysate were detected by spraying the acid aniline phthalate (0.325% 0-pthalic acid dissolved in water saturated n-butanol containing 0.2 mL aniline). Galactose as the sole sugar was identified in the cell wall of strain MB18 (Fig. 3.29). Polar lipids were isolated from the strain MB18 by the method described earlier (Minnikin *et al.*, 1984). The isolated polar lipids were detected on aluminum backed silica gel 60 F_{254} plate (Merck, Germany) by one and two-dimensional thin-layer chromatography (TLC) according to the method (Counsel and Murray, 1986). Polar lipid profile (Fig 3.30) contained, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) phospholipids as found in the other members of *Brevibacterium*. For detection of mycolic acid, method described by Minnikin *et al.* (1980) was used. No mycolic acid was detected in cell wall of the strain MB18. Menaquinones were extracted from lyophilized cells and analyzed by HPLC following methods described by Collins *et al.* (1977) and Groth *et al.* (1997) respectively.

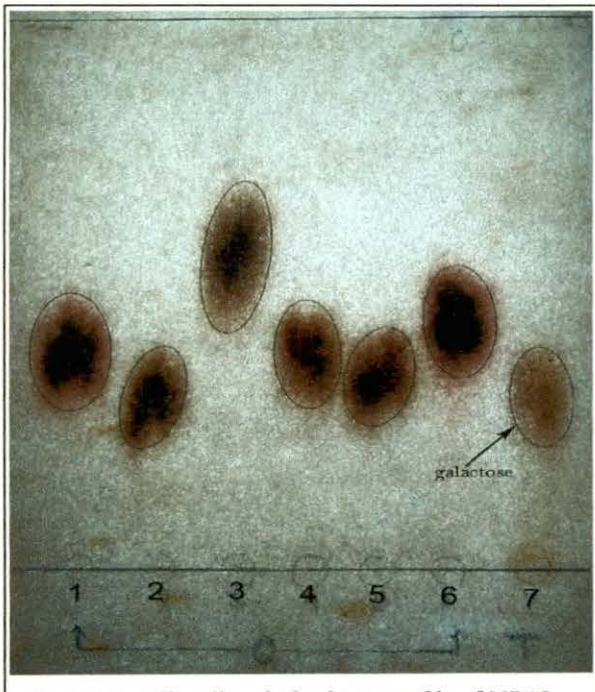


Fig. 3.29: Cell wall carbohydrate profile of MB18. 1, arabinose; 2, galactose; 3, rhamnose; 4, mannose; 5, glucose; 6, xylose; 7, Acid hydrolysate of *Brevibacterium siliguriense* sp. nov. strain MB18^T. Top gray colored line showed solvent front.

Fig. 3.30: The lipid profile of strain MB18

PG, phosphatidylglycerol;
DPG, diphosphatidylglycerol

For analysis of fatty acids, fatty acid methyl esters (FAMEs) were extracted from 36 h old (exponentially growing) cells grown in Tryptone-soy-agar (M290; HiMedia, India) at 28 °C. It was then analyzed by gas chromatography (Hewlett Packard 5890 II plus, Palo alto, CA, USA) and the Sherlock Microbial Identification System using version 4.10 of the TSBA40 library (Microbial ID, Newark, DE, USA). The analyzed FAME peaks are shown in Fig 3.31. Fatty acids of strain MB18^T [anteiso-$C_{15:0}$ (50.82%), anteiso-$C_{17:0}$ (26.73%), iso-$C_{15:0}$ (14.04%), iso$C_{16:0}$ (4.07%); iso-$C_{17:0}$ (3.16%); and trace amount of $C_{16:0}$ (0.48%); iso$C_{14:0}$ (0.47%) and $C_{14:0}$ (0.22%)] were typical of the genus *Brevibacterium*, but the proportion differed from those reported for *B. epidermidis* DSM 20660^T

{anteiso-$C_{15:0}$ (70.0%), anteiso-$C_{17:0}$ (21.5%), iso-$C_{15:0}$ (2.0%), iso$C_{16:0}$ (3.0%); and trace amount of anteiso-A $C_{17:1}$ (2.0%), $C_{18:0}$ (1.5%), and $C_{16:0}$ (1.0%) [Collins *et al.*, 1983]}.

For determination of G+C content, the genomic DNA of the strain was prepared by disrupting cells with French pressure cell and purified on hydroxyapatit following standard procedure (Cashion *et al.*, 1977). The purified DNA was hydrolyzed with p1 nuclease and the nucleotides de-phosphorylized with bovine alkaline phosphates (Mesbah *et al.*, 1989). The resulting deoxyribonucleside were analyzed by HPLC system (Shimdzu Corp. Japan,) having LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-20A automatic sample injector and a SPD-6A UV spectrophotometric detector. The system was calibrated with non-methylated lambda DNA (Sigma) (49.86 mol% G+C) and three sample DNAs, *Bacillus subtillis* DSM 402 (43.52 mol% G+C), *Xanthomonas campestris* pv. *campestris* DSM 3586^T (65.07 mol% G+C), and *Streptomyces violaceoruber* DSM 40783 (72.12 mol% G+C). GC was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah *et al.* (1989). The DNA G+C content of the strain MB18 was found to be 64.6 mol% which was very near to its neighbour, *B. epidermidis* DSM 20660^T (63.5 mol %).

Table 3.3: Differential phenotypic characteristics between *B. siliguriense* sp. nov. strain MB18^T and *B. epidermidis* LMG 21455^T, the nearest phylogenetic neighbor.
Symbols: –, negative result; +, positive result.

Characteristics	MB18^T	*B. epidermidis*
Growth at :.		
37°C	poor	good
40°C	–	+
Hydrolysis of Casein	–	+
Hydrolysis of Xanthine	–	+
Acid from Phenyl acetate	+	–
Carbon utilization:		
Glucose	–	+
Arabinose	–	+
Succinic acid	–	+
Xylose	–	+
Inositol	–	+
Mannose	+	–
Raffinose	–	+
Glycerol	+	–
Galactose	–	+
Gluconate	–	+
Enzymes:		
Alkaline phosphatase	–	+
b-glucoronidase	–	+
Fermentation of D-mannitol	+	–
DNA G+C content (mol%)	64.6	63.5
Origin of isolation	River water	Human skin

In spite of the similarities, strain MB18 can be readily differentiated from *B. epidermidis* with reference to some physiological and biochemical characteristics (Table 3.3). Strain MB18 can also be distinguished from *B. epidermidis* NCDO 2286^T by 16S rRNA gene sequence similarity, DNA G+C content, cellular fatty acids, and other chemical analysis. On the basis of the data obtained with our polyphasic taxonomic approach, strain MB18 merits recognition as a member of a novel species of the genus *Brevibacterium*, for which the name *Brevibacterium siliguriehse* sp. nov. was proposed.

3B.1. Description of *Brevibacterium siliguriense* sp. nov.

Brevibacterium siliguriense: si.li.gu.ri.en'se. N.L. neut. adj. siliguriense of or pertaining to town Siliguri, the location from where the water sample was collected.

Cells are Gram-positive, non-motile, non-spore-forming, rods, 2.0 ±0.23 µm long and 0.4±0.06 µm wide, catalase-positive and oxidase-negative. Colonies are off-white, circular and low convex with entire margin. Growth is observed at 20-37°C (optimum 28 °C; no growth at 40 °C), pH 5–12 (optimum 7.0); and 0-15% (w/v) KCl and NaCl (optimum 2 %). The supplementation of KCl in PY media shows better growth than the NaCl. Urease, gelatinase, arginine dihydrolase 1, β–galactosidase, β-glactopyranosidase, leucine arylamidase, L-proline arylamidase, α-galactosidase, alanine arylamidase and tyrosine arylamidase are positive. Nitrate is reduced to nitrite. Tests for α-glucosidase, ala-phe-pro arylamidase, L-aspartate, arylamidase, α-mannosidase, phosphatase, β-glucoronidase, H_2S production, indole production, L-pyrrolidonyl-arylamidase and fermentation of

D-xylose, D-sorbitol, D-galactose, D-ribose, lactose, N-acetyl D-glucosamine, D-maltose, D-raffinose, sacchorose, D-trehalose, D-amygdalin are negative. Additional detailed phenotypic characteristics and antibiogram are given in Table 3.4A and 3.4B. Strain MB18^T contains *mDAP* in peptidoglycan. Phosphatidylglycerol and diphosphatidylglycerol are the major polar lipids. The major menaquinone is MK-8(H2) and fatty acid profile contains predominantly anteiso-$C_{15:0}$ (50.82%), anteiso-$C_{17:0}$ (26.73%) and iso-$C_{15:0}$ (14.04%).The G+C content of the type strain is 64.6 mol%.

The type strain MB18^T (=DSM **23676**^{T} = LMG **25772**^{T}) was isolated from water sample of river Mahananda, Siliguri, West Bengal, INDIA.

3.4. Conclusion

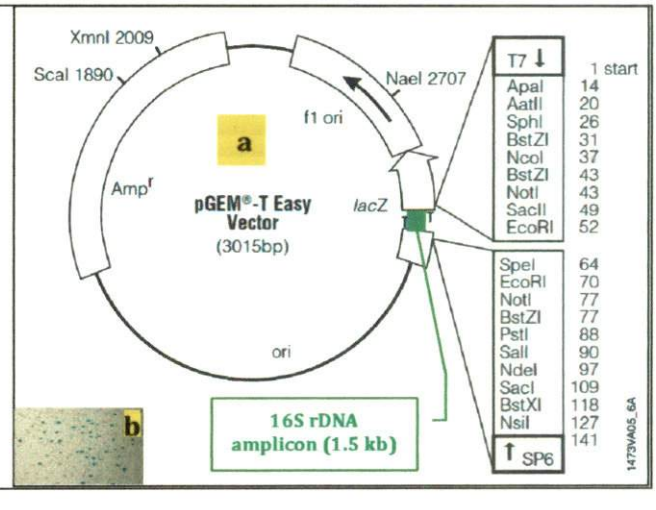
Analyses of phenotypic characteristics and 16S rRNA gene sequence phylogenies of the facultatively oligotrophic isolates with class 1 integron carriages indicated the diversity of oligotrophs in the Mahananda River at Siliguri. They belonged to different families like *Comamonadaceae, Moraxellaceae, Pseudomonadaceae, Aeromonadaceae,* and *Enterobacteriaceae.* These families fell under two super classes: *Betaproteobacteria* and *Gammaproteobacteria.* Despite of the several isolates placed in a specific genera, nine isolates, MB25, MB28, MB41, MB44, MB48, MB54, MB81, MB83 and MB12 could not be assigned to any of the known genera. The 16S rRNA gene sequences of these isolates (the isolates which could not be assigned any genus) poorly shared with the known genera of different classes. Each of these isolates demand further studies for establishment of novel genus. However, on examining their positions in the dendogram (based on numerical taxonomy), they were found to branch separately with isolates clustered under the family *Enterobacteriaceae.* Majority of the isolates were detected from the family *Enterobacteriaceae* comprising of genera: *Shigella, Kluyvera, Klebsiella, Salmonella, Citrobacter, Serratia, Enterobacter, Proteus, Providencia* and *Escherichia. Acinetobacter* was the only genus identified amongst the isolates that was under the family *Moraxellaceae.* The only culturable oligotrophic Gram positive strain isolated in the course of this study, MB18, was identified as a novel species of the genus *Brevibacterium* on the basis of 16S rRNA gene sequence similarity, DNA G+C content, cellular fatty acids, and other chemical analysis. On the basis of the data obtained with our polyphasic taxonomic approach, strain MB18 merits recognition as a member of a novel species of the genus *Brevibacterium,* for which the name *Brevibacterium siliguriense* sp. nov. was proposed.

Thus it may be concluded that exploring river water for culturable oligotrophic bacteria provides not only an opportunity to discover novel bacteria but also strengthens the study of microbial biodiversity at large.

Remaining figures with their legends:

Fig. 3.2: a, Cloning of 16S rDNA-amplicon in pGEMT-easy vector vector-II and b, Blue-white screening of recombinant clone on Luria agar plate containing ampicillin (100 mg/L), X-gal and IPTG. Red color shows the gene of interest, ligated within the multiple cloning site of T-vector.

Amp^r, gene for ampicillin resistance; *ori*, orgin of replication; *lacZ*, gene for beta-galactosidase synthesis.



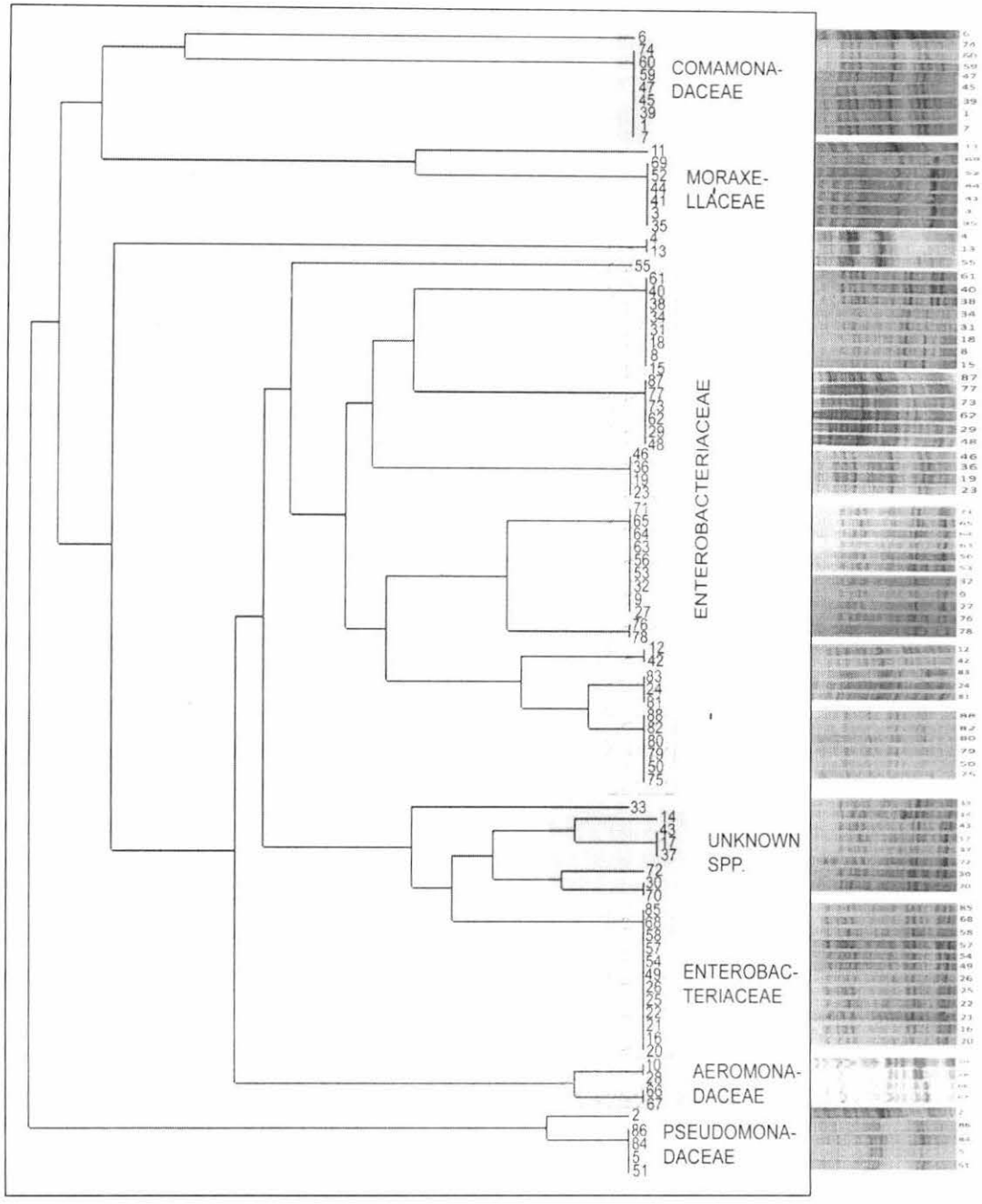


Fig. 3.3: Dendogram based on similarity matrix (numerical analysis) computed from phenotypic characters and One-dimensional SDS-PAGE analysis of total proteins extracted from bacteria possessing class 1 integrons. MB12 and MB18 are excluded from this analysis, for detail please see manuscript. Similarity matrix of phenotypic data is available as supplementary table in the soft copy of the thesis (Annexure-I).

Numbers corresponding to the isolates: 1, OB 05; 2, OB 12; 3, MB 03; 4, MB 05; 5, MB 08; 6, MB 09; 7, MB 16; 8, MB 19; 9, MB 20; 10, MB 21; 11, MB 22; 12, MB 23; 13, MB 24; 14, MB 25; 15, MB 26; 16, MB 27; 17, MB 28; 18, MB 29; 19, MB 30; 20, MB 31; 21, MB 32; 22, MB 33; 23, MB 34B; 24, MB 35; 25, MB 36; 26, MB 37A; 27, MB 38; 28, MB 39; 29, MB 40; 30, MB 41; 31, MB 42; 32, MB 43; 33, MB 44; 34, MB 45; 35, MB 46; 36, MB 47; 37, MB 48; 38, MB 49; 39, MB 50; 40, MB 51; 41, MB 52; 42, MB 53; 43, MB54; 44, MB 55; 45, MB 56; 46, MB 57; 47, MB 58; 48, MB 59; 49, MB 60; 50, MB 61; 51, MB 62; 52, MB 63; 53, MB 64; 54, MB 65; 55, MB 66; 56, MB 67; 57, MB 68; 58, MB 69; 59, MB 70; 60, MB 71; 61, MB 72; 62, MB 73; 63, MB 74 ; 64, MB 75; 65, MB 76; 66, MB 77; 67, MB 78; 68, MB 79; 69, MB 80; 70, MB 81; 71, MB 82; 72, MB 83; 73, MR 01; 74, MR 02; 75, MR 03; 76, MR 04; 77, SR 19; 78, NV 66; 79, OD 05; 80, OD 08; 81, OD 10; 82, OC 16; 83, OC 24; 84, OC 74; 85, OC 75; 86, OC 78; 87, OD 21; 88, OD 24.

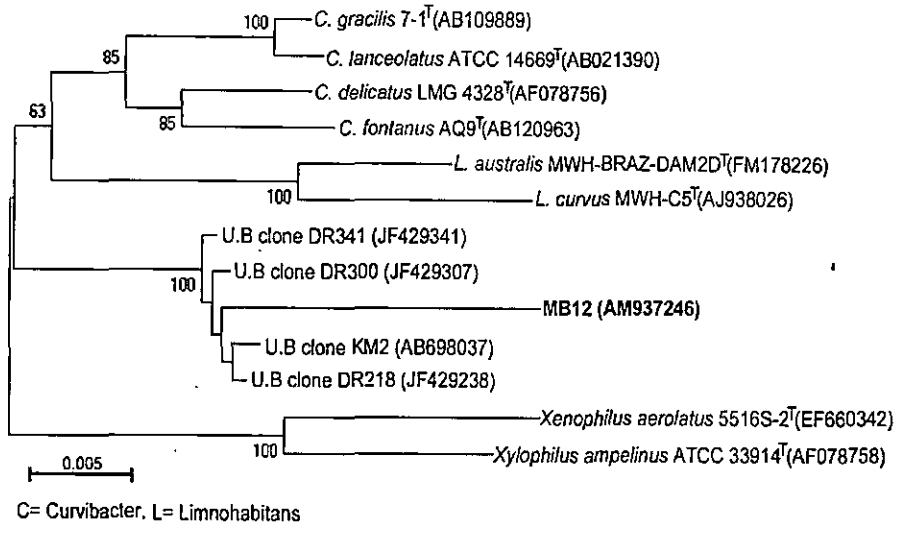
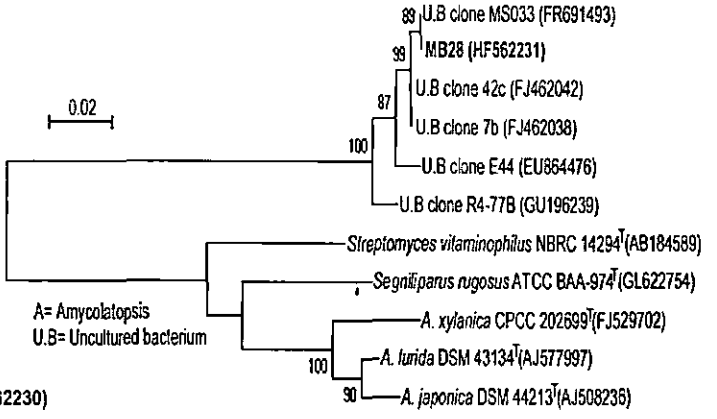


Fig. 3.4: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-12 (Bold face) within uncultured bacteria and related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are indicated at branching nodes. Accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

Fig. 3.5: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB28 (Bold face) within the uncultured bacterium and members of related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.



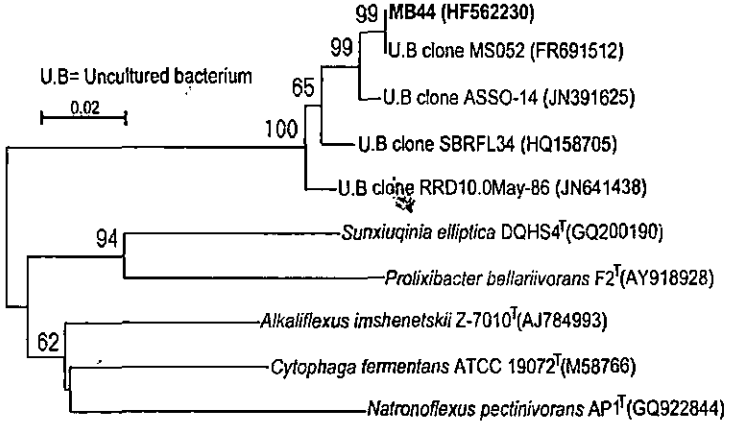
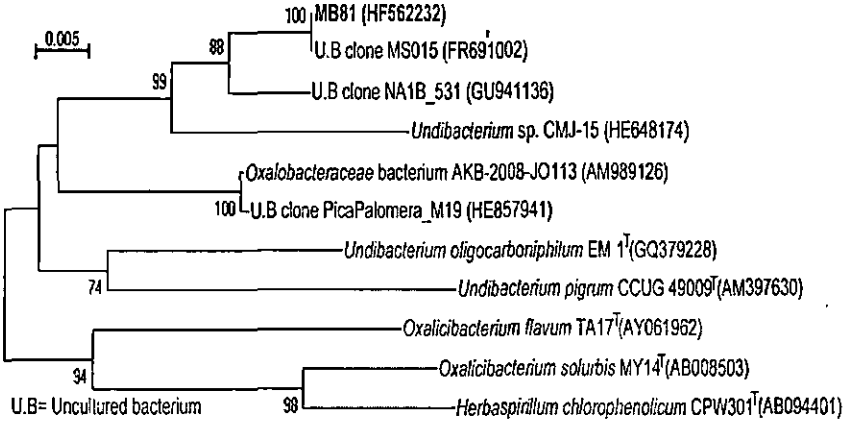


Fig. 3.6: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB28 (Bold face) within the uncultured bacterium and members of related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

Fig. 3.7: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains MB-81 (Bold face) within uncultured bacteria and related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes.. Bar, 0.005 substitutions per nucleotide.



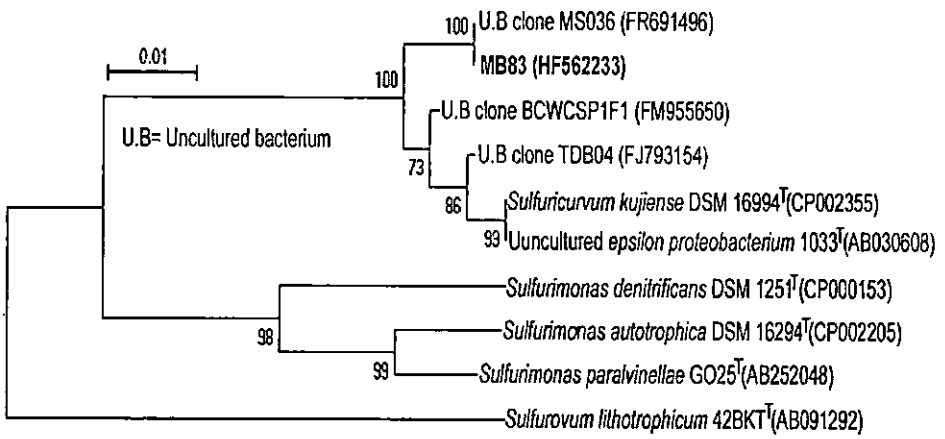


Fig. 3.8: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-83 (Bold face) within uncultured bacteria and related genera. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide.

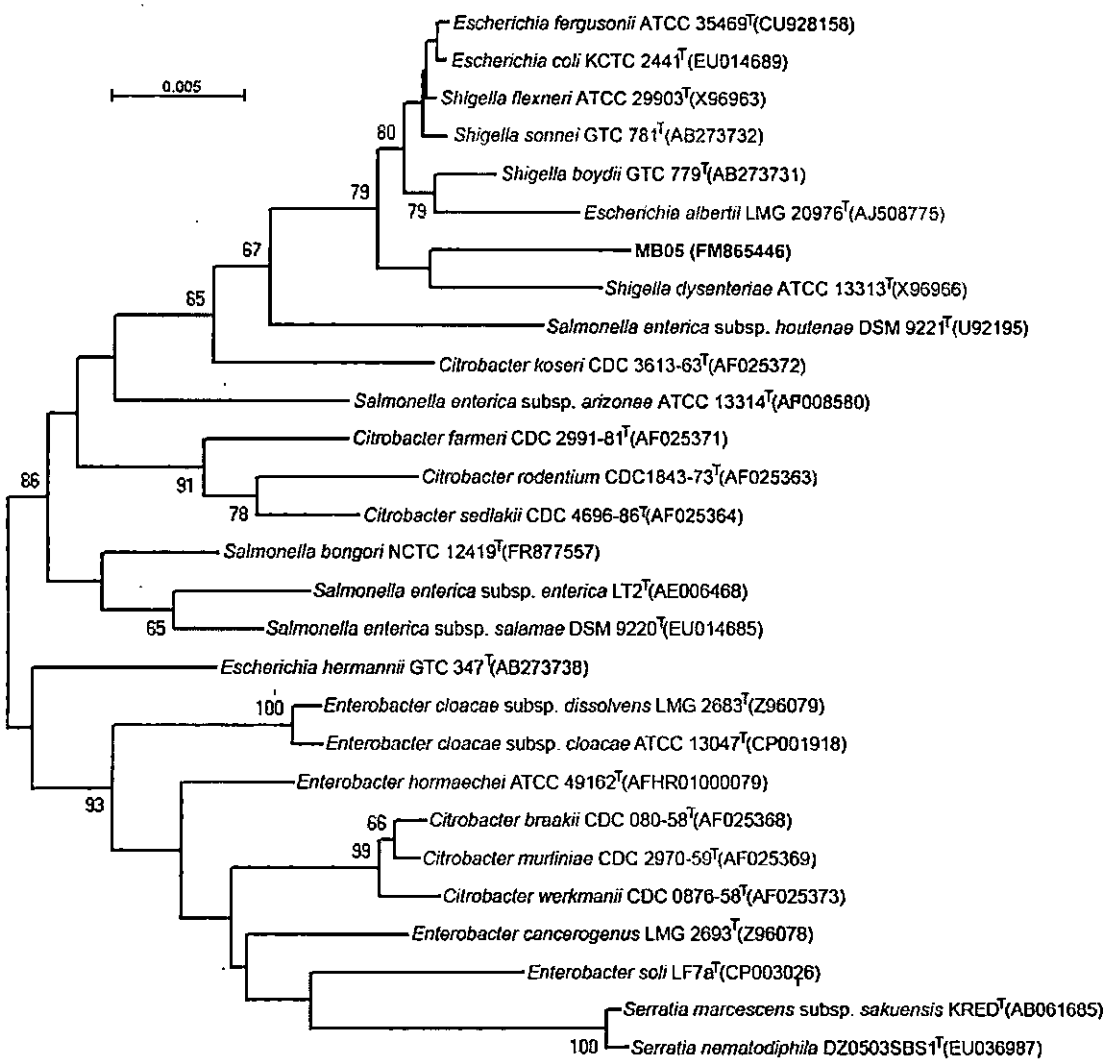


Fig. 3.9: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-05 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

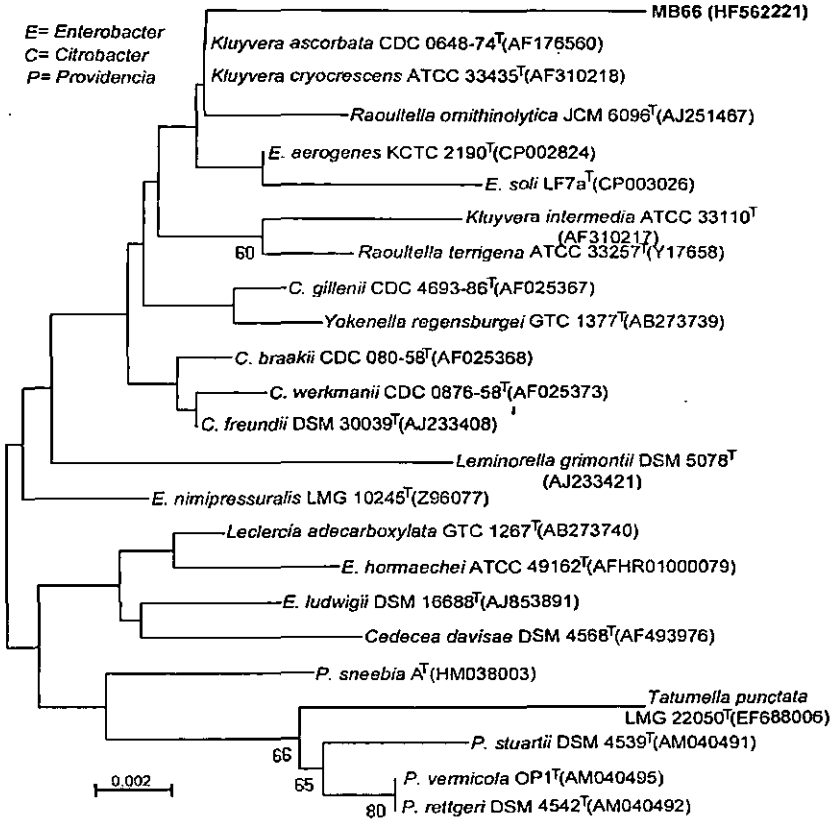


Fig. 3.10: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB66 (Bold face) within the members of family *Enterobacteriaceae.* Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

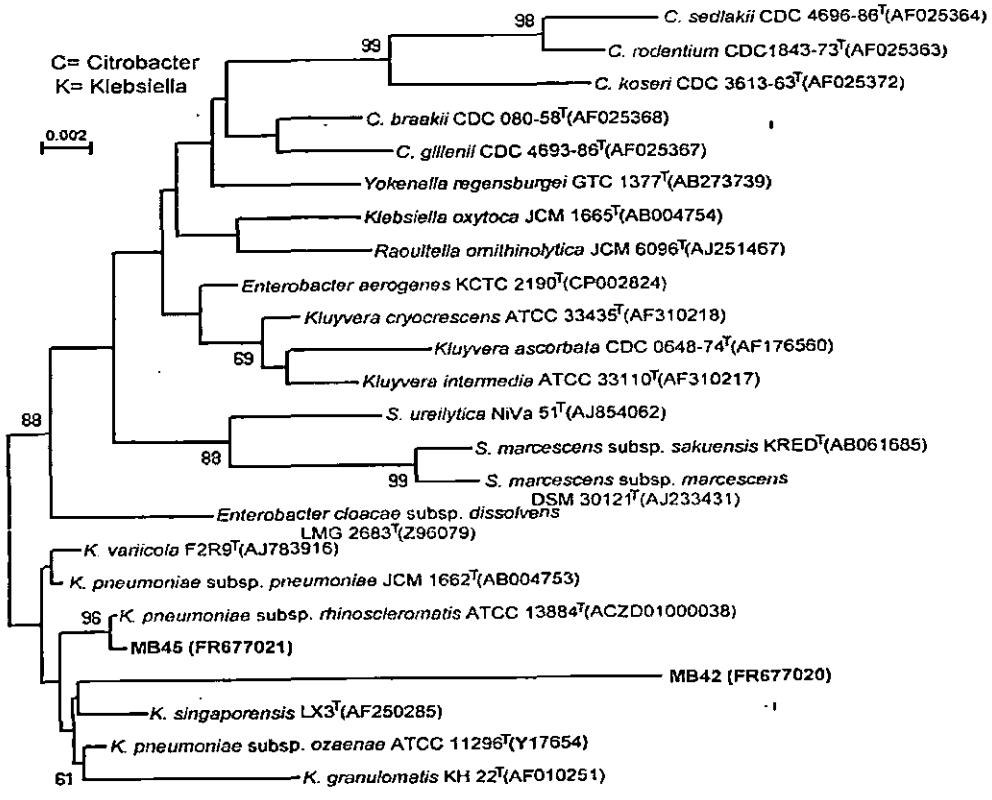


Fig. 3.11: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strains, MB42 and MB45 (Bold face) within the members of family *Enterobacteriaceae.* Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

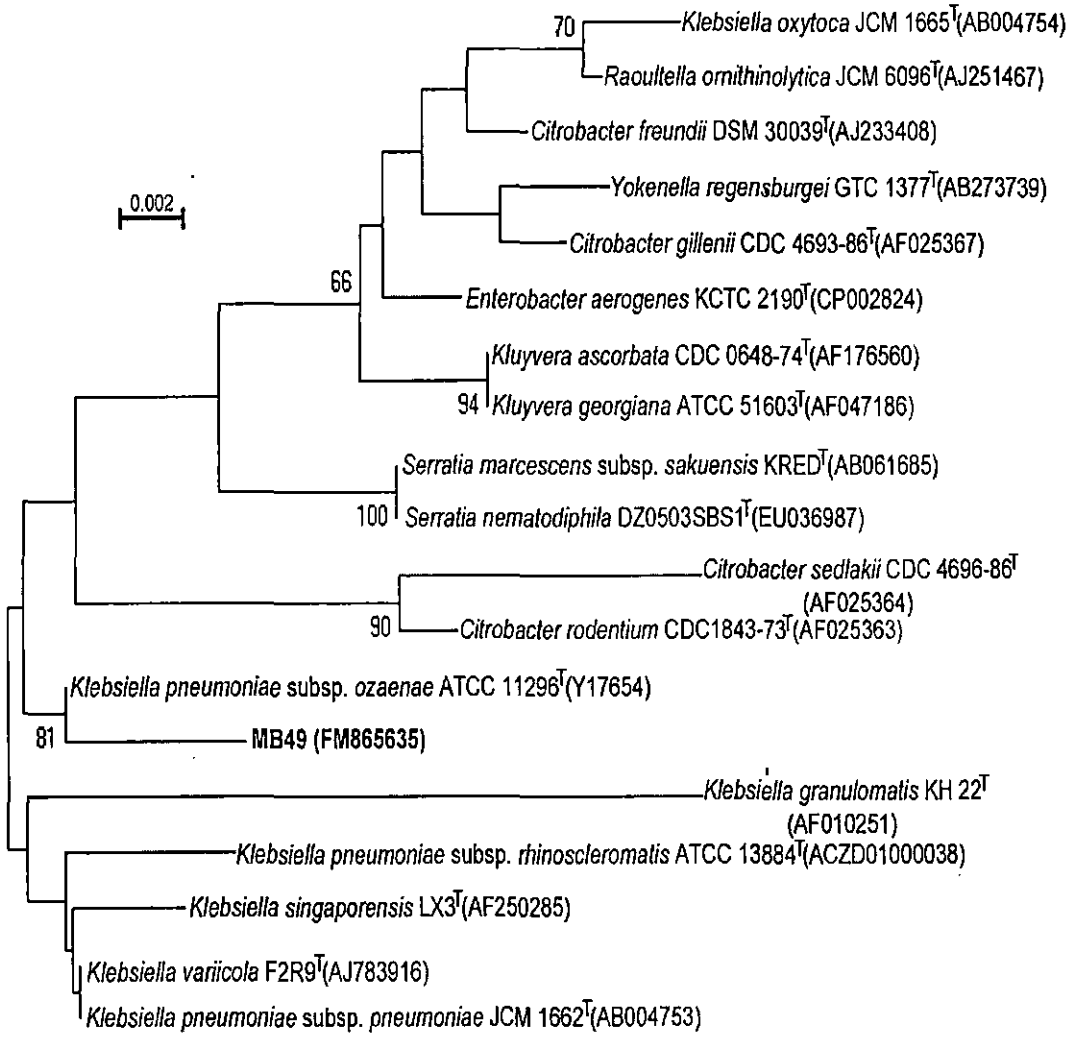


Fig. 3.12: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB49 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

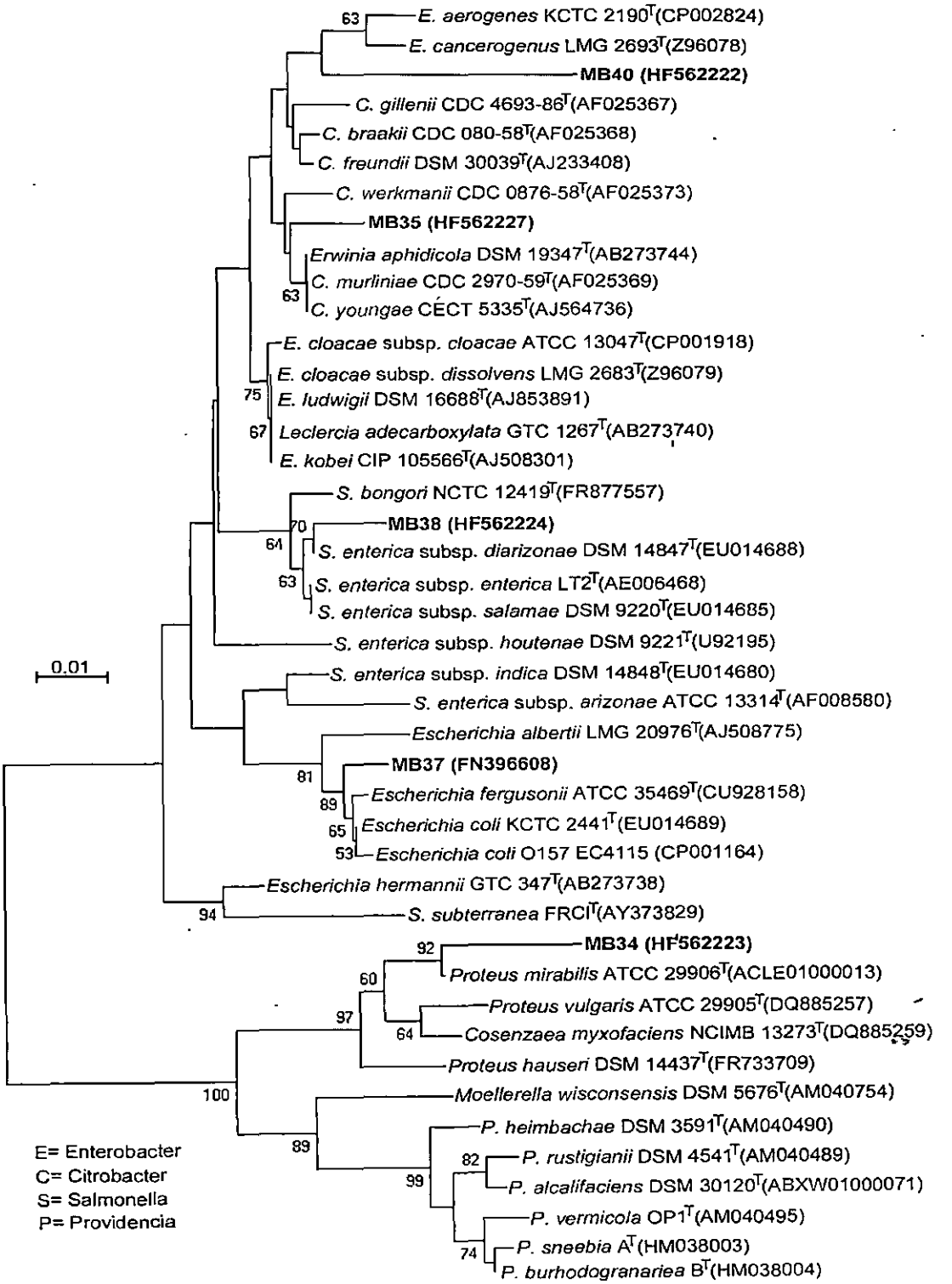


Fig. 3.13: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strains, MB34, MB35, MB37, MB38, and MB40 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide.

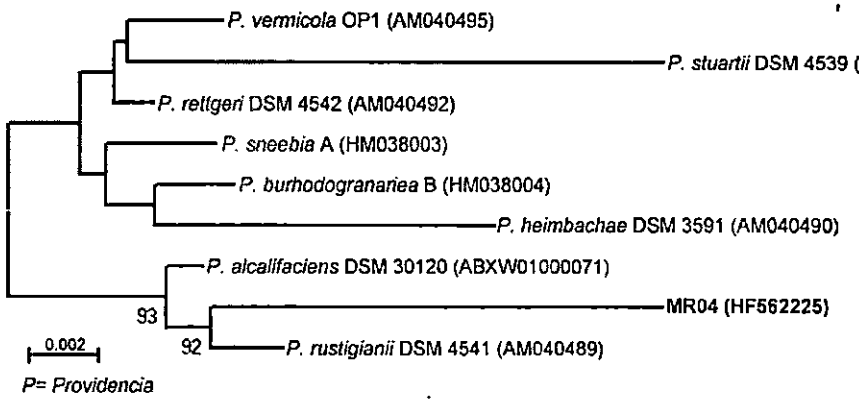


Fig. 3.14: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MR-04 (Bold face) within the members of genus *Providencia*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are indicated at branching nodes. Accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

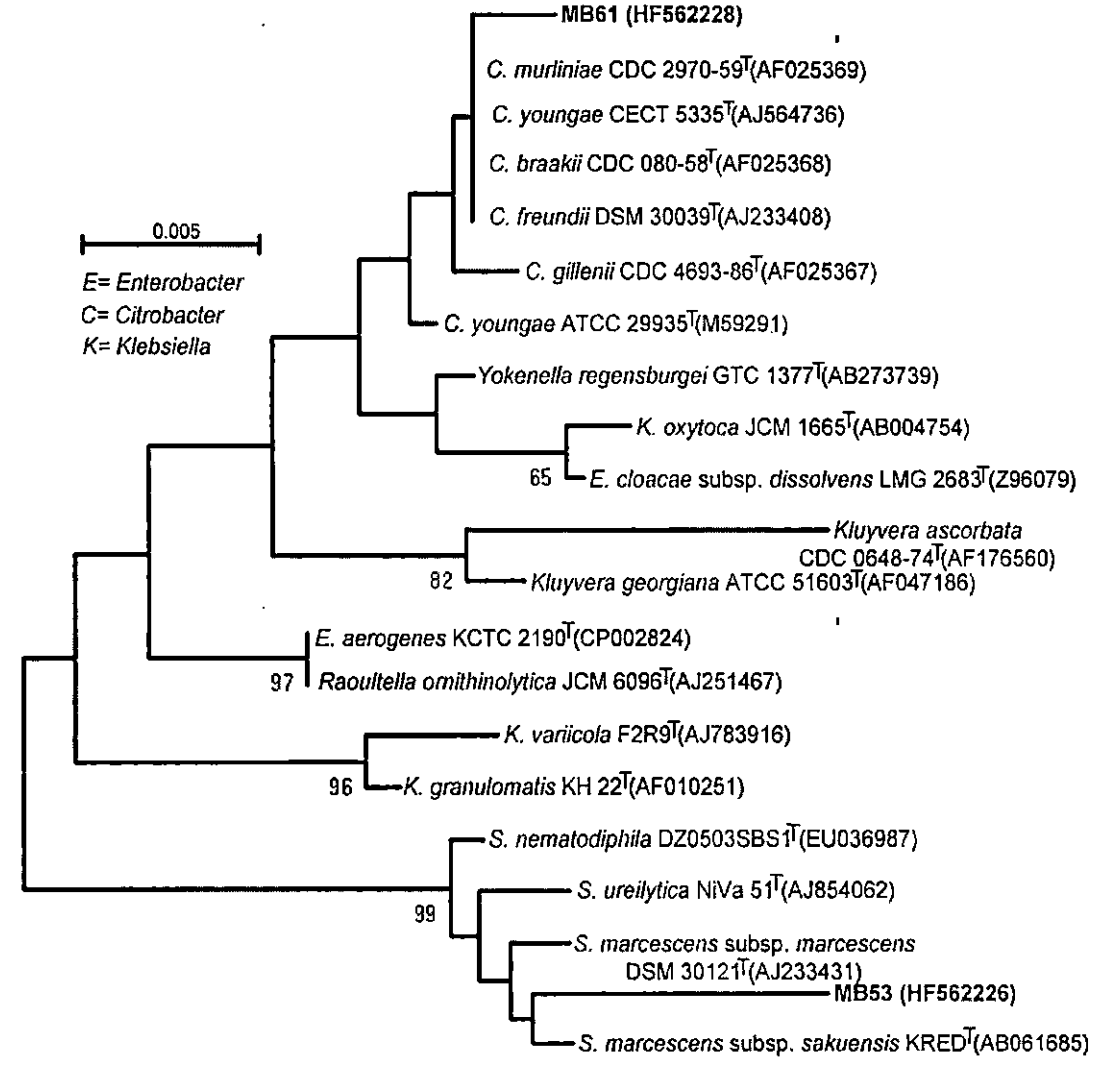


Fig. 3.15: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strains, MB53 and MB61 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. Accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

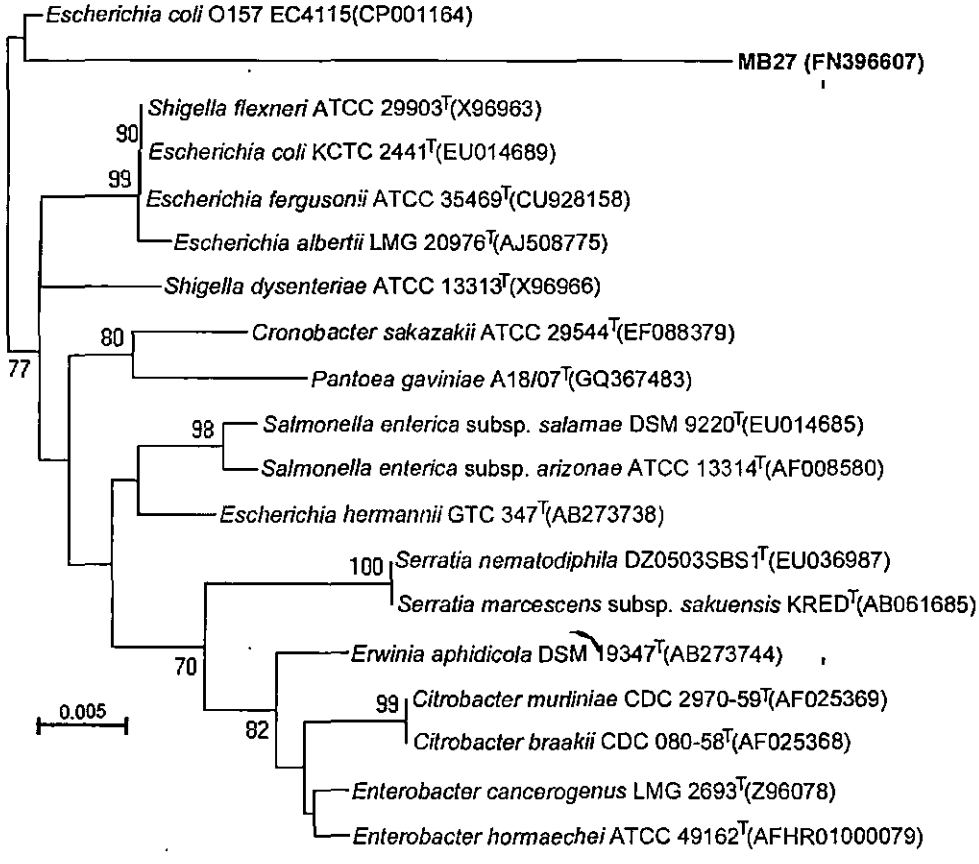


Fig. 3.16: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB27 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

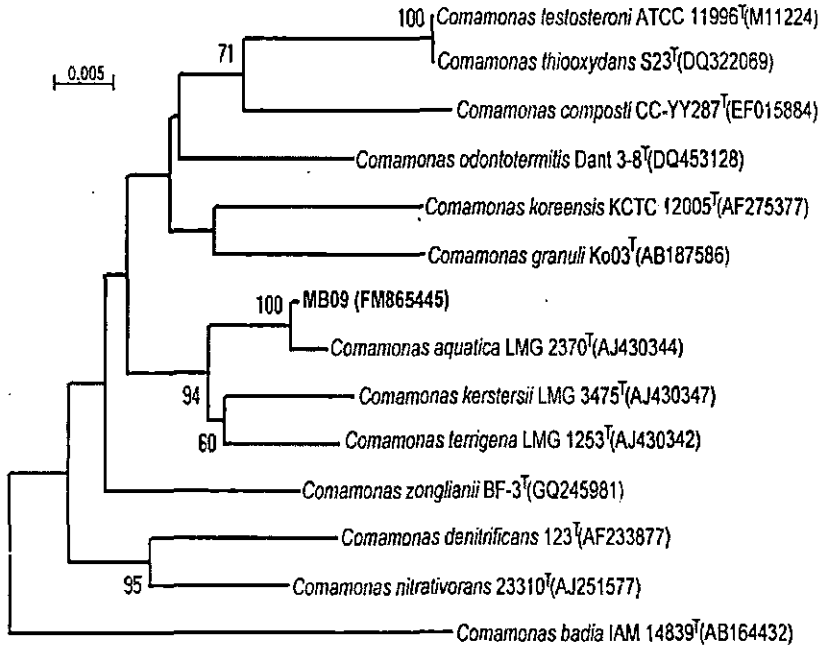


Fig. 3.17: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-05 (Bold face) within the members of family *Enterobacteriaceae*. Bootstrap values (≥60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

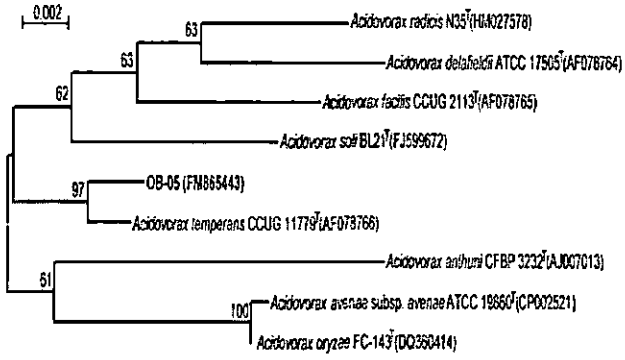


Fig. 3.18: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain OB-05 (Bold face) within the members of genus *Acidovorax*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

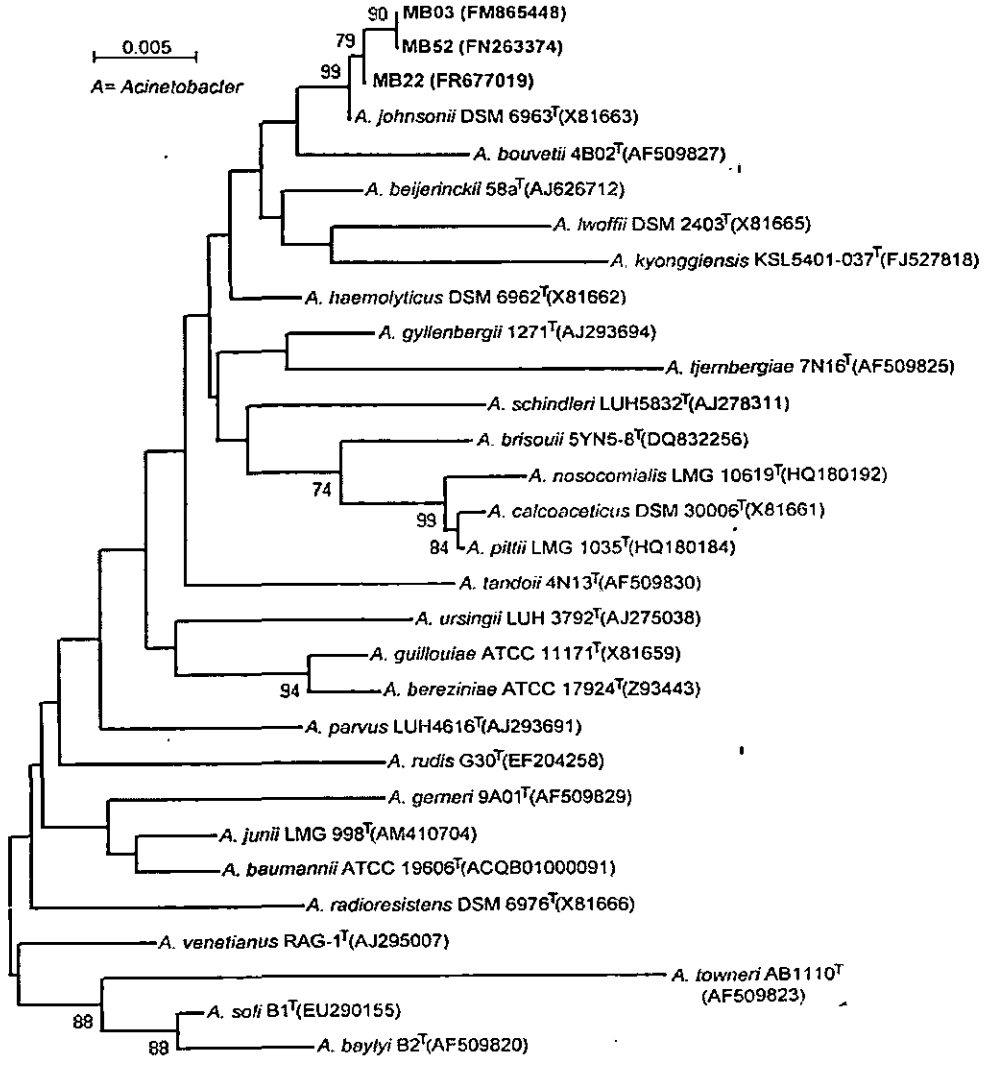


Fig. 3.19: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains, MB03, MB22, and MB52 (Bold face) within the members of genus *Acinetobacter*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide.

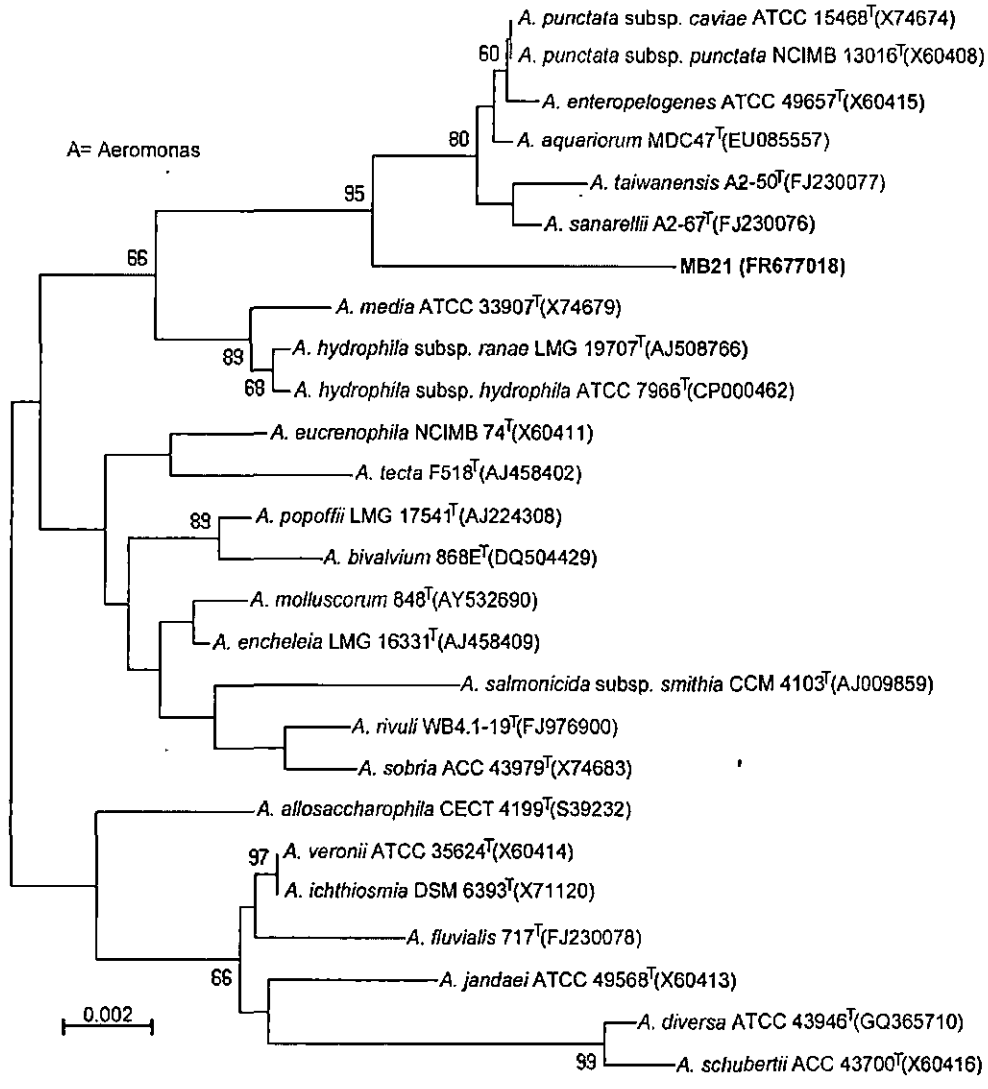
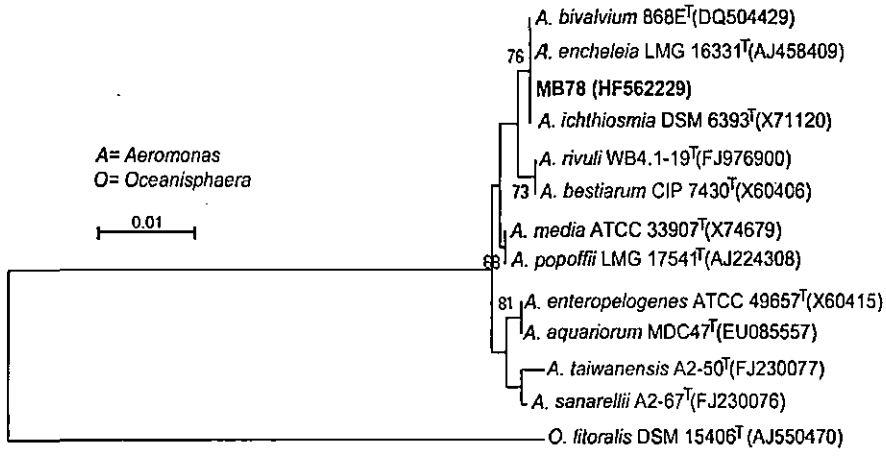


Fig. 3.20: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB21 (Bold face) within the members of genus ***Aeromonas***. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

Fig. 3.21: Rooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MB-78 (Bold face) within the members of genus *Aeromonas*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. *Oceanisphaera litoralis* DSM 15406^T was used as out group. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotides.



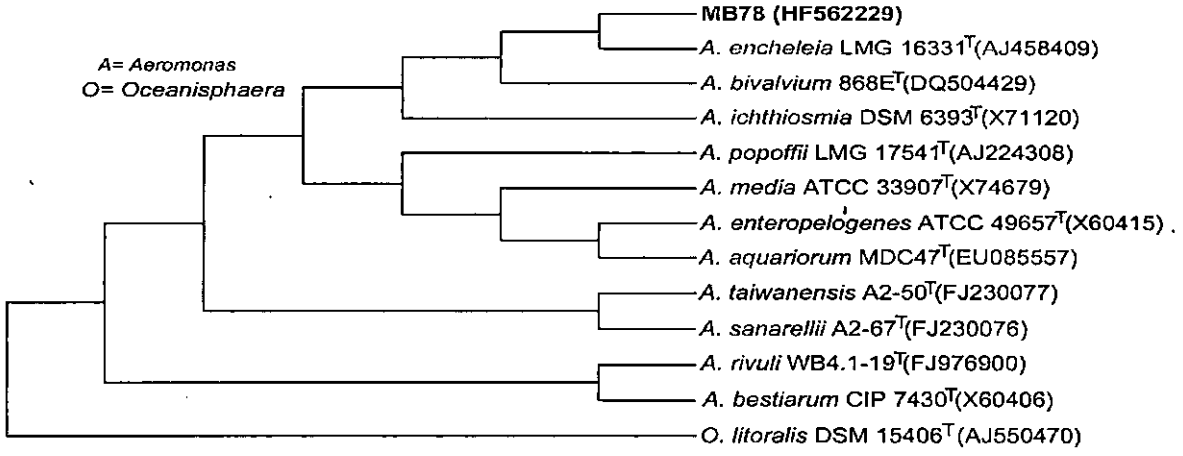


Fig. 3.22: Maximum parsimony tree (based on 16S rRNA gene sequences) showing the position of strain MB-78 (Bold face) within the members of genus *Aeromonas*. *Oceanisphaera litoralis* DSM 15406T (Ac. No. AJ550470) was used as an out group.

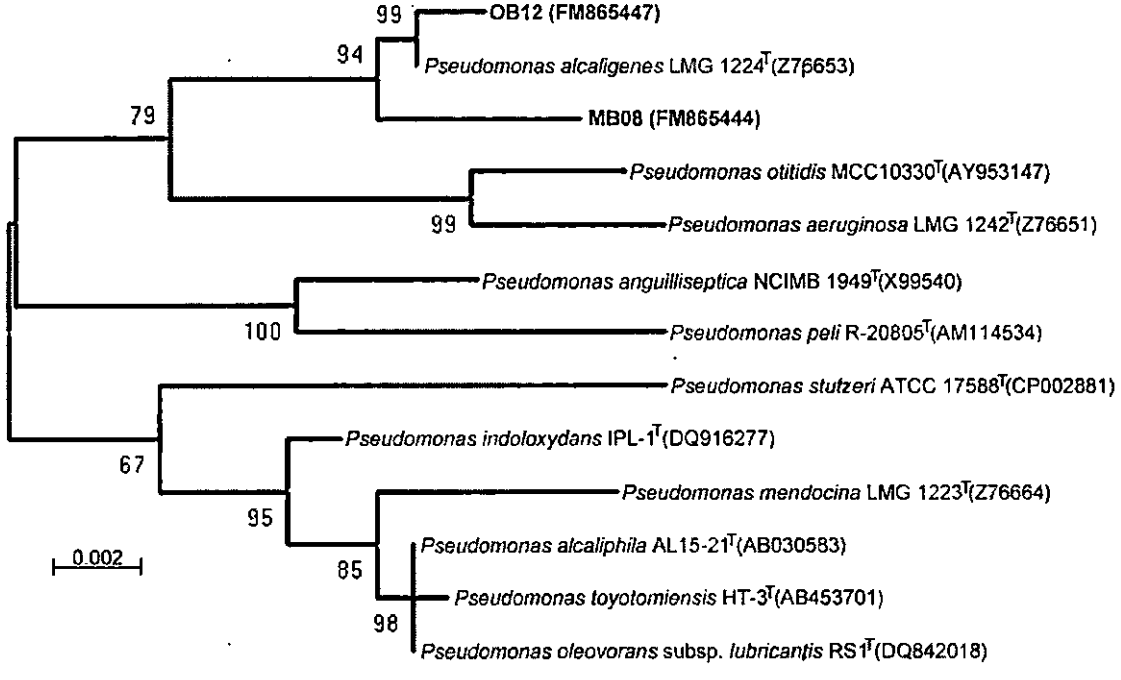


Fig. 3.23: Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains OB-12 and MB08 (Bold face) within the members of genus *Pseudomonas*. Bootstrap values (>60%), expressed as a percentage of 1000 replications, are given at branching nodes. EMBL/GenBank accession numbers are given in parentheses. Bar, 0.002 substitutions per nucleotide.

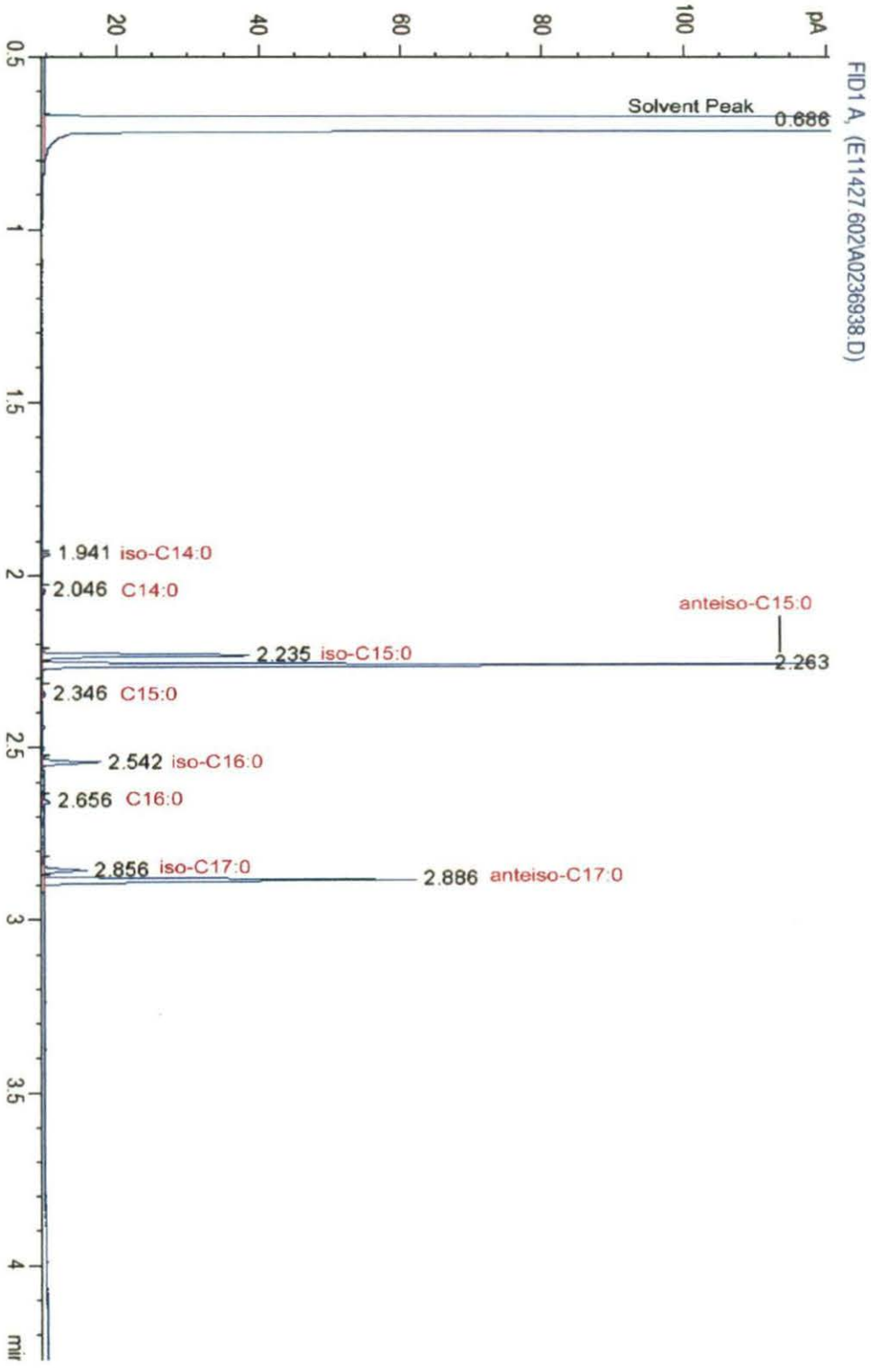


Fig. 3.31: Fatty acid composition analysis of *Brevibacterium siliguriense*. Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography with flame ionization detection. The analyzed fatty acids corresponding to the FAME peaks are indicated with red text.

Table 3.1: Phenotypic characteristics exhibited by class 1 integron bearing facultatively oligotrophic bacteria.

Tests	Isolates									
	OB05	OB12	MB03	MB05	MB08	MB09	MB16	MB18	MB19	MB20
Indole	-	-	-	+	-	-	-	-	-	+
MR	-	-	-	+	-	-	-	-	-	+
VP	-	-	-	-	-	-	-	+	+	-
Citrate	-	+	+	-	+	-	-	+	+	+
Catalase	+	-	+	+	-	-	+	+	+	+
Casienase	-	+	-	-	+	-	-	-	-	-
Gelatinase	-	-	-	-	-	+	-	+	-	+
Amylase	-	-	-	-	+	-	-	-	-	-
H2S	-	+	-	-	+	-	-	-	-	-
Oxidase	+	+	-	-	+	+	+	-	-	-
Acid from										
Dextrose	-	-	-	-	-	-	-	-	+	+
Dulicitol	-	-	-	+	-	-	-	-	-	-
Adonitol	-	+	-	-	+	-	-	-	-	+
Cellobiose	-	-	-	-	-	-	-	-	+	-
Melibiose	-	-	-	-	-	-	-	-	+	+
Mannose	-	+	-	-	+	-	-	+	+	-
Trehalose	-	-	-	-	-	-	-	-	+	+
Maltose	-	-	-	-	-	-	-	-	+	+
Sorbitol	-	+	-	-	+	-	-	-	+	+

Table 3.1: continue.....

Tests	Isolates									
	MB21	MB22	MB23	MB24	MB25	MB26	MB27	MB28	MB29	MB30
Indole	-	-	-	+	+	-	+	+	-	-
MR	+	-	+	+	+	-	+	+	-	-
VP	-	-	-	-	-	+	-	-	+	-
Citrate	+	+	+	-	-	+	-	-	+	+
Catalase	+	+	+	+	-	+	+	+	+	+
Casienase	+	-	+	-	-	-	-	-	-	-
Gelatinase	+	+	-	-	-	-	+	-	-	+
Amylase	+	-	-	-	-	-	-	-	-	-
H2S	-	-	-	-	-	-	-	-	-	+
Oxidase	+	-	-	-	-	-	-	-	-	-
Acid from										
Dextrose	+	-	+	-	+	+	+	+	+	+
Dulicitol	-	-	+	+	-	-	+	-	-	-
Adonitol	-	-	+	-	-	-	-	-	-	+
Cellobiose	-	-	+	-	-	+	-	-	+	+
Melibiose	-	-	+	-	+	+	+	+	+	+
Mannose	-	-	+	-	+	+	+	+	+	+
Trehalose	+	-	+	-	+	+	+	+	+	+
Maltose	+	-	+	-	+	+	+	+	+	+
Sorbitol	-	-	+	-	+	+	+	+	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB31	MB32	MB33	MB34	MB35	MB36	MB37	MB38	MB39	MB40
Indole	+	+	+	-	-	+	+	+	-	-
MR	+	+	+	-	+	+	+	+	+	-
VP	-	-	-	-	-	-	-	-	-	+
Citrate	-	-	-	+	+	-	-	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	-	-	+	-
Gelatinase	+	+	+	+	-	+	+	+	+	-
Amylase	-	-	-	-	+	-	-	-	+	+
H2S	-	-	-	+	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	+	-
Acid from										
Dextrose	+	+	+	+	+	+	+	+	+	+
Dulicitol	+	+	+	-	+	+	+	-	-	-
Adonitol	-	-	-	+	+	-	-	+	-	+
Cellobiose	-	-	-	+	+	-	-	-	-	+
Melibiose	+	+	+	+	+	+	+	+	-	+
Mannose	+	+	+	+	+	+	+	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	-	+	+	+	+	-	-

Table 3.1: continue.....

Tests	Isolates									
	MB41	MB42	MB43	MB44	MB45	MB46	MB47	MB48	MB49	MB50
Indole	+	-	+	-	-	-	-	+	-	-
MR	+	-	+	+	-	-	-	+	-	-
VP	-	+	-	-	+	-	-	-	+	-
Citrate	-	+	+	-	+	+	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	+	-	-	-	+	-	-	-
Amylase	-	-	-	-	-	-	-	-	-	-
H2S	-	-	-	-	-	-	+	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	+
Acid from										
Dextrose	+	+	+	+	+	-	+	+	+	-
Dulicitol	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	+	-	-	-	+	-	-	-
Cellobiose	-	+	-	-	+	-	+	-	+	-
Melibiose	+	+	+	+	+	-	+	+	+	-
Mannose	-	+	-	+	+	-	+	+	+	-
Trehalose	+	+	+	+	+	-	+	+	+	-
Maltose	+	+	+	-	+	-	+	+	+	-
Sorbitol	+	+	+	+	+	-	-	+	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB51	MB52	MB53	MB54	MB55	MB56	MB57	MB58	MB59	MB60
Indole	-	-	-	+	-	-	-	-	-	+
MR	-	-	+	+	-	-	-	-	-	+
VP	+	-	-	-	-	-	-	-	+	-
Citrate	+	+	+	-	+	-	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	+	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	+	-	-	+
Amylase	-	-	-	-	-	-	-	-	+	-
H2S	-	-	-	-	-	-	+	-	-	-
Oxidase	-	-	-	-	-	+	-	+	-	-
Acid from										
Dextrose	+	-	+	+	-	-	+	-	+	+
Dulicitol	-	-	+	-	-	-	-	-	-	+
Adonitol	-	-	+	-	-	-	+	-	+	-
Cellobiose	+	-	+	-	-	-	+	-	+	-
Melibiose	+	-	+	+	-	-	+	-	+	+
Mannose	+	-	+	+	-	-	+	-	-	+
Trehalose	+	-	+	+	-	-	+	-	+	+
Maltose	+	-	+	+	-	-	+	-	+	+
Sorbitol	+	-	+	+	-	-	-	-	-	+

Table 3.1: continue.....

Tests	Isolates									
	MB61	MB62	MB63	MB64	MB65	MB66	MB67	MB68	MB69	MB70
Indole	-	-	-	+	+	+	+	+	+	-
MR	+	-	-	+	+	+	+	+	+	-
VP	-	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	-	+	+	-	-	-
Catalase	+	-	+	+	+	+	+	+	+	+
Casienase	-	+	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	+	+	-	+	+	+	-
Amylase	+	+	-	-	-	-	-	-	-	-
H2S	+	+	-	-	-	-	-	-	-	-
Oxidase	-	+	-	-	-	-	-	-	-	+
Acid from										
Dextrose	+	-	-	+	+	+	+	+	+	-
Dulicitol	+	-	-	-	+	+	-	+	+	-
Adonitol	+	+	-	+	-	-	+	-	-	-
Cellobiose	+	-	-	-	-	+	-	-	-	-
Melibiose	+	-	-	+	+	-	+	+	+	-
Mannose	+	+	-	-	+	-	-	+	+	-
Trehalose	+	-	-	+	+	+	+	+	+	-
Maltose	+	-	-	+	+	+	+	+	+	-
Sorbitol	+	+	-	+	+	-	+	+	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB71	MB72	MB73	MB74	MB75	MB76	MB77	MB78	MB79	MB80
Indole	-	-	-	+	+	+	-	-	+	-
MR	-	-	-	+	+	+	+	+	+	-
VP	-	+	+	-	-	-	-	-	-	-
Citrate	-	+	+	+	+	+	+	+	-	+
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	+	+	-	-
Gelatinase	-	-	-	+	+	+	+	+	+	-
Amylase	-	-	+	-	-	-	+	+	-	-
H2S	-	-	-	-	-	-	-	-	-	-
Oxidase	+	-	-	-	-	-	+	+	-	-
Acid from										
Dextrose	-	+	+	+	+	+	+	+	+	-
Dulicitol	-	-	-	-	-	-	-	-	+	-
Adonitol	-	-	+	+	+	+	-	-	-	-
Cellobiose	-	+	+	-	-	-	-	-	-	-
Melibiose	-	+	+	+	+	+	-	-	+	-
Mannose	-	+	-	-	-	-	+	+	+	-
Trehalose	-	+	+	+	+	+	+	+	+	-
Maltose	-	+	+	+	+	+	+	+	+	-
Sorbitol	-	+	-	+	+	+	-	-	+	-

Table 3.1: continue.....

Tests	Isolates									
	MB81	MB82	MB83	MR01	MR02	MR03	MR04	SR19	NV66	OD05
Indole	+	+	+	-	-	-	-	-	-	-
MR	+	+	+	-	-	+	+	-	+	+
VP	-	-	-	+	-	-	-	+	-	-
Citrate	-	+	-	+	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Casienase	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	+	-	-	-	-	-	-	-	-
Amylase	-	-	-	+	-	+	-	+	-	+
H2S	-	-	-	-	-	+	-	-	-	+
Oxidase	-	-	-	-	+	-	-	-	-	-
Acid from										
Dextrose	+	+	+	+	-	+	+	+	+	+
Dulicitol	-	-	-	-	-	+	-	-	-	+
Adonitol	-	+	-	+	-	+	+	+	+	+
Cellobiose	-	-	-	+	-	+	-	+	-	+
Melibiose	+	+	+	+	-	+	+	+	+	+
Mannose	-	-	-	-	-	+	-	-	-	+
Trehalose	+	+	+	+	-	+	+	+	+	+
Maltose	+	+	-	+	-	+	+	+	+	+
Sorbitol	+	+	+	-	-	+	+	-	+	+

Table 3.1: continue.....

Tests	Isolates								
	OD08	OD10	OC16	OC24	OC74	OC75	OC78	OD21	OD24
Indole	-	-	-	-	-	+	-	-	-
MR	+	+	+	+	-	+	-	-	+
VP	-	-	-	-	-	-	-	+	-
Citrate	+	+	+	+	+	-	+	+	+
Catalase	+	+	+	+	-	+	-	+	+
Casienase	-	-	-	-	+	-	+	-	-
Gelatinase	-	-	-	-	-	+	-	-	-
Amylase	+	+	+	+	+	-	+	+	+
H2S	-	+	-	+	+	-	+	-	+
Oxidase	-	-	-	-	+	-	+	-	-
Acid from									
Dextrose	+	+	+	+	-	+	-	+	+
Dulicitol	+	+	+	+	-	+	-	-	+
Adonitol	+	+	+	+	+	-	+	+	+
Cellobiose	+	+	+	+	-	-	-	+	+
Melibiose	+	+	+	+	-	+	-	+	+
Mannose	+	+	+	+	+	+	+	-	+
Trehalose	+	+	+	+	-	+	-	+	+
Maltose	+	+	+	+	-	+	-	+	+
Sorbitol	+	+	+	+	+	+	+	-	+

Table 3.2: Putatively assigned genera (on the basis of partial 16S rRNA gene sequences) of integron carrying isolates

Sl. No	Isolate	Genus	Ac. No.	Sl. No	Isolate	Genus	Ac. No.
1	[a] OB 05	*Acidovorax* sp.	FM865443	46	MB 57		-f-
2	OB 12	*Pseudomonas* sp.	FM865447	47	MB 58		-a-
3	[b] MB 03	*Acinetobacter* sp.	FM865448	48	MB 59		-e-
4	[c] MB 05	*Shigella* sp.	FM865446	49	MB 60		-o-
5	[r] MB 08	*Pseudomonas* sp.	FM865444	50	[k] MB 61	*Citrobacter* sp.	HF562228
6	MB 09	*Comamonas* sp.	FM865445	51	MB 62		-r-
7	MB 16		-a-	52	MB 63		-b-
8	MB 19		-d-	53	MB 64		-g-
9	MB 20		-g-	54	MB 65		-o-
10	[p] MB 21	*Aeromonas* sp.	FR677018	55	MB 66	*Kluyvera* sp.	HF562221
11	MB 22	*Acinetobacter* sp.	FR677019	56	MB 67		-g-
12	MB 23		-i-	57	MB 68		-o-
13	MB 24		-c-	58	MB 69		-o-
14	MB 25		-l-	59	MB 70		-a-
15	MB 26		-d-	60	MB 71		-a-
16	[o] MB 27	*Escherichia* sp.	FN396607	61	MB 72		-d-
17	[m] MB 28	Un. B	HF562231	62	MB 73		-e-
18	MB 29		-d-	63	MB 74		-g-
19	MB 30		-f-	64	MB 75		-g-
20	MB 31		-o-	65	MB 76		-g-
21	MB 32		-o-	66	MB 77		-q-
22	MB 33		-o-	67	[q] MB 78	*Aeromonas* sp.	HF562229
23	[f] MB 34B	*Proteus* sp.	HF562223	68	MB 79		-o-
24	[j] MB 35	*Citrobacter* sp.	HF562227	69	MB 80		-b-
25	MB 36		-o-	70	[n] MB 81	Un. B	HF562232
26	MB 37A	*Escherichia* sp.	FN396608	71	MB 82		-g-
27	[g] MB 38	*Salmonella* sp.	HF562224	72	MB 83	Un. B	HF562233
28	MB 39		-p-	73	MR 01		-e-
29	[e] MB 40	*Enterobacter* sp.	HF562222	74	MR 02		-a-
30	MB 41		-n-	75	MR 03		-k-
31	[d] MB 42	*Klebsiella* sp.	FR677020	76	[h] MR 04	*Providencia* sp.	HF562225
32	MB 43		-g-	77	SR 19		-e-
33	MB 44	Un. B	HF562230	78	NV 66		-h-
34	MB 45	*K. pneumoniae*	FR677021	79	OD 05		-k-
35	MB 46		-b-	80	OD 08		-k-
36	MB 47		-f-	81	OD 10		-j-
37	MB 48		-m-	82	OC 16		-k-
38	[d] MB 49	*Klebsiella* sp.	FM865635	83	OC 24		-j-
39	MB 50		-a-	84	OC 74		-r-
40	MB 51		-d-	85	OC 75		-o-
41	MB 52	*A. johnsonii*	FN263374	86	OC 78		-r-
42	[i] MB 53	*Serratia* sp.	HF562226	87	OD 21		-e-
43	MB 54		-m-	88	OD 24		-k-
44	MB 55		-b-	89	MB 12	Un. B	AM937246
45	MB 56		-a-	90	MB 18	*B. siliguriense*	AM937247

Un.B, Unclassified bacterium;

Isolates exhibiting similar phenotype: a, *Acidovorax* sp.; b, *Acinetobacter* sp.; c, *Shigella* sp.; d, *Klebsiella* sp.; e, *Enterobacter* sp.; f, *Proteus* sp.; g, *Salmonella* sp.; h, *Providencia* sp.; i, *Serratia* sp.; j and k, *Citrobacter*.; l-n, Unknown bacterium; o, *Escherichia* sp.; p and q, *Aeromonas* sp.; r, *Pseudomonas* sp.; *A. johnsonii, Acinetobacter johnsonii; B. siliguriense, Brevibacterium siliguirense; K. pneumoniae, Klebsiella pneumoniae*; rDNA, ribosomal deoxyribonucleic acid.

Table 3.4A: Detailed phenotypic characteristics of *Brevibacterium siliguriense* sp. nov strain MB18^T. Symbols: +, positive; –, negative; W, weak reaction; R, resistant; S, sensitive

Characteristics	MB18^T	Characteristics	MB18^T
NaCl tolerance (% w/v)	15	Citrate utilization	+
Oxidase	–	Utilization of D–arabinose	–
Hydrolysis of Esculin	–	Utilization of L–arabinose	–
Hydrolysis of Gelatin	+	Utilization of Sodium gluconate	–
Phenylalanine deamination	–	**Acid from:**	
Haemolysis	–	2,3 butylene glycol	–
H_2S production	–	D–Arabinose	–
Voges proskaurs	+	D–mannitol	+
Methyl Red	–	D–Xylose	–
Indole	–	D–galactose	–
Amylase	–	D–ribose	–
Lipase production	–	Lactose	–
α–Glucosidase	–	D–Maltose	–
Pyrrolidone peptidase	–	D–Sorbitol	–
Pyrazinamidase	+	D–mannose	+
Phosphatidyl-inositol-Phospholipase	–	D–Raffinose	–
Arginine Dihydrolase1	+	D–Trehalose	–
Arginine Dihydrolase 2	–	Saccharose/Sucrose	–
β–Galactosidase	+	Fructose	w
α–galactosidase	+	Dextrose	w
L–aspartate arylamidase	–	Melibiose	–
β galactopyranosidase	+	L–Arabinose	–
α–mannosidase	–	Inulin	–
Phosphatase	–	Glycerol	+
Leucine arylamidase	+	Salicin	–
L–proline arylamidase	+	Dulicitol	–
β –glucuronidase	–	Inositol	–
Ala–Phe–Pro Arylamidase	–	Xylitol	–
β –glucuronidase	+	Adonitol	–
Alanine arylamidase	+	Melezitose	–
Tyrosine arylamidase	+	Sorbose	–
D–amygdalin	–	Rhamnose	–
L–Lactate alkalization	–	DNA G+C content (mol%)	64.6
N–Acetyl–D–Glucosamine	–	**Antibiotic susceptibility test:**	
Methyl– β –D–Glucopyronside	–	Bacitracin Resistance (0.0006 mg)	R
α–methyl–D–mannoside	–	Novobiocin Reistance (0.000075 mg)	S
α–methyl–D–glucoside	w	O/129 Resistance (0.0084 mg)	S
Glucosamine	–	Optochin (0.000399 mg)	R
Pullulan	–	Ampicillin (<S/R≥, 25 mg/L)	R
Salicin	–	Cefepime (<S/R≥, 7.5 mg/L)	R
ONPG	–	Chloramphenicol (<S/R≥, 30 mg/L)	R
Lysine utilization	–	Co-trimoxazole (<S/R≥, 15 mg/L)	R
Ornithine utilization	+	Netilmicin (<S/R≥, 3.75 mg/L)	R

Table 3.4B: Detailed phenotypic characteristics of *Brevibacterium siliguriense* sp. nov. strain MB18^T. Symbols: +, positive; –, negative; W, weak reaction; V, variable reaction

Characteristics	MB18^T	Characteristics	MB18^T	Characteristics	MB18^T
α-Cyclodextrin	–	β-Methyl-D-Galactosidee	–	Succinic Acid	–
β-Cyclodextrin	–	3-Methyl Glucose	–	N-Acetyl-L-Glutamic Acid	–
Dextrin	w	β-Methyl-D-Glucoside	–	L-Alaninamide	–
Glycogen	–	α-Methyl-D-Mannoside	–	D-Alanine	–
Mannan	+	Palatinose	–	L-Alanine	–
Tween 40	+	D-Psicose	–	L-Alanyl Glycine	–
Tween 80	–	L-Rhamnose	–	L-Asparagine	–
N-Acetyl-D-Mannosamine	–	Stachyose	–	L-Glutamic Acid	–
N-Acetyl-β-D-Mannosamine	–	D-Tagatose	–	Glycyl-L-Glutamic Acid	–
Amygladin	–	Turanose	–	L-Pyroglutamic Acid	–
D-Arabitol	–	Acetic Acid	+	L-Serine	v
Arbutin	–	α-Hydroxybutyric Acid	–	Putrescine	w
D-Cellobiose	–	β-Hydroxybutyric Acid	–	2,3 butandiol	–
D-Fructose	–	γ-Hydroxybutyric Acid	–	Adenosine	–
L-Fucose	–	p-Hydroxy-Phenylacetic Acid	–	2'-Deoxy Adenosine	–
D-Galactose	–	α-Ketoglutaric Acid	–	Inosine	–
D-Galacturonic Acid	–	α-Ketovaleric Acid	–	Thymidine	–
Gentibiose	–	Lactamide	–	Uridine	–
D-Gluconic Acid	–	D-Lactic Acid Methyl Ester	–	Adenosine-5'-Momonophosphate	–
α-D-Glucose	–	L-Lactic Acid	–	Thymidine-5'-Momonophosphate	–
m-Inositol	–	D-Malic Acid	–	Uridine-5'-Momonophosphate	–
α-D-Lactose	–	L-Malic Acid	–	D-Fructose-6-Phosphate	–
Lactulose	–	Pyruvatic Acid Methyl Ester	+	α-D-Glucose-1-Phosphate	–
Maltotriose	–	Succinic Acid Mono-methyl Ester	+	D-Glucose-6-Phosphate	w
D-Melezitose	–	Propionic Acid	+	D-L-α-Glycerol Phosphate	–
D-Melebiose	–	Pyruvic Acid	+		
α-Methyl-D-Galactoside	–	Succinamic Acid	–		

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