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TAXONOMICAL STUDIES OF BIOACTIVE BACTERIAL ISOLATE-MSB-6

M D A G CHANDRA SEKHAR[1] AND CH. MURALI MOHAN[2]

[1]Department of Biochemistry, GITAM Institute of Science, GITAM University, Visakhapatnam-530 045, A.P., India
[2]Department of Biotechnology, GITAM Institute of Technolog, GITAM University, Visakhapatnam-530 045, A.P.,India

ABSTRACT

In order to identify the resulted bioactive strain MSB-6 from primary and secondary screening of marine soil samples, various morphological, physiological and biochemical tests are performed. Further confirmation is done by the 16S rRNA gene analysis. The reports confirmed strain MSB-6 as *Pseudomonas fluorescens* and it is deposited to Gene Bank with the accession number KC852045. It is mesophilic, alkaliphilic and moderately salt tolerant in nature. It ferments dextrose, galactose, inulin, xylose, mannose, melibiose, L-arabinose and showed positive results to citrate utilization test, cytrochrome oxidase test, catalase test, gelatin hydrolysis, casein hydrolysis and nitrate reduction. Altogether, the results once again proved that the natural marine environment is also good source for isolation of novel varieties of antagonistic bacteria.The culture conditions are optimizing for the production of antimicrobial metabolites by *MSB-6* bacteria. The metabolite production is started after 24 h of incubation and reached its maximum levels after 48 h and decreased gradually. The pH is adjusted to 7.0 and temperature to 37^0C supported the production of antimicrobial metabolites. The best proved carbon source was dextrose respectively as basal medium.

KEY WORDS: ***MSB-6*, optimization, antimicrobial metabolites, marine.**

M D A G CHANDRA SEKHAR
Department of Biochemistry, GITAM Institute of Science, GITAM University, Visakhapatnam-530 045, A.P., India

***Corresponding author**

INTRODUCTION

Microbes are characterized by its extraordinary diversity in shape, size, physiology and life style. It is essential to classify them into groups based on their similarities and differences. Marine bacteria are responsible for the production of various biochemical products including majority of clinically useful antibiotics. It is established that proper identification and characterization of microorganisms is very important because it also broadens the scope for exploration of industrially important microbial products. Among the bacterial isolates isolated from marine sediment MSB-6 was found to exhibit highest biological activity. MSB-6 was identified and characterised by conducting a series of microscopic and biochemical tests. The strain identification was identified by tests like production of pyocyanin on King's A medium (King *et al.*, 1954), cytochrome oxidase (Kovacs, 1956), arginine dihydrolase (Thomley, 1960), tobacco HR (Klement, 1963), liquefaction of gelatin (Dye, 1968), levan production, nitrate reduction, growth at 4 and 41°C, (Shaad *et al.*, 2001) and the growth efficiency on carbon sources like D-galactose, L-arabinose, meso-inositol, sorbitol, L-tartrate, trehalose,was tested with the basal medium of Ayers *et al.*, (1919). It is perhaps not surprising that novel marine bacteria were providing to be such a valuable source of new bioactive compounds (Fiedler and Bruntner *et al.,* 2005) as bacteria systematic is providing a taxonomic road map to genes hence products, including the discovery of first-in-class drug candidates (Blunt and Copp, 2007; Kumar and Goodfellow, 2008). Main aim of present study is the isolation, identification and characterization of potent antagonistic, alkaliphilic marine sediment bacteria from Visakhapatnam coast of Bay of Bengal and to screen for their antagonistic activity against selective human pathogenic microorganisms. The strain is identified and deposited at Microbial Type Culture Collection and Gene bank, IMTECH, Chandigarh, India.

EXPERIMENTAL

The bacteria isolated from marine sediment were biologically active and also taxonomically diverse (Rheinheimer, 1992). The promising isolate should be identified systematically in order to enhance the potency and yield of the bioactive compound produced by it. The identification was done according to Bergey's manual of systematic bacteriology (Krieg and Holt, 1984 and Sneath *et al*., 1986).

Morphological and physiological characterization

The potential isolate MSB-6 in pure form was streaked on nutrient agar plate in order to study its colony morphology like size, configuration, opacity, surface and pigmentation (Kenneth, 1958). The individual bacterial cell was also observed under microscope for its size, shape, staining and motility (Pathiranana *et al*., 1991). The physiological parameters like favourable growth temperature, pH, salinity and aeration are also studied to know the optimal conditions for bacterial cell growth. The existence of bacterial cell growth was observed in the temperature range of 0-65^{o}C, pH range of 5.0 – 11.0, percentage of sodium chloride range 1% -10%. Simultaneously the metabolic activity of promising isolate was tested i.e. whether it is aerobic or anaerobic.

Biochemical characterization

Biochemical characterisation of the promising isolate MSB-6 was done by performing a series of biochemical tests like Indole test, Methyl Red test, Voges Proskauer test, Citrate Utilization test, Arginine dihydrolase , Casein hydrolysis, Catalase test, Cytochrome Oxidase, growth on MacConkey agar, H_2S Production, Gelatin hydrolysis, Lysine decarboxylase, Starch hydrolysis, Nitrate Reduction, Oxidation/Fermentation and Urea hydrolysis. All the tests were carried out according to standard protocol given for each individual test.

Acid production from carbohydrates

It is also one of the biochemical tests but read separately due to its broad spectrum of reactants. The carbohydrate utilization by the bacterial isolate was tested by including various carbohydrates in culture media i.e. adonitol, L-arabinose, cellobiose, dextrose, dulcitol, fructose, galactose, inositol, inulin, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, mannose, and xylose (Pridham and Gottlieb, 1948).

Molecular characterization

The morphological, physiological, cultural and biochemical characteristics of the bioactive isolate MSB-6 were compared with existing related genera and species cited in the literature. The literature includes Bergey's Manual of systematic Bacteriology (Krieg and Holt, 1984 and Sneath *et al*., 1986), Laboratory Manual of Fundamental Principles of Bacteriology (Salee, 1948), Bergey's Manual of Determinative Bacteriology (Nandy *et al.,* 2007), Mackie and McCartney Practical Medical Microbiology (Collee *et al.,* 1969) and Biological and microbiological abstracts. Examination of these characteristics has suggested the tentative genus and species of the bioactive isolate but it should be accompanied by molecular characterization (Grimont *et al.,* 1996 and Hartung, 1998).

Isolation of genomic DNA and PCR amplification

The isolated bioactive bacterium MSB-6 was incubated for 48 hours and the genomic DNA was isolated according to the method described by Wang *et al*., (2001). 16S rDNA was amplified from the obtained genomic DNA with the primers PS16f 5′-TGGCTCAGATTGAACGCTGGCGG-3′ (forward) and PS16r5′-GATCCAGCCGCAGGTTCCCCT AC-3′ (backword). PCR amplification was carried out in 20 µl reaction mixtures containing 4 µl of lysed bacterial suspension, 1× PCR bovine serum albumin, 5% dimethyl sulfoxide, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.40 µM of each primer and 1.4 U of Taq DNA polymerase Amplifications were performed with a Corpet research g001 cycler. The initial denaturation (2 min at 94 °C) was followed by 30 PCR cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s) and a final extension at 72 °C for 10 min. The amplified DNA was purified using polymerase chain reaction (PCR) purification kit (Promega, Madison, WI, USA), diluted to 200 ng µ1-1, and was sequenced using Microsynth Inc. (Balgach, Switzerland)(haritha meruvu et al., 2013).

Determination of 16 S rRNA gene sequence

The 16S rRNA gene sequence of bacteria MSB-6 was deposited in the EMBL/GenBank/DDBJ databases and compared with close relatives using the BLAST search tool (Altschuf *et al.,* 1997; Thompson *et al.,* 1994). The distance matrices of the aligned sequences are calculated by using the two parameters method of Kimura (1980). The maximum likelihood method is used for constructing a phylogenetic tree. The robustness for individual branches is estimated by boot strapping with 100 replicates. Basic Local Alignment Tool (BLAST) (Altschul *et al.*, 1990) can be used to quickly compare the sequence data in hand to a sequence database like GenBank. Different BLAST algorithms are available including BLASTn, that compares two nucleotide sequences and BLASTX that compares translated nucleotide sequence data to the available protein sequence database. BLAST does not match proteins over their entire length but rather look for any regions of similarity within proteins that are closely related. The information collected from the genome sequence analyses are used to deduce which genes are present and what their function may be. Thereafter, each gene-encoding sequence can be sorted according to their assigned role.

PCR amplification and cloning

For bacteria, PCR was performed using 100 ng of DNA with universal 16S rRNA gene primers 8-27f (Weisburg *et al*., 1991) and 1492r (Reysenbach *et al*., 1992). The DNA amplification was carried out using Hi-Fi Platinum Taq (Invitrogen, San Diego, Calif.). Cycling conditions were as follow: initial denaturation at 94ºC for 5 min, 20 cycles of 94ºC

for 30 sec, 48ºC for 2 min, 72ºC for 1.5 min, and a final extension of 5 min at 72ºC in a PTC-200 MJ-research thermal cycler (Bio-Rad, Hercules, Calif). For archaea, PCR was performed using 100 ng of 16S rRNA gene primers 21f and 951r (DeLong, 1992). Cycling conditions were: initial denaturation at 94ºC for 5 min, 25 cycles of 94° C for 1 min, 54° C for 30 sec, 72° C for 2 min, and a final extension of 5 min at 72° C. PCR products were purified by electrophoresis in a 1% (wt/vol) agarose gel and bands of approximately 1500 bp were excised and recovered using a gel extraction kit (Qiagen, Inc., Chatsworth, Calif.). Purified PCR products were cloned with a TOPO-XL cloning kit according to the manufacturer's instructions (Invitrogen).

Sequencing, phylogenetic and rarefaction analysis

Sequencing of clones was done using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, Calif.) with M13 forward and reverse primers. Sequences were compared to those in the GenBank database with the Basic Local Alignment Search Tool (BLAST) algorithm to identify known closely related sequences. Sequences were examined for the formation of chimeras using the program CHECK_CHIMERA (Maidak *et al.*, 1997). Sequences of 142 clones, with a length of at least 500 bp, were manually compiled and aligned with Phydit software (Chun, 1995). Trees were generated by the neighbor-joining (Saitou & Nei, 1987) algorithm (Kluge & Farris, 1969) implemented in Phydit. The robustness of inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data and only values >50% were shown. The assemblage of 16S rRNA gene sequences in each library was analyzed by rarefaction analysis using EcoSim (Gotelli & Entsminger, 2006) to assess the extent to which the diversity of microbial communities was represented by the library at the class and species level. The number of species in each clone library was determined by comparing closely related sequences using bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast 2.cgi) (Tatusova & Madden, 1999). 16S rRNA gene sequences exhibiting a percentage of similarity of 97% or lower (Devereux *et al.*, 1990) were considered two different species.

RESULTS & DISCUSSION

Morphological and physiological characterization

The bioactive bacterial isolate MSB-6 was isolated on nutrient agar medium. Isolated bacterium was Gram-negative, short, rod shaped, produces pale greenish pigmentation with high mol% A+T content. They were taxonomically diverse, biologically active and colonize all marine habitats from deep oceans to the shallow west estuaries (Rheinheimer, 1992). It exhibited optimum growth under aerobic conditions at temperature 37^{o}C and pH at 7.0. It is mesophilic and alkaliphilic in nature which showed growth range from 15^{o}C to 42^{o}C. Optimum growth is observed at 2.5% (w/v) sodium chloride (NaCl), but maximum tolerance of sodium chloride concentration is exhibited growth up to 5.0% (w/v), indicating it is indigenous to marine environment and moderate salt tolerance in nature.

Table 1
Morphological & Physiological characterization of MSB-6 bacteria

Colony Morphology	Result
Size	Large
Configuration	Circular
Opacity	Opaque
Surface	Moist
Pigment	Pale Green
Cell Shape	Rod
Cell Size	Small
Gram Staining	Negative
Spore	Negative
Motility	Positive
Temperature (^{0}C)	15-37
pH	5-9
NaCl (%)	2.5-5.0

Biochemical characterization

The bactria MSB-6 could utilize dextrose, mannitol, melibiose, xylose, mannose as the carbon source along with acid production; however, adonitol, L-arabinose, inulin, cellobiose, dulcitol, fructose, inositol, lactose, maltose, raffinose, rhamnose, salicin, sorbitol, sucrose and trehalose were not utilized by the organism and there is growth occurred on MacConkey agar. The biochemical tests like citrate utilization, casein hydrolysis, cytochrome oxidase, catalase test, gelatin hydrolysis and Arginine dihydrolase are positive.

Table 2
Biochemical characterization of MSB-6 bacteria

Biochemical test	Result
Indole Test	Negative
Methyl Red Test	Negative
Voges Proskauer Test	Negative
Growth On Mac-Conkey Agar	Positive
Citrate Utilization Test	Positive
Arginine Dihydrolase	Negative
Casein Hydrolysis	Positive
Catalase Test	Positive
Cytochrome Oxidase	Positive
Gelatin Hydrolysis	Positive
H_2S Production	Negative
Lysine Decarboxylase	Negative
Nitrate Reduction	Positive
Oxidation/Fermentation	Oxidative
Starch Hydrolysis	Negative
Urea Hydrolysis	Negative

Acid production of MSB-6 bacteria

The MSB-6 bacteria is able to ferment carbohydrates dextrose, galactose, melibiose, xylose and mannose for acid production but unable to ferment adonitol, inulin, cellobiose, dulcitol, fructose, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose and trehalose. The carbohydrate fermentation of MSB-6 isolate is shown in the Table-5.

Table 3
Acid production from carbohydrate utilisation by MSB-6 bacteria

Carbohydrate source	Result
Adonitol	Negative
L- Arabinose	Positive
Cellobiose	Negative
Dextrose	Positive
Dulcitol	Negative
Fructose	Negative
Inulin	Positive
Galactose	Positive
Inositol	Negative
Lactose	Negative
Mannose	Positive
Maltose	Negative
Melibiose	Positive
Raffinose	Negative
Rhamnose	Negative
Salicin	Negative
Sorbitol	Negative
Sucrose	Negative
Trehalose	Negative
Xylose	Positive

16S rRNA analysis of MSB-6 bacteria

The 16S rRNA of MSB-6 is coded by 1444 bp long gene and the sequence is deposited in Gene Bank with an accession number KC852045. The homology studies have shown that MSB-6 16S rRNA sequence exhibits more than 99% similarity with the *Pseudomonas fluorescens* compared to other closely related *Pseudomonas* sp. **(**Figure-5).

ACCESSION: BankIt1618020-KC852045
DEFINITION: Pseudomonas fluorescens strain MSB-6.

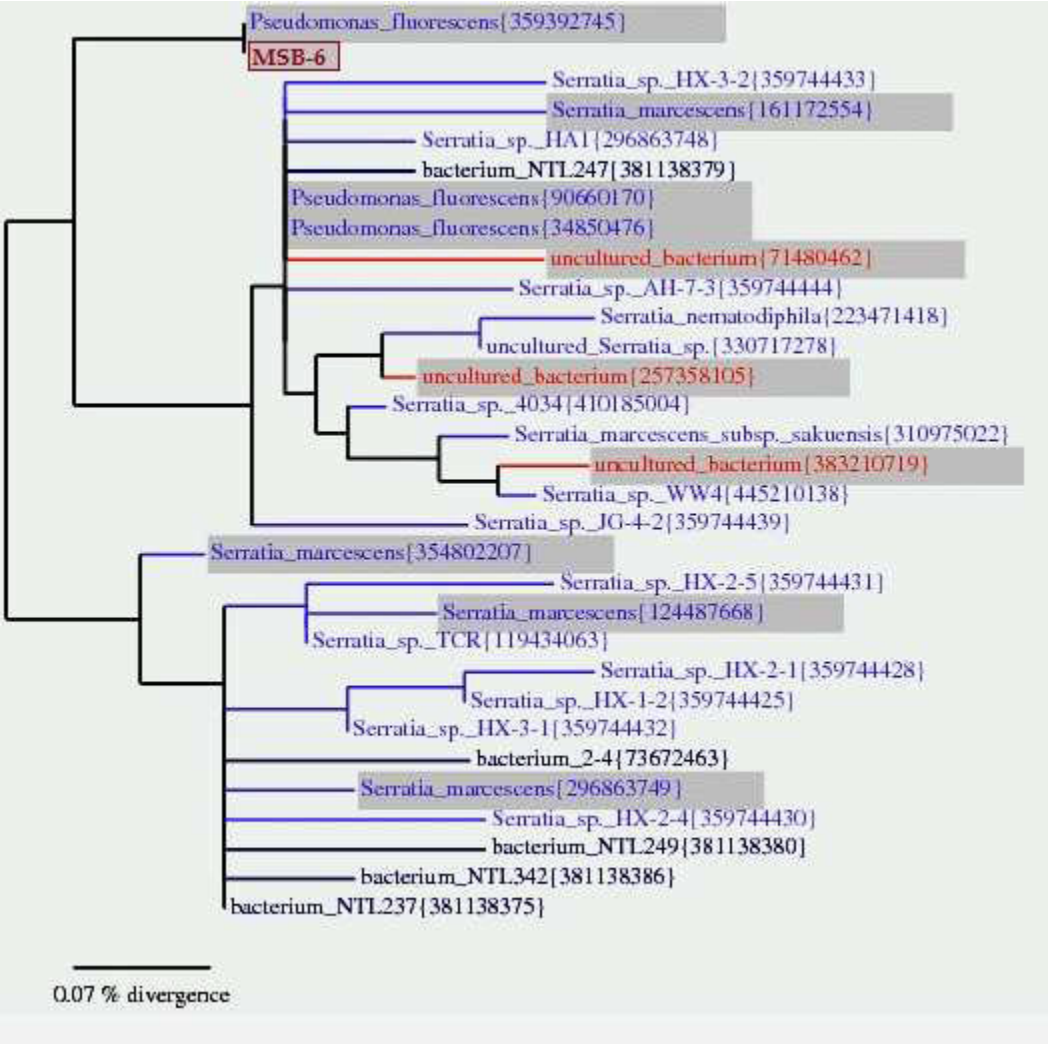

Figure 1
PHYLOGENETIC TREE

Among the microbial population from marine soil, *Pseudomonas* species is reported to be the most abundant form. Many of the *Pseudomonas* strains had major importance in bacteriocin research since this genus produces a diverse array of antimicrobial peptides with several different basic chemical structures (Stein, 2005; Pakpitcharoena *et al*., 2008). They were the producers of most of the known bioactive metabolites. They include numerous potentially useful compounds providing the widest range and most promising array of pharmacologically and agriculturally active compounds. There were wide spread in nature and can be found in greater or less frequency in most ecological niche (Takahashi and Omura, 2003). They were widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics (Bibb, 2005).

CONCLUSION

On the basis of morphological, physiological, biochemical and phylogenetic analysis, isolate MSB-6 is identified as genus *Pseudomonas*. The isolate showed closest similarity to the species *fluorescens*. Identity of the isolate is

further confirmed by the Institute of Microbial Technology, Chandigarh, India and the culture has been deposited in the Gene Bank, Chandigarh, India as *Pseudomonas fluorescens.* Further the strain is subjected for Optimization for enhancing the antimicrobial activity of the antibiotic metabolite.

REFERENCES

1. Blunt, J.W.,B.R.Copp, W.Hu, M.H.G.Munro, P.T.Northcote and M.R.Prinsep, 2007. Marine natural products. Nat. Prod. Rep., 24:31-86.
2. Collee, J.G., Fraser, A.G., Marmion, B.P., Simmons, A., 1969. Mackie and McCartney Practical Medical Microbiology, 14th edition. Churchill Livingstone, New York.
3. Fiedler, H.P., C. Bruntner, A.T. Bull, A.C. Ward and M. Goodfellow et al., 2005. Marine actinomycetes as a source of novel secondary metabolites. Antonie van Leeuwenhoek, 87: 37-42.
4. Grimont, P.A.D. Vancanneyt, M. Lefévre, M. Vandemeulebroecke, K. Vauterin, L. Brosch, R. Kersters, K. and Grimont, F. (1996) Ability of biolog and Biotype-100 systems to reveal the taxonomic diversity of the *pseudomonads.* Systematic Applied Microbiology, 19: 510-527.
5. Haritha meruvu and sri Rami reddy donthireddy (2013) Biochemical and molecular taxonomy of chitinolytic *citrobacter freundii* str. nov. haritd11, isolated from indian marine environment, Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 1168 - 1175
6. Hartung, J. S. (1998) Molecular probes and assays useful to identify plant pathogenic fungi, bacteria, and marked bio control agents. In: Boland, GJ and Kuykendall, LD (eds) Plant microbe interactions and biological control. Marcel Dekker, Inc. New York, Basel and Hongkong. 393-413.
7. Kenneth, L.K., 1958. Prepared research paper RP 2911, Central nations for the revised ISCC-NBS color name blocks. J. Res. NBS, 16: 427-427.
8. Krieg, N. R. and Holt, J. G. 1984. Bergey's manual of systematic bacteriology. 2: 999-1071. Williams and Wilkins Baltimore, USA.
9. Kumar, Y. and M. Goodfellow, 2008. Five new members of the 16S rRNA gene clade: Streptomycetes violaceusniger sp. nov., Streptomyces himastatinicus sp. nov., Streptomycetes mordarskii sp. nov., Streptomyces rapamycinicus sp. nov and Streptomyces ruanii sp. nov. Int. J. Syst. Evol. Microbial., 58: 1369-1378.
10. Bibb, M.J. Regulation of secondary metabolism in Streptomycetes. Curr. Opin. Microbiol., 8: 208-215 (2005).
11. Nandy, P., Thakur, A.R., Chaudhuri, S.R., 2007. Characterization of bacterial strains isolated though microbial profiling of urine samples. Online J. Biol. Sci. 71, 44–51.
12. Pakpitcharoena, A. Potivejklub, K. Kanjanavasa, P. Areakita, S. and Chansir, K. Biodiversity of thermo tolerant Bacillus sp. producing biosurfactants, biocatalyst and antimicrobial agents. Sci. Asia., 34: 424-431 (2008).
13. Pathiranana, C., D. Tapiolas, P.R. Jensen, R. Dwight and W. Fenical, 1991. Structural determination of moduralide: A new 24-membered ring macrolide glycoside produced by a marine bacterium (Actinomycetes). Tetrahedron Lett., 32: 2323-2326.
14. Pathiranana, C., D. Tapiolas, P.R. Jensen, R. Dwight and W. Fenical, 1991. Structural determination of moduralide: A new 24-membered ring macrolide glycoside produced by a marine bacterium (Actinomycetes). Tetrahedron Lett., 32: 2323-2326.
15. Pridham, T.G. and D. Gottlieb, 1948. The utilization of carbon compounds by some actinomycetales as an aid for species determination. J. Bacteriol., 56: 107-114.

16. Rheinheimer, G. Aquatic microbiology, 4th N.Y. edn. Wiley, L.L. Richardson, W.M. Goldberg, K.G. Kuta, Floridas mystery coral-killer identified. *Nature*., 392: 557-558 (1992).
17. Salle, A.J., 1948. Laboratory Mannual on Fundamental Principles of Bacteriology, 3rd ed. McGraw-Hill Book Company, INC, London.
18. Sneath, P. H., Mair, N. S. and Sharp, E. 1986. Bergey's manual of systematic bacteriology. 2: 999-1071. Williams and Wilkins Baltimore, USA.
19. Stein, T. Bacillus subtilis antibiotics: structures, syntheses and specific functions. Mol. Microbial., 56: 845-857 (2005).
20. Takahashi, Y. and Omura, S. Isolation of new actinomycete strains for the screening of new bioactive compounds. J. Gen. Applied Microbiol., 49: 141-154 (2003).