Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 7, 2014

Original Article

INTERGENUS PROTOPLAST FUSION STUDIES BETWEEN MARINE *STREPTOMYCES* SP. ANS4 AND *ESCHERICHIA COLI*

BALAJI S., M. RADHAKRISHNAN[2], R. BALAGURUNATHAN[3*]

[1]Department of Bacteriology, National Institute for Research in Tuberculosis, Chennai 600031 Tamil Nadu, [2]Centre for Drug Discovery and Development, Sathyabama University, Chennai 600119 Tamil Nadu, [3]Department of Microbiology, Periyar University, Salem 636011 Tamil Nadu, India
Email: rbalaguru@yahoo.com

Received: 08 May 2014 Revised and Accepted: 18 June 2014

ABSTRACT

Objective: To study the intergenus protoplast fusion bioactive marine *Streptomyces* sp ANS4 with the *Escherichia coli* for the strain improvement.

Methods: Protoplast of both the organisms was prepared by lysozyme treatment and fused using polyethylene glycol (PEG). Fusant colonies were regenerated using regeneration medium where all the regenerated colonies are powdery in consistency. Microscopic, cultural, physiological, enzymatic and antibiotic susceptibility characteristics of one fusant culture and both the parent cultures are studied. Growth curve and bioactive metabolite production by both the parent and fusant cultures was also studied.

Results: oval shaped protoplasts of both the parent cultures are clearly seen under the phase contrast microscope. All the colonies developed after protoplast fusion on regeneration medium looked like one of the parent culture *Streptomyces* sp ANS4. One among the 10 antagonistic colonies selected from the regeneration medium was expressed the characteristics of either or both of the parental cultures and certain new characteristics. The results of growth curve study showed that the fusant culture possess 10 hours reduction in attaining stationary phase compared to parent *Streptomyces* sp ANS4. The antibiotic activity of fusant culture was also higher than the parent cultures.

Conclusion: The intergenus protoplast fusion between the *Streptomyces* sp ANS4 and the *E.coli* strain results in the reduction in time for secondary metabolite production and also increase in antimicrobial activity. This suggested that the potential bioactive compounds may be obtained in increased quantity from the fusant culture with lesser fermentation period.

Keywords: Protoplast fusion, Strain improvement, *Streptomyces, E. coli.*

INTRODUCTION

Actinomycetes are virtually unlimited sources of novel compounds with many therapeutic applications and hold a prominent position due to their diversity and proven ability to produce novel bioactive compounds [1]. Traditionally, these bacteria have been isolated from terrestrial sources, although the first report of mycelium-forming actinomycete from the marine sediments appeared several decades ago [2]. The actinomycetes are active components of marine microbial communities [3] and form stable, persistent populations in various marine ecosystems. Marine actinobacteria are the best sources of secondary metabolites and the vast majority of these compounds are derived from the single genus *Streptomyces*, whose species are distributed widely in the marine and terrestrial habitats and are of commercial interest due to their unique capacity to produce novel metabolites [4].

Various methods of genetic manipulation enable the microbial geneticists to create new combinations of genetic material in microbes. The genetic manipulation techniques such as inducing mutation, protoplast fusion, gene amplification, recombinant DNA technology etc., are used in the field of microbiology. Protoplast fusion is an efficient method among the various genetic manipulation approaches to induce a high frequency of recombination [5].

Protoplast fusion is accomplished by removing the cell walls of organisms of two strains and mixing the resulting protoplasts. This allows the fusion of the cell and genetic material before new cell wall is produced. By mixing two strains, each of which has desirable characteristics, the new strains that have both characteristics can be produced. After protoplast fusion, some organisms are probably fast growing with good producers of the substances. A 200-10,000-fold increase in recombination frequency is obtained through protoplast fusion when compared to other conventional mating procedures [6].

As noted, the fusion technique may provide improvement of an antibiotic producing strain or by the gene recombination involving interspecific fusion leads to the production of novel antibiotics [7]. Studies on interspecies mating were initiated in recent years to apply genetic recombination technique to the isolation of hybrid strains producing new antibiotics.

The ability of the hybrid strains producing new antibiotic will depend in part on proper choice of mating pair, and selective procedures imposed during regeneration [6]. Even though there are some reports available on the intraspecific fusion, but there are no reports available on the intergenus fusion with actinomycetes.

During the course of our antimicrobial screening program, we isolated an actinomycete, *Streptomyces sp* ANS4 from Andaman marine sediments. Crude extract prepared from this strain showed promising activity against Gram positive and Gram negative bacterial pathogens. In this present study, we report the first successful intergenus fusion between actinomycete and eubacteria and how the parental characters are distributed in fusant.

MATERIALS AND METHODS

Description of *Streptomyces sp* ANS4 and *Escherichia coli*

Streptomyces sp ANS4 was isolated from sediment samples collected from Andaman marine ecosystem using starch casein agar [8]. This strain produced small powdery colonies with grayish aerial mycelium on ISP2 agar medium. Both non-fragmented aerial and substrate mycelium was observed under bright field microscope. Strain ANS4 showed promising antibacterial activity against Gram positive and Gram negative bacteria. Viability of strain ANS4 is maintained on ISP2 agar slants [9] as well as in 20% glycerol broth. Another parent culture *E. coli* was isolated from sewage sample. Viability of *E. coli* is maintained as slant culture and stab culture using nutrient agar medium.

Preparation of parent cultures

Spores of *Streptomyces* sp ANS4 from ISP2 agar medium was inoculated in to 100 ml of medium S (gm/l: glucose 10; peptone 4; yeast extract 4; MgSo4 0.5; KH2Po4 2; K2HPo4) for 48 hours at 28^{0}C in rotary shaker. The cells were collected and further transferred into medium "S" supplemented with 0.8% glycine. The flask was once again kept at 28^{0}C in rotary shaker until the middle of the exponential phase. Finally, the mycelium was harvested by centrifugation and washed with a solution of 0.3M sucrose solution [10]. Another parent strain, *E. coli* was grown on LB broth (gm/l: tryptone 10; yeast extract 5; sodium chloride 10). After sterilization, 10µg/ml of filter sterilized chloramphenicol was added from a stock solution (34 mg/ml in absolute alcohol) for the inhibition of protein synthesis [11]. After 24 hours of incubation, the cells were recovered by centrifugation at 3000 rpm for 10 minutes at 4^{o}C.

Preparation of protoplasts

For the preparation of protoplasts, *Streptomyces* sp ANS4 harvested from medium 'S' was transferred into medium 'P' (gm/l sucrose 103; potassium sulphate 0.25; trace element solution 2 ml; K2HPo4 0.05; MgCl2 2.03; CaCl2 3.68; and 0.25M TES buffer 100 ml) and incubated for 2 hours [10]. Lysozyme was added in the final concentration of 1 mg/ml of medium 'P' and incubated at 32^{o}C for 2 hours. Then the suspension was filtered through cotton and then through Wattman paper having 5 µm pore size to exclude mycelial forms. The filtered protoplasts were washed three times with cold medium 'P' by means of centrifugation at 1000 rpm for 15 minutes. The pellet of *E. coli* was obtained from LB medium and resuspended in 2.5ml of ice-cold solution of 20% (w/v) sucrose in 50mm Tris-Cl (pH 8.0). Five hundred microlitre of fresh lysozyme solution (5mg/ml in 250 mM Tris-Cl pH 8.0) was added. The suspension was incubated at 4^{o}C for 5 minutes. Again 1ml of ice-cold solution of 0.25M EDTA (pH 8.0) was added to the above mixture and incubated on ice for 5 minutes. Finally 1 ml of ice-cold 50 mM Tris-Cl was slowly added and the suspension was incubated at 37^{o}C for 15 minutes [11].

Fusion of parental protoplasts & regeneration

About 500 µl of protoplast suspension from each parental strain was mixed and centrifuged. The pellet was resuspended in 0.1ml of medium 'P' and mixed with 0.9 ml of 50% (w/v) filter sterilized poly ethylene glycol 400 (PEG 400) in medium 'P'. The suspension was allowed to stand 30 seconds for the fusion of protoplasts [12]. The fused protoplasts were diluted with medium 'P' and 0.05ml of the dilution was spread on a regeneration (R_2) medium (gm/l sucrose 103; potassium sulphate 0.25; trace element solution 2 ml; K2HPo4 0.05; MgCl2 10.12; CaCl2 2.95; glucose 10; L-proline 3; casaminoacids 0.1; agar 22; 0.25M TES buffer 100 ml; pH 7.0) plates and incubated at 28^{o}C [10]. After enumerating the colonies the fusant cultures were randomly selected and streaked on ISP2 medium for further studies. One fusant culture was selected for characterization.

Table 1: Properties of parent and fusant cultures

Characteristics	Parent cultures		*Fusant culture*
	Streptomyces sp ANS4	***E. coli***	
Colony morphology	Gray colour powdery colonies	Colourless Mucoid colonies	Gray colour powdery colonies
Micromorphology	Filamentous	Rod shaped	Filamentous
Aerial and substrate mycelium	Present	-	Present
Antibiotic sensitivity			
Penicillin	S	S	R
Ampicillin	S	S	S
Chloramphenicol	S	S	R
Gentamycin	S	S	R
Enzyme activity			
Amylase	+	+	+
Protease	+	+	+
Lipase	+	+	+
Nitrate reduction	-	+	+
Gelatin liquefaction	+	+	+
Melanin production	+	-	+
Carbon utilization			
Glucose	+	+	+
Fructose	+	-	+
Sucrose	+	+	+
Inositol	-	+	+
Mannitol	-		+
Rhamnose	-	+	+
Biomass quantity (gm/200 ml)			
Wet weight	10.30	3.20	21.50
Dry weight	2.80	1.05	5.10
Quantity of Ethyl acetate extract (mg/200 ml)	25	12	40
Antimicrobial activity*			
Methicillin resistant *Staphylococcus aureus*	12.3±0.57	-	13.3±0.58
Staphylococcus aureus	10.3±0.58	-	10.3±0.57
Bacillus subtilis	14.7±0.58	-	13.3±1.15
Escherichia coli	8.0±1.0	8.3±0.58	-
Salmonella typhi	9.7±0.57	-	10.3±0.59
Vibrio cholerae	6.7±0.58	-	10.7±0.57

S – sensitive; R – resistant; * Zone of Inhibition represented with mean ± standard deviation (n = 3).

Characterization of fusant and parent cultures

Cultural, microscopic and carbon utilization characteristics of fusant and *Streptomyces* sp ANS4 was studied by adopting the method described by Shirling and Gottileb [9] whereas the same characteristics of *E. coli* were studied by adopting the standard microbiological procedures.

The antibiotic susceptibility of *Streptomyces* sp ANS4 and fusant culture was determined on ISP2 agar plates whereas antibiotic susceptibility of *E. coli* was determined using Muller Hinton agar [13]. Antibiotic discs (Himedia) used for this includes chloramphenicol, penicillin, gentamycin and ampicillin. Zone of inhibition was measured after 48 hours and expressed in millimeter in diameter.

The parental and fusant cultures were tested for the enzyme activities such as amylase, protease and lipase using starch agar, skim milk agar and Tween 80 agar, respectively. All the plates were incubated at 28^{0}C for 3 days. After incubation, the amylase activity was observed by adding iodine solution; protease activity was observed directly by zone of clearance whereas the lipase activity was determined by opaque zone around the colonies [14].

Growth curve studies

Hundred ml of LB medium was inoculated with 10ml of one-hour culture of *E. coli* and each 100 ml of sporulation medium was inoculated with 10ml of *Streptomyces sp* and fusant culture grown on ISP2 medium. Then the flasks were kept in rotary shaker at 28^{0}C. The OD value was measured for every 2 hours in a spectrophotometer at 550nm. Uninoculated medium was used as a blank [15]. The OD value was taken for *Streptomyces sp* and fusant cultures between 0 and 120 hours and between 0 and 40 hours for *E. coli*.

Production of bioactive metabolites

The parent strain *Streptomyces* sp ANS4 and the fusant culture were grown on ISP2 agar for 5 days at 28^{0}C. The spores were collected using 20% sterile Tween 80 solution. The collected spores were transferred into each 50ml of soybean meal inoculation medium and kept in rotary shaker for 48 hours at 28^{0}C. Then 10% of inoculum was added to the soybean meal production medium. The culture broths of production medium were centrifuged separately at 6,000 rpm for 20 minutes at 4^{0}C and the resultant cell free supernatant was used for the extraction of bioactive compounds. The mycelial cake was collected and weighed to determine total biomass. The fermentation broth was extracted with ethyl acetate in pH levels of 4,7 and 10 and concentrated. About 250 microgram of crude extract was impregnated on the filter paper disc and tested against different clinical bacterial pathogens by disc diffusion method. Zone of inhibition was measured after 24 hours of incubation at 37^{0}C and expressed in millimeter in diameter [8].

RESULTS AND DISCUSSION

Protoplast preparation, fusion and regeneration

The first work on the protoplast fusion of *Streptomyces* using poly ethylene glycol (PEG) was reported by Hopwood et al. [16] and recombination frequencies achieved by this technique are so high that selectable marker could be dispensed with at least in certain kinds of strain improvement programmes. In the present study, the efficient formation of protoplast of *Streptomyces sp* and *E. coli* was confirmed by roundness of the cells. Okanishi et al. [10] have demonstrated that *S. griseus* and *S. venezulae* become highly sensitive to enzymatic hydrolysis by lysozyme and lytic enzyme No. 2 after the growth in a medium containing a partially growth-inhibitory concentration of glycine. Baltz [12] also found that lysozyme treatment was effective on fusion of *S. fradiae* and *S. griseofuscus* protoplasts. The effect of lysozyme in the present study was found satisfactory with the successful isolation of protoplasts.

The effective protoplast fusion among various species of *Streptomyces* was achieved using 40 to 60% PEG for 30-60 seconds at 23^{0}C (Baltz, 1981; Ochi et al., 1979; Hopwood and Wright 1981)[7,17,18]. Imada et al. [5] achieved intergenus protoplast fusion between *Streptomyces* and *Micromonospora* using 40% PEG for 3 minutes at room temperature. In the present study, the fusion was undertaken by using 50% PEG (w/v) and good result was obtained. The effect of Mg^{2+} and Ca^{2+} on the stabilization of protoplast was described. The Mg^{2+} prevents the release of lipid residues from plasma membrane and controlling the liberation of mesosome vesicles from protoplast during the removal of cell wall by lysozyme. The Ca^{2+} prevents the lysis of lysozyme-induced protoplast in hypertonic medium. The combinations of 10mM-$MgCl_2$ and 25mM-$CaCl_2$ was effective for preparing and stabilizing the protoplasts of streptomycete in hypertonic solutions was reported by Okanishi et al. [10] as like in the present study.

In this study the first visible colonies appeared from regeneration medium after 4 days of incubation, while the last appeared on 10 days of incubation. The process of cell regeneration from protoplast was generally slow and asynchronous. In this intergenus experiment, there are about 147 colonies were appeared and almost all the fusants resemble Streptomycete colonies in cultural and micromorphology. Among 147 total colonies, 10 colonies were randomly selected based on their morphological differences. Imada et al. [5] also suggested that the protoplast fusion technique would be promising for obtaining new clones through intergenus protoplast fusion. Among these 10 colonies, only one colony was selected for further characterization based on the bioactivity.

Characterization of parent and fusant cultures

The morphology and spore arrangements of fusant was found similar to the parent *Streptomyces* sp ANS4 when observed under bright field microscope. In antibiotic sensitivity testing, both parental culture of *Streptomyces sp* ANS4 and *E. coli* was found sensitive to the antibiotics penicillin, gentamycin, choloramphenicol and ampicillin. Whereas the fusant culture showed resistance to the penicillin, chloramphenicol and ampicillin. It is also observed that the fusant culture was found sensitive to gentamycin (Table 1). It was already mentioned that protoplast fusion technique may result in expression of new protein, which is not expressed in either parental strains [19]. In the present study also the fusant culture, showed resistant to penicillin chloramphenicol and ampicillin but neither of the parents showed this resistance. It is discussed that the genes responsible for resistance were already present in any one of the parents but under strict regulation. The fusion might have inactivated one or more regulatory genes and resulted in newly found resistance character in fusant.

All the cultures of both parent as well as fusant cultures showed positive to all the enzyme activities tested viz. amylase, protease and lipase. So there is no change among enzyme activities. The parent strain of *Streptomyces sp* showed negative result for nitrate reduction test but the fusant showed positive as in *E. coli.* It clearly demonstrates that the gene coding for nitrate reduction was transferred to fusant and successfully expressed in it (Table 1). Both the parents and fusant cultures were found to exhibit gelatin liquefaction. Both the parent *Streptomyces* sp ANS4 and fusant culture was found to produce melanin. The melanin pigment production was observed in *Streptomyces sp* but not in fusant and *E. coli.* It may be attributed to alteration in *Streptomyces* genome by recombination or deletion or blocking of genes responsible for melanin production during fusion.

By using glucose as a positive control of carbon source, the following results were observed. The *Streptomyces sp* utilizes sucrose, and fructose, and *E. coli* utilizes sucrose, inositol and rhamnose. But the fusant culture utilizes mannitol, sucrose, fructose, inositol and rhamnose. It was observed that fusant possessed enhanced capabilities of degrading sugars as their sole source of carbon when compared to parental strains. The fusant was found to be utilizing all the 6 sugars tested, while the capability of parent strain *Streptomyces sp* was 3 and *E. coli* was 4 and none of them utilized raffinose. *E. coli* can degrade inositol and rhamnose but not *Streptomyces sp.* But the fusant can degrade both sugars. It shows that *E.coli* provides the genes responsible for utilization of these sugars and successfully expressed in fusant. In the case of mannitol neither of parental strains can utilize it but the fusant efficiently utilizes the mannitol (Table 1). It may be due to inactivation of one or more regulatory proteins that resulted in expression of genes responsible for degradation of mannitol.

Growth curve studies

From the growth curve studies it was observed that there are about 10 hours of reduction in growth time of fusant culture. In *Streptomyces sp* the stationary phase attained at 68 hours but in fusant it was at 58 hours. The stationary phase of *E. coli* was found at 23 hours of incubation. It is concluded that the fusant culture showed maximum growth between 68 and 23 hours. During the studies of growth factors of parental and fusant culture strains, we observe that the growth characteristics of fusant were similar to *Streptomyces sp* but the time of reaching stationary phase was significantly reduced. There is a possibility that the cell cycle components of *Streptomyces sp*

responding to cyclins and kinases encoded by *E.coli* genome. The mixing of cell cycle components from two different genera with different growth kinetics might have resulted in intermediate growth kinetic properties. This theory correlates properly with the readings found in the present experiment i.e., *E. coli* at 22 hours, *Streptomyces sp* at 68 hours and fusant 58 at hours.

Isolation of bioactive compound

The supernatant and mycelial pellet of parent and fusant cultures from production medium were collected for further analysis. The result showed that fusant culture was produced double the biomass when compared to parental culture. Ethyl acetate extract prepared from the supernatants of parent and fusant cultures were taken for total quantity of bioactive compounds and activity against various clinical pathogens. About 40 and 25 mg of crude extract were obtained from the cell free supernatant of fusant culture and *Streptomyces* sp ANS4, respectively using ethyl acetate. The results showed that the fusant extract having wide spectral activity against both Gram positive and Gram negative bacteria in addition to MRSA strain. One particular point of interest in this study was the antagonistic activity of fusant against methicillin resistant *Staphylococcus aureus* (MRSA) while parental *Streptomyces sp* cannot show such inhibition effect on MRSA (Table 1). It may be due to alteration in secondary metabolite production in fusant or may attributed to modification made by *E. coli* encoding enzymes in the structure of bioactive substance encoded by *Streptomyces sp* which resulted in novel bioactive substance.

In the present study we have successfully isolated a fusant strain by protoplast fusion with the faster growth rate than parental strain with high antibiotic activity especially against MRSA. As effective antibiotics are not available nowadays to treat the disease caused by MRSA strains, this fusant culture obtained from the present study may be a good source for isolating more effective antibiotics with short fermentation time compared to wild strains.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Berdy J. Thoughts and facts about antibiotics:where we are now and where we are heading. J of Antibiotics 2012;65:385-95.
2. Weyland H. Actinomycetes in North Sea and Atlantic Ocean sediments. J Nature 1969;223:858.
3. Jensen PR, Gontang E, Mafnas C, Mincer TJ, Fenical W. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. J Environmental Microbiology 2005;7(7):1039-48.
4. Manivasagan P, Venkatesan J, Sivakumar K, Kim S. Pharmaceutically active secondary metabolites of marine actinobacteria. J Microbiological Res 2013.
5. Imada C, Okanishi M, Okami Y. Intergenus protoplast fusion between Streptomyces and *Micromonospora* with reference to distribution of parental characteristics in the fuants. J Bioscience and Bioengineering 1999;88(2):143-47.
6. Godfrey O, Ford L, Huber MLB. Interspecies matings of *Streptomyces fradiae* with *Streptomyces bikiniensis* mediated by conventional and protoplast fusion technique. Canadian J of Microbiology 1978;24:994-97.
7. Ochi K, Hitchcock MJM, Katz E. High frequency fusion of *Streptomyces parvulus* or *Streptomyces antibioticus* protoplasts induced by poly ethylene glycol. J of Bacteriology 1979;139(3);984-92.
8. Balagurunathan R. and Subramanian A. Antagonistic streptomycetes from marine sediments. J Advances in Biosciences 2001;20(2):71-6.
9. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. Int J of Systematic Bacteriology 1966;16(3):313-40.
10. Okanishi M, Suzuki K, Umezawa H. Formation and reversion of Streptomycete protoplasts:cultural condition and morphological Study. J of General Microbiology 1974;80:389-400.
11. Maniatis T, Sambrook J, Fritsch EF. Expression of cloned genes in cultured mammalian cells. A laboratory manual of molecular cloning. J Cold Spring harbor laboratory Press 1989.
12. Baltz RH. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. J of General Microbiology 1978;107;93-102.
13. Bauer AW, Kirby M, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disc method. American J of Clinical Pathology 1966;45:493-97.
14. Ranjekar MK, Sridhar KR. Occurrence and extracellular enzymatic potential of actinomycetes of a thermal spring, Southern India. Asian J Microbiol Biotechnol Environ Sci 2002;4(1):59-64.
15. Atlas RM, Parks LC, Brown AE, Inc USA. Laboratory manual of experimental microbiology. Mosby Year book 1995.
16. Hopwood DA, Wright HM, Bibb MJ. Genetic recombination through protoplast fusion in *Streptomyces.* J Nature 1977;268:171-4.
17. Baltz RH, Matsushima P. Protoplast fusion in *Streptomyces*: conditions for efficient genetic recombination and cell regeneration. J of Gen Microbiol 1981;127:137-46.
18. Hopwood DA, Wright HM. Protoplast fusion in *Streptomyces*: Fusions involving ultraviolet irradiated protoplasts. J Gen Microbiol 1981;126:2-7.
19. Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, Fujii I *et al*. Production of hybrid antibiotics by genetic engineering. J Nature 1985;314;642-44.