Isolation & Characterization of Tn-induced Mutants of Biosurfactant Producing *Pseudomonas* Strains

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Transposon mutagenesis system has been developed for biosurfactant producing *Pseudomonas* strains. In present study a suicide plasmid based Tn10 mutagenesis was successfully used for a biosurfactant producing *Pseudomonas aeruginosa* strain K3. Tetracycline resistant transconjugants were detected at the frequency of 10^{-4}-10^{-6} per recipient. Transposon insertions yielded mutants of several different phenotypes including hexadecane negative (Hex$^-$), biosurfactant non-producer (BSNP), and biosurfactant hyperproducer(BSHP). With reversion rates 10^{-10}, these Tn10 induced mutants were quite stable.

Biosurfactants (BS) are of increasing interest commercially as substitutes of chemical surfactants which are widely used in pharmaceutical, cosmetic, petroleum and food industries. Biosurfactants are biodegradable and can be produced on renewable resource substrates and thus have immense potential to replace chemically synthesized surfactants. Molecular genetic studies of BS producing *Pseudomonas* have been limited due to unavailability of proper mutants. Transposon mutagenesis is a powerful technique for genetic analysis in bacteria (6). Transposon provide physical and genetic markers for inducing mutations at single sites in the genes of interest. Transpositional mutagenesis has been successfully applied to a wide range of gram negative bacteria. The present study was undertaken to understand the molecular genetics of BS producing strains of *P. aeruginosa*. Transposon mutagenesis using suicide plasmid-based transposon delivery vectors for one of the previously isolated biosurfactant producing strain K3 (7) have been developed (3). In this report, we assess the potential for the introduction of transposons into *P. aeruginosa* using suicide vectors. Isolation and characterization of Tn10 induced mutants will be discussed.

MATERIALS AND METHODS

Bacterial strains & plasmids. Bacterial strains and plasmids used in this study are shown in Table 1.
Minimal inhibitory concentration (MIC) determination. Minimal inhibitory concentrations (MICs) of BS producing strains were tested against varying concentrations of different antibiotics. The antibiotics and the concentration (μg/ml) used for each was as follows. Kanamycin (Km) 150, Ampicillin (Am) 150, 500 Streptomycin (Sm) 150,500, Tetracycline (Tc) 50,150, Chloramphenicol (C) 50,150, Nalidixic acid (Nd) 25,50.
Media and growth conditions. *E. coli* and *P. aeruginosa* strains were maintained on LB agar at 4°C and grown at 37°C in LB broth supplemented with appropriate antibiotics. A minimal BH medium (1) containing 1 % hexadecane and 1% glucose was used in some experiments. Transposon induced mutants were selected on selective plates supplemented with 150 μg/ml Tc and 50 μg/ml Nd.

Proceedings of International Symposium on Biotechnology for Sustainable Development, Dec. 1520, 1993. Eds. Kauser A. Malik, Anwar Nasim & Ahmad M. Khalid, NIBGE, 1995, Faisalabad, Pakistan.

TABLE 1: Bacterial Strains and Plasmid Used in this Study

STRAIN/PLASMID	CHARACTERISTICS	SOURCE
Pseudomonas Strains		
EB26	BS+, Wild Type	Our Lab
EB27	"	"
EB28	"	"
EB45	"	"
EB48	"	"
K3	"	"
S8	"	"
EBN8	BS+, Gamma Ray Mutant	"
E. coli Strains		
S17-1	Thi, Pro, recA-, hsdR-, Tp^r, Sm^r,	Imperial College (U.K.)
HB101	Thr, Leu, Pro, supE, hsdM-	"
ED8645	metB, supE, hsd+	"
Plasmids		
pSUP2021	Suicide plasmid, Km^r AP^r Cm^r,Tn5 donor, rep ColE1	"
pSX2	Suicide plasmid, Km^r, Tc^r,Tn10 donor, rep ColE1	"
pME9	rep Ts, Suicide plasmid, Km^r,Tc^r, Ap^r.	"

Transposon mutagenesis of *P. aeruginosa.* For transfer of suicide plasmid to *P. aeruginosa*, procedure described by Khan (5) was adopted. *E. coli* cells harboring plasmid pSX2 and *P. aeruginosa* K3 strain were grown overnight in LB medium containing Tc and Nd at 37°C respectively. The donor (*E. coli*) and recipient (*P. aeruginosa*) K3 strain were subcultured in LB and grown for 2-3 hours. Then 1 ml from each of the culture was centrifuged in an Eppendorf tube at 7,000 rpm for 4 min and the pellets were washed with LB medium twice. The washed pellets were resuspended in 1 ml of LB medium, mixed together in appropriate ratio and centrifuged. The pellet was resuspended again, vortexed and incubated at 37°C overnight and the conjugation mixture was plated on appropriate selective media by serial dilutions. The frequency of conjugation was expressed as per recipient in conjugation mixture.

Screening of Tn10 induced mutants. Transconjugants resulting from matings *E. coli* x *P. aeruginosa* were individually picked on to master plates of same composition as used for postmating selection. After overnight incubation master plates were replica plated on the following media. BH + 1% hexadecane (to detect their ability to use hexadecane as energy source), BH + 1% glucose (to test their ability to use glucose), selective plates Tc (150ug/ml), ND (50ug/ml), Km (200ug/ml) to confirm the absences of suicide plasmid containing Km^R gene out side Tn region and blood agar plates (to test their biosurfactant production ability) because biosurfactant producing bacteria have the ability to lyse red blood cells forming clear zones around the colonies (9).

RESULTS AND DISCUSSION

The main objective of this research work was to determine the possibility of applying random transposon mutagenesis for genetic studies of biosurfactant producing bacterial strains.

MICs of parental strains. Determination of antibiotic sensitivities of both, transposon donor and recipient

strains, is essential for selection of Tn-induced transconjugants. The MICs of different antibiotics for biosurfactant producing strains was determined by plate assay method. All these strains were found to be resistant to one or more antibiotics. The result indicated that concentration of more than 20ug/ml of Nd in selection plates was enough to eliminate the *E. coli* donor strains in selection plates.

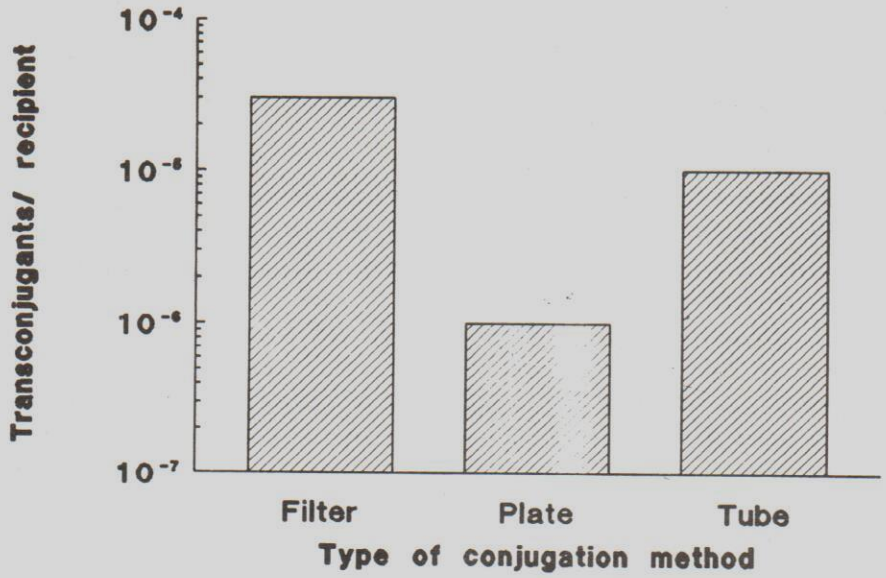

FIG 1: Comparison of transconjugation frequencies following mating *E. coli* HB101 (pSX2) with *Pseudomonas aeruginosa* strain K3 using different mating techniques.

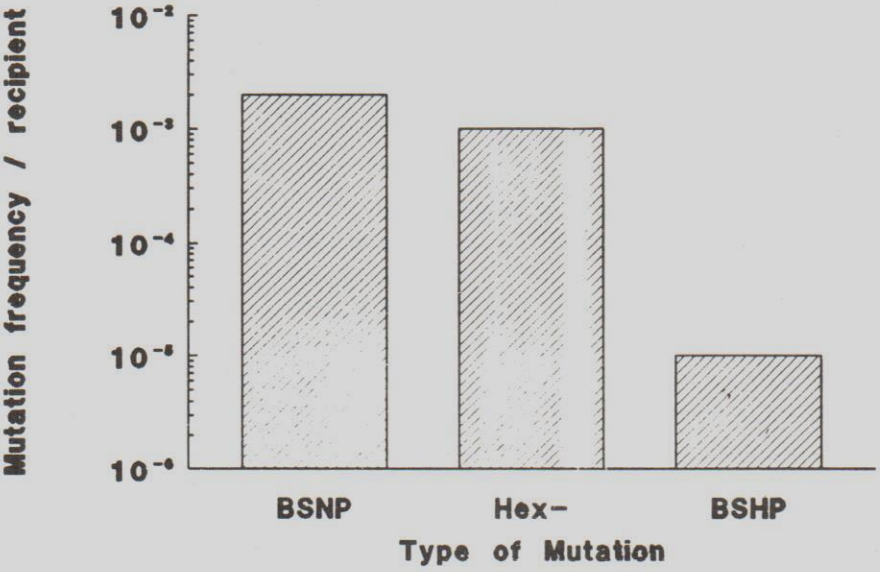

FIG 2: Mutation frequency of various Tn-mediated mutants

TABLE 2: Stability of Tn10 mediated mutants

MUTANT	CHARACTERISTICS	RATE OF REVERSION
BSNP35	BS^-, Hex^-	10^{-10}
BSNP25	BS^-, Hex^-	10^{-9}
BSNP17	BS^-, Hex^-	10^{-10}
BSHP5	BS hyper producer	10^{-10}
BSHP8	BS Hyper producer	10^{-9}

Each value is an average of 6 experiments

Optimization of conditions for Tn10 insertion. Transpositional frequencies may be influenced by various factors such as method and duration of conjugation process, temperature, donor to recipient ratio or nature of the conjugation medium used. To improve transposition frequencies, effects of such physiological factors on transposition frequencies were studied.

The optimized conditions for maximum transposition were as follows: donor to recipient ratio 1:5, duration of mating 24 hours and mating temperature 37°C. By following these conditions the highest transconjugation frequency was achieved when conjugation was performed on membrane filter (Fig 1).

Spontaneous mutation rate. While using Tn10 mutagenesis and selecting for Tc^R transconjugants it became important to know the conditions which prevent or reduce the growth of any spontaneous Tc^R mutations. Different concentrations of Tc were tested and concentration of 150 μg/ml was found optimum for use. Because the frequency of spontaneous mutants was lower than 10^{-10} per cell at this concentration.

Characterization of Tn10-mediated strains. The transconjugation frequencies achieved by using a suicide plasmid system in biosurfactant producing *P. aeruginosa* K3 strain for filter and broth matings were within the range of 10^{-4}-10^{-6} /recipient (Fig 1). This is the first report of successful use of Tn10 in any biosurfactant producing strain. The possible explanation for this high frequency could be the optimization of transposon mutagenesis conditions described earlier. Also the vector plasmid pSX2 (Km^R, Tc^R, Tra+), is a Tn10HH104 containing derivative of pRK2013 (4). This HH104 is a promoter up mutation that enhances transcription from the transposase promoter in IS10R, resulting in a 100 fold increase in the frequency of transposition of the element (10). Since Tn10 transposes at lower frequency, approximately 10^7 per element per cell generation (8), use of high-hopper derivative facilitates the collection of inserts. These findings also suggest that transposon mutagenesis based techniques could work effectively in these bacteria.

More than 5000 Tc^R transconjugants obtained after *E. coli* x *P. aeruginosa* matings were tested for various mutations. On the basis of their characteristics, the Tn-induced strains were divided into three main groups: biosurfactant non-producers (BSNP), biosurfactant hyper producers (BSHP) and hexadecane negative(HEX^-). The mutation frequency of these strains are given in Fig 2. The representative strains from each group were further characterized for their biosurfactant production by testing hemolytic activity, surface tension and interfacial tension of cell free supernatant of these cultures. On the basis of these parameters 8 strains (BSHP) showed higher BS production than wild type strain. The results of two such strains BSHP3 and BSHP5 are shown in Fig 3 and Fig 4. It was found that about 50 mutants (BSNP) were unable to use hexadecane (Hex) as energy source. Result of two such strains BSNP19 and BSNP23 are shown in Fig 3 and Fig 4. These mutants were unable to form clear zones on blood agar plates showing the loss of biosurfactant productivity. However, addition of 10µg/ml of crude BS (rhamnolipid) in minimal medium restored their ability to grow on 1% hexadecane which show that rhamnolipids play a major role in hexadecane utilization by *P. aeruginosa* K3.

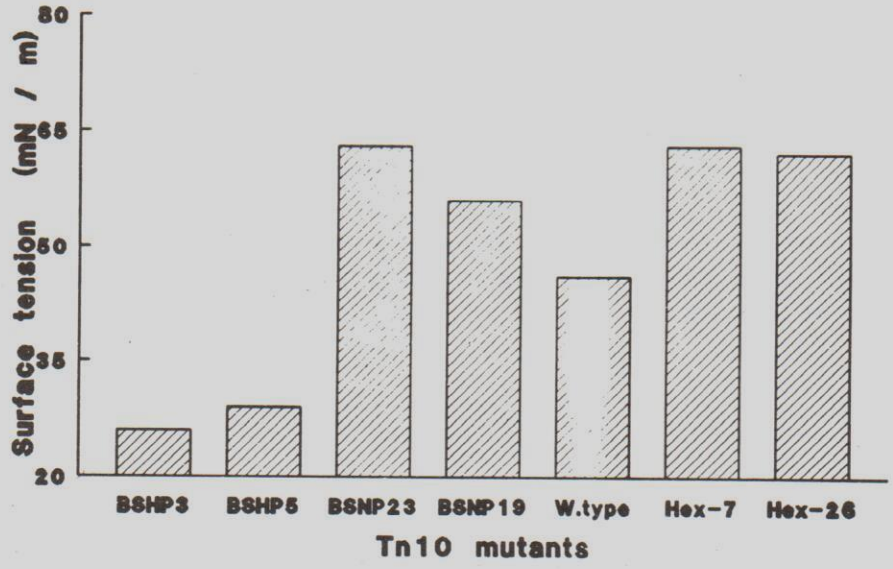

FIG 3: Comprison of biosurfactant producing abilities of Tn-induced strains on the basis of the basis of their hemolytic activity.

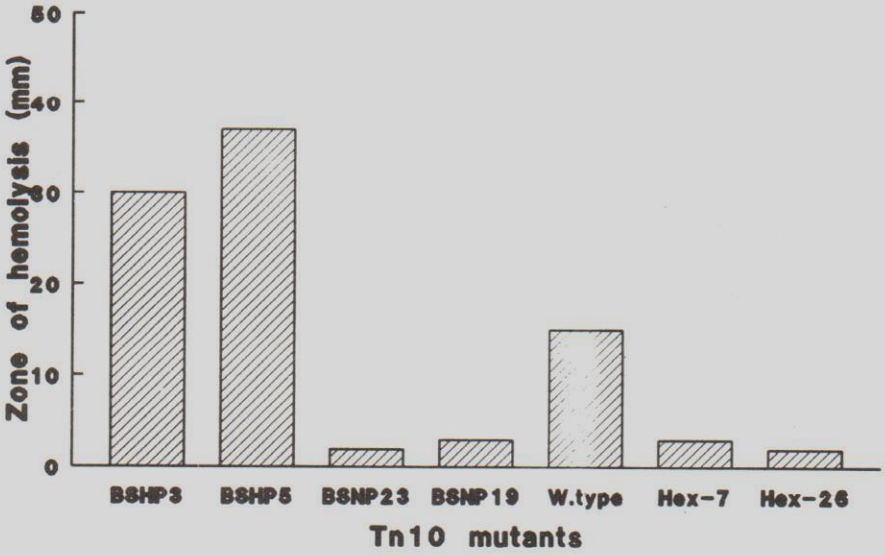

FIG 4: Comparison of biosurfactant producing abilities of Tn-induced strains on the basis of the basis of their surface tension measurements.

Stability of Tn constructed strains. To investigate the stability of Tn10 induced mutants in *P. aeruginosa*, the reversion of auxotroph to prototroph state was tested. The reversion frequency was

extremely low (10^{-10}) (Table 2) which indicates the high stability of Tn10 insertion in the mutant strains.

Elimination of Delivery Vector. Transconjugants (Tc^R) obtained after *E. coli* X *P. aeruginosa* matings were tested for Km^R to confirm if pSX2 (Tc^R, Km^R) can behave as a suicide plasmid in *P. aeruginosa*. It was found that all Tc^R transconjugants were Km^S, indicating that plasmid could not be stably maintained in these transconjugants. This instability was probably due to lack of replication, since pSX2 is derived from pRK2013 and therefore contains a ColE1 replicon (4). Plasmids which rely on ColE1 replicons are unable to replicate in non-enteric hosts (2).

The present study shows that Tn10 insertion mutation of biosurfactant producing *Pseudomonas* strains can be successfully used to isolate Tn-induced mutants affected in BS production and hexadecane utilization. These studies would greatly facilitate investigations on the genes involved in biosurfactant production which will subsequently help in better understanding of the biosynthetic pathway to exploit commercial potential of these bacteria.

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