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Plasmid-Mediated Biodegradation of the Anionic Surfactant Sodium Dodecyl Sulphate, by *Pseudomonas aeruginosa* S7

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Abstract Sodium dodecyl sulphate (SDS), an anionic surfactant, has been used extensively due to its low cost and excellent foaming properties. Fifteen different bacterial isolates capable of degrading SDS were isolated from detergent contaminated soil by enrichment culture technique and the degradation efficiency was assessed by Methylene Blue Active Substances (MBAS) assay. The most efficient SDS degrading isolate was selected and identified as *Pseudomonas aeruginosa* S7. The selected isolate was found to harbor a single 6-kb plasmid. Acridine orange, ethidium bromide, SDS and elevated temperatures of incubation failed to cure the plasmid. The cured derivatives of SDS degrading *Pseudomonas aeruginosa* were obtained only when ethidium bromide and elevated temperature (40°C) were used together. Transformation of *E. coli* DH5α with plasmid isolated from S7 resulted in subsequent growth of the transformants on minimal salt media with SDS (0.1%) as the sole source of carbon. The SDS degradation ability of S7 and the transformant was found to be similar as assessed by Methylene Blue Active Substance Assay. The antibiotic resistance profiles of S7, competent DH5α and transformant were analyzed and it was noted that the transfer of antibiotic resistance correlated with the transfer of plasmid as well as SDS degrading property.

Keywords Sodium dodecyl sulphate · *Pseudomonas aeruginosa* · Curing · Transformation

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Man's inborn craving for improved standards of living, has resulted in the indiscriminate usage and disposal of various chemicals, which ultimately find their way into the ecosystem. Detergents constitute one among these pollutant chemicals, equally contributed by small households and large industrial establishments. Due to extensive use as cleaning agents as well as their use in pharmaceuticals, laboratories, cosmetics, foods, paints, in agriculture etc., the degree of detergent pollution is increasing at an alarming rate (Swisher 1987; Marchesi et al. 1994). After use large quantities of surfactants and their derivatives are released to the aquatic or terrestrial environment. Synthetic detergents are reported to be acutely toxic to fish in concentrations between 0.4 and 40 mg/L (Abel 1976).

Sodium dodecyl sulphate (SDS) (Fig. 1), an anionic surfactant, has been used extensively due to its low cost and excellent foaming properties. The molecule has a tail of 12 carbon atoms, attached to a sulphate group giving it amphiphilic properties required of a detergent. The extensive disposal and toxicity of SDS necessitates its effective remediation. Biodegradation of surfactants is most often performed by soil or aquatic microorganisms and leads to generation of water and carbon dioxide gas (Schleheck et al. 2000). Several species of *Pseudomonas* are known to biodegrade SDS. The role of Alkylsulphatases, the primary SDS degrading enzymes which hydrolyse SDS to sulphate and 1-dodecanol is reviewed (Gadler and Faber 2007). Sometimes biodegradative enzymes are encoded on the chromosome but often they are plasmid encoded. Many of these plasmids are transmissible among *Pseudomonas* species and compatible with one another (Chakrabarty 1976). Plasmid encoded pathway for salicylate degradation in *Pseudomonas putida* R1 was identified (Chakrabarty 1972).

The present investigation was undertaken to determine the genetic basis of SDS biodegradation. An understanding

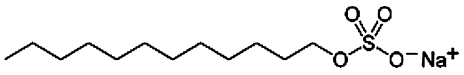

Fig. 1 Structure of sodium dodecyl sulfate

of the genetic basis of such activity could then provide a basis for predicting environmental fate of detergents. When working with some plasmid containing bacteria, it is often desirable to obtain a plasmid-cured derivative. This allows a direct comparison to be made between the plasmid containing and plasmid cured cells.

Materials and Methods

Fifteen bacterial isolates capable of utilizing Sodium Dodecyl Sulphate (SDS) as sole source of carbon were isolated from surfactant contaminated soil (Meenachil river basin, Kottayam, Kerala) by enrichment in mineral salt medium (MSM).It contained SDS, Na_2HPO_4 (1.6 g/L), KH_2PO_4 (1.0 g/L), NH_4Cl (0.5 g/L), K_2SO_4 (0.06 g/L), $CaCl_2$ (0.025 g/L), trace element solution (2.0 mL/L). The trace element solution contained $FeSO_4{\cdot}7H_2O$ (0.1 g/L), $MnCl_4{\cdot}4H_2O$ (0.1 g/L) and ZnSO4·$7H_2O$ (0.1 g/L). Incubation was carried out at room temperature (30 ± 2°C) for 24 h with shaking at 150 rpm. The most efficient SDS degrading isolate was selected and identified as *Pseudomonas aeruginosa* according to Bergey's Manual of Systematic Bacteriology and further confirmed by the MTCC, Chandigarh.

The SDS degradation efficiency of a microorganism, growing in a media containing SDS as the sole carbon source, can be assessed by finding out the level of SDS left over in the media. The concentrations of anionic surfactant (SDS) were determined by a modified version of the Methylene blue active substance (MBAS) Assay (Hayashi 1975). The method is based on the formation of a complex between the anionic surfactant and an excess of the cationic dye Methylene blue, followed by extraction of the complex (but not excess dye) into chloroform and measurement of the absorbance of the blue chloroform layer at 655 nm. Alioquots of methylene blue solution (0.1 mL), 0.4 mL of 0.825 Mm phosphate buffer (pH 7.2), and an appropriate volume of sample (up to 1 mL, in triplicate) were mixed in acid-washed, optically matched glass tubes (12 cm by 1 cm (internal diameter) and vortexed intermittently five times for 3 s each time. Chloroform was added to each tube and the contents were vigorously vortexed for 5 s. The tubes were allowed to stand at 4°C for 5 min, followed by centrifugation at 2,000 rpm for 4 min. The tubes were allowed to warm to room temperature, and the absorbance of the chloroform layer was measured at 655 nm using a UV-spectrophotometer, against an appropriate blank.

The small scale alkaline lysis method (Maniatis et al. 1982) was used for the isolation of plasmid DNA from 1.5 mL culture broths of treated as well as untreated cells of the SDS degrading organism. To monitor spontaneous plasmid loss cells were grown in nutrient broth for approximately 50 cell divisions with frequent media replacement. Cultures were then diluted and spread on nutrient agar plates. 520 individual colonies were picked and tested for growth on Sodium dodecyl sulphate.

The curing of plasmid (Trevors 1986) was carried out using ethidium bromide, acridine orange, SDS (10%) as well as elevated temperature. The concentration range of mutagens was 50–600 μg mL^{-1} with increments of 50 for ethidium bromide and 100 μg mL^{-1} to 800 μg mL^{-1} with increment of 100 for acridine orange. For curing by heat treatment, cultures were grown in LB at the desired temperature, i.e; 38, 40 or 42°C overnight, reinoculated in fresh LB medium and reincubated at the corresponding temperature until late log phase. Diluted samples of the culture displaying observable turbidity were plated on LB agar. SDS negative colonies were selected by preparing patch plates of the cultures on two medias viz, SBS medium and modified SBS media (SBS- SDS + DEXTROSE) containing dextrose as sole carbon source instead of SDS. The protocol for plasmid curing using the combination of acridine orange or ethidium bromide and elevated temperature was as follows; to 100 mL LB broth containing 700 μg mL^{-1} acridine orange or 500 μg mL^{-1} ethidium bromide, overnight grown culture of organism was inoculated. This was incubated on a rotary shaker (120 rev min^{-1}) at 40°C for 48 h. Cell broth was then suitably diluted and spread on nutrient agar plates and incubated at 30°C for 24 h. Isolated colonies were patch plated onto SBS agar and modified SBS agar plates and incubated for 24 h at 30°C. Colonies missing from SBS agar plates were picked up from the master modified SBS agar plates and checked further for disappearance of plasmid DNA. The percentage curing efficiency was expressed as number of colonies with cured phenotype per 100 colonies tested.

Escherichia coli competent cells were prepared (Maniatis et al. 1982). Competent *E. coli* DH5α cells were then transformed with plasmid DNA isolated from the bacterial culture by the heat shock method.Antibiotic resistance profile was determined by the disc diffusion method. The discs for the antibiotics amikacin, gentamicin, erythromycin, tetracycline and chloramphenicol (Himedia Laboratories, Mumbai) were used. The plasmid DNA profiles were separated on an agarose gel (0.8%w/v) prepared in TAE buffer. The electrophoresis was carried out at 75 V for 1 h to study plasmid DNA profiles of cured variants and transformants.

The data represent the arithmetical averages of at least three replicates. One-way analysis of variance with post-test

($p < 0.05$) was performed with the experimental results to test the significance of the mean values.

Results and Discussion

An understanding of the genetic basis of bioremediation activity could provide a basis for predicting the environmental fate of compounds like SDS. The ability to transfer a function to recipient cells are generally accepted properties that provide genetic evidence for the presence of a plasmid involved in conferring a particular function to a cell (Wiliams 1978).The presence of a plasmid was demonstrated by gentle cell lysis followed by agarose gel electrophoresis. Loss of the ability of cells to utilize SDS and gaining of this ability via plasmid transformation was associated with the disappearance and appearance of plasmid bands of the same size as those from the original isolate (Kostal et al. 1998).These observations are in agreement with the conclusion that *Pseudomonas aeruginosa* S7 harbours single 6-kb plasmid that encodes genes for the SDS degradation.

The potential spontaneous loss of plasmid was tested by screening individual colonies from cultures grown for approximately 50 generations in nutrient media with no selection. Screening was for the loss of the ability to grow on SDS. No colony was found that had spontaneously lost either of these degradative abilities. The plasmid was found to be refractory to the effect of Acridine orange, ethidium bromide and SDS and no cured derivatives were obtained. Growth at elevated temperatures up to 42°C also gave the same results. However, the curing effect of ethidium bromide was found to be enhanced at high temperature. The cured derivatives of SDS degrading *Pseudomonas aeruginosa* were obtained at a frequency of 8.1% by incubation with ethidium bromide (500 μg/mL) at 40°C (Table 1). Deshpande et al. 2001 also obtained cured variants of dimethoate degrading *Pseudomonas aeruginosa* MCMB-427 using a combination of ethidium bromide (500 μg/mL) and incubation temperature of 40°C. Similarly curing of plasmid in *Pseudomonas mendocina* involved in biodegradation of monocrotophos (a pesticide) using ethidium bromide and elevated incubation temperature (40°C) was reported (Bhadbhade et al. 2002), while only the phenotype cured but the plasmid was not cured when acridine orange (100 μg/mL) and elevated temperature were used together (Kulkarni and Kanekar 1998). Curing of the cells indicated that SDS degradation was plasmid borne.

The plasmid DNA preparation from *Pseudomonas aeruginosa* was used to transform $CaCl_2$ treated competent cells of *Escherichia coli* DH5α to investigate the role of plasmid in SDS degradation. Transformation of *E. coli* DH5α resulted in subsequent growth of the transformants on SBS agar plate with SDS (0.1%) as the sole source of carbon. The untransformed *E. coli* DH5α cells however, did not grow on SBS agar plate. The transformant harboured a plasmid that gave a band at 6 kb position on agarose gel (Fig. 2). Transformation of *E. coli* DH5α strain by plasmid DNA of *P. aeruginosa* S7 and its expression in a distant host conclusively indicated host-chromosome independent SDS metabolism. The analysis of the antibiotic resistance profiles of S7, competent DH5α and

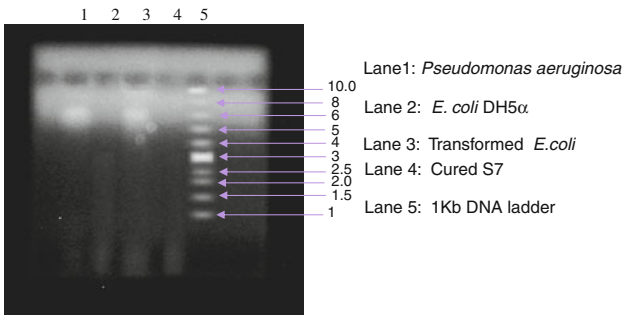

Fig. 2 Agarose gel electrophoresis of plasmid DNA isolated from *Pseudomonas aeruginosa* S7, *E. coli* DH5α, transformed DH5α and cured S7. *Lane 1 Pseudomonas aeruginosa, Lane 2 E. coli* DH5α, *Lane 3* Transformed *E. coli*, *Lane 4* Cured S7, *Lane 5* 1Kb DNA ladder

Table 1 Curing of *Pseudomonas aeruginosa* Plasmid by different curing agents

Curing agent	Sub inhibitory concentration	Total no: of colonies examined	Colonies with lost phenotype	Frequency of curing (%)
Acridine orange	700 μg/mL	141	0	0
Ethidium bromide	500 μg/mL	111	0	0
SDS	0.1 g/Ml	72	0	0
Elevated temperature	38°C	62	0	0
	40°C	84		
	42°C	77		
Elevated temperature and acridine orange	40°C + 700 μg/mL	102	0	0
Elevated temperature and ethidium bromide.	40°C + 500 μg/mL	148	12	8.1%

Table 2 Antibiotic resistance pattern of S7, DH5α and transformed DH5α

Antibiotics used	*Pseudomonas aeruginosa*		*E. coli* DH5α		Transformed *E. coli* DH5α	
	Zone of inhibition	Result	Zone of inhibition	Result	Zone of inhibition	Result
Amikacin (30 mcg)	26 mm	S	28 mm	S	27 mm	S
Chloramphenicol (10 mcg)	22 mm	S	25 mm	S	25 mm	S
Erythromycin (15 mcg)	4 mm	R	23 mm	S	4 mm	R
Gentamicin (30 mcg)	28 mm	S	25 mm	S	28 mm	S
Tetracycline (30 mcg)	6 mm	R	17 mm	MS	6 mm	R

S sensitive, *R* resistant, *MS* moderately sensitive

Table 3 Comparison of degradation of SDS by MBAS assay

Culture	Percentage of anionic surfactant left over*
Pseudomonas aeruginosa S7	29.73 ± .53
Cured strain	94.32 ± .28
Transformant	27.67 ± .37
E. coli DH5α	98.72 ± .17

* Values represent means ± SD for triplicate cultures

transformant revealed that the transfer of antibiotic resistance correlated with the transfer of plasmid as well as SDS degrading property (Table 2). *E. coli* transformant carried out significant degradation of SDS comparable to that by *Pseudomonas aeruginosa* S7 (Table 3). Non-transformed *E. coli* DH5α and the cured strain did not show any notable degradation of SDS. These results clearly proved that the genes encoding SDS degradation were located on the plasmid. The information generated by detailed studies on SDS catabolic plasmid will form an excellent source for genetic manipulations leading to construction of genetically modified microbial strains (GMS) or genetically engineered microorganisms (GEM). These efficient strains can be used to augment the potential of the resident populations at the contaminated site. The use of a bioreactor appears to be a better approach as there are very strict rules governing release of modified microorganisms to the environment (Stephenson and Warnes 1996).

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