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A proteomic approach for exploring biofilm in *Streptococcus mutans*

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Abstract:
Biofilm formation by *Streptococcus mutans* is considered as its principal virulence factor, causing dental caries. Mutants of *S. mutans* defective in biofilm formation were generated and analyzed to study the collective role of proteins in its formation. Mutants were characterized on the basis of adherence to saliva-coated surface, and biofilm formation. The confocal laser microscopy and scanning electron microscopy images showed that the control biofilms had cluster of cells covered by layer of exo-polysaccharide while the biofilms of mutants were thin and spaced. Two-dimensional protein electrophoresis data analysis identified 57 proteins that are either up (44 proteins) or down (13 proteins) regulated. These data points to the importance of up and down regulated proteins in the formation of biofilm in *Streptococcus mutans*.

Background:
Streptococcus mutans is now regarded as the principal microbiological aetiological agent of dental caries and a target of novel preventive strategies. Beyond initial adherence, it appears that a variety of genes are required for the proper maturation of biofilms formed by *S. mutans* and other oral streptococci. By the use of specific- and random-mutagenesis strategies, many different types of genes that are required for these organisms to transition from adherent microcolonies to complex, three-dimensional biofilms have been identified. These include those for intercellular communication systems and environmental sensing systems, components of the general stress response pathway involved in protein repair and turnover, global regulators of carbohydrate metabolism, and adhesion-promoting genes **[1]**.

Interestingly, many of the genes identified to affect biofilm formation affect the expression of a large panel of genes, many of which are either unidentified or have no known function **[2]**. There are a large number of hypothetical proteins of as-yet-unknown function present in the annotated genome of *S. mutans* (http://www.stdgen.lanl.gov/oragen). Among these proteins, there could be other unknown surface proteins that play an important role in adhesion and maturation of biofilms. Techniques like 2D protein analysis and microarray are now being used to screen out the expression of all genes involved in the formation of a biofilm **[3, 4]**. An integrated approach to study protein-function relationship was also employed to study the variation in *S. mutans* genome **[5]**.

Chemical mutagenesis induces change in morphological and characteristic traits of *S. mutans*, had been demonstrated earlier by Murchison *et al.* **[6, 7]**. However, a deeper insight into the effect of EMS on the adherence and biofilm formation by *S. mutans* was lacking. The biofilm formation by *S. mutans* on saliva-coated surface is believed to be a multi-step process **[8]**. The initial adherence of *S. mutans* on the saliva-coated surface is mainly due to the interaction of its cell-surface adhesin with the salivary agglutinin **[9]**. The later stages of biofilm formation are marked by active formation of exopolysaccharides that mediate the clumping and effective biofilm formation of *S. mutans* **[10]**.

The transition from planktonic phase to biofilm phase of *S. mutans* is a resultant of many differential and altered genes **[2]**. To explore this, we isolated the EMS-induced adherence defective mutants of *S. mutans* and characterized them on their defects in biofilm formation. The protein profile of the mutants that show defective initial adherence and biofilm formation was then evaluated.

Methodology:
Bacterial strains:
The strain of *Streptococcus mutans* UA159 (MTCC#497), was purchased from Microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh

Generation and isolation of mutants:
Mutants from UA159 strain of *S. mutans* were generated by the method of Murchison *et al.* **[7]**. PCR amplification for was performed by the method of Yano *et al*. **[11]** to confirm of *S. mutans*

Glass-dependent adherence and initial adherence of *S. mutans*:
Glass surface adherence assay was performed by the method of Hamada *et al.* with slight modifications **[12]**. For checking the adherence of *S. mutans* on saliva-coated surface, human saliva from four healthy individuals was collected and clarified by the method of Shellis *et al.* **[13]**. Assessment of the adherence of bacteria to saliva coated surface was done on 96 well flat-bottomed microtitre plate, using the protocol of Jakubovics *et al.* with slight modifications **[14]**.

Confocal microscopy:
The biofilms were developed on saliva-coated glass cover slips for comparison between parent strain and mutants. The 9-well microtitre plate with seeded saliva-coated cover slips, containing 10 ml of TSB with 0.25% sucrose was inoculated with 100 µl of mid-exponential grown cultures of parent strain and mutants BSM3, BSM5 and BSM61 respectively. The

cover slips were removed and noninvasive confocal analysis was performed as described elsewhere **[15]**.

Scanning electron microscopy:
The cells were fixed with 25% SEM grade glutaraldehyde in PBS, pH 7.2 for 15 minutes. Fixed samples were then dehydrated through a graded series of ethanol concentrations, mounted, and sputter coated with gold-palladium. Samples were analyzed by SEM (Hitachi S-3000 N; High Technology Operation, Japan) at several magnifications (×500 to ×13,000) at the EM core laboratory of JALMA, Agra.

Isolation of total cellular proteins of *Streptococcus mutans*:
Cells of *S. mutans* UA159 and mutants BSM3, BSM5 and BSM61 were grown at 37ºC in an anerobic environment up to an OD 600nm of 1.5. The washed cell pellets were resuspended in lysis buffer containing 5 M Urea, 4% (w/v) CHAPS, 50 mM DTT and 0.5% (v/v) IPG buffer (pH 4-7) (GE healthcare, NJ, USA), and left on ice for 10 minutes with intermittent sonication. The cell lysate was centrifuged at 12,000 g for 10 minutes at 4ºC. The supernatant was collected and protein in it was measured by the method of Lowry *et al.* **[16]**.

Two-dimensional protein electrophoresis:
The total protein isolated by the above described method was analyzed by 2D electrophoresis using the method of O'Farrell **[17]**. Gels were then silver stained, digitally scanned and analyzed using automated software Decodon Delta 2D version 3.6. The gel pictures of parent strain and mutants BSM3, BSM5 and BSM61 were loaded on the Delta 2D platform and warped for the 2D image analysis. The spot detection was done using the software and a synthetic fusion image was constructed of all the four images. The spots were transferred from the fusion image to the individual images and were then quantified **[18]**.

Statistical analysis:
All the values were calculated as the mean of individual experiments in triplicate, compared with control groups. Comparison between the thickness of control and mutant biofilms was done using SPSS 11.0.0 statistical software using one-way ANOVA and posthoc tests (LSD); statistically significant tests were set at a P-value of <0.05. Absorbance from adherence assays was evaluated for statistically significant differences using Student's unpaired two-sided *t* test (assuming unknown and unequal variances).

Discussion:
The EMS-induced mutagenesis had earlier been done by Murchison *et al.* **[6, 7]**, for the isolation of adherence defective mutants of *S. mutans*. A similar methodology was followed in this study and mutagenized cells were subcultured four times for isolation of stable mutants, containing chains of genotypically similar cells **[6]**. In our experiments, the survival frequency after mutagenesis was 14% whereas Murchinson *et al*. **[6]** reported it to be 10%. Nearly 70% of the picked mutagenized cells showed some amount of decrease in adherence to the glass surface. The *S. mutans* cells showing nearly or more than 50% decrease in adherence to the glass surface were selected for further characterization.

The salivary proteins act as a receptor of *S. mutans* as they carry the anchor sites for the wall proteins of *S. mutans* **[19]**. The initial adherence of selected *S. mutans* mutant cells on the saliva-coated hydrophobic surface, specifically polystyrene microtitre plates was examined for further characterization of the mutants **(Table 2 see Supplementary material)**. 40% of the isolated mutants showed more than 50% decrease in initial adherence to saliva-coated polystyrene surface. The decrease in initial adherence of these mutants implies a possible mutation in bacterial proteins that interact with the saliva.

The interaction of *S. mutans* cells with saliva plays a key role in the biofilm formation on the dental plaque **[20]**. The biofilm formation by *S. mutans* on the saliva-coated surface was reduced in all the mutants **(Table 3 see Supplementary material)**. Also, the biofilm formation was found to be least in the mutants showing the least initial adherence (BSM3) and maximum in the mutants showing maximum initial adherence (BSM61). This implies that the biofilm formation is dependent on the initial adherence of the mutants.

The biofilm formation by *S. mutans* is a phase dependent process. The time-dependent assay **(Table 4 see Supplementary material)** reveals that the sucrose-independent adherence defective mutants show less biofilm formation (61% of the control) even in the initial phase. The initial phase is marked by initial adherence of the bacteria to the substratum, here saliva-coated polystyrene plate (8). However, the sucrose-dependent adherence defective mutants show just 1.5% inhibition at initial phase while showing a distinct inhibition at the multiplication (40%) and maturation (42%) phase of biofilm formation. The later stages of biofilm are marked by the synthesis of exopolysaccharide (glucan). The time-dependent analysis of biofilm formation thus implies that sucrose-independent mutants are altered at the stage of saliva-bacteria interaction. However, sucrose-dependent mutants showed an alteration in glucan mediated adherence of the cells for the biofilm formation.

The thickness and the architecture of the biofilm of some sucrose-independent mutants were studied by confocal microscopy. Quantitative estimations show that amongst the mutants BSM3 showed the least biofilm formation while BSM61 showed the maximum biofilm formation **(Table 5 see Supplementary material)**. BSM5 also showed stable and relatively more adherence defective character; hence we selected these three sucrose-independent adherence defective mutants for Confocal Microscopic analysis. The micrographs suggest that the biofilm formed by the parent strain covered a larger surface area and had a definite architecture **(Figure 1)**. However, the biofilms formed by the mutants showed more or less spread cells with no distinct pattern of arrangement. The biofilm formed by BSM61 showed more clumped and aggregated cells than mutants BSM3 and BSM5. The thickness of the biofilm was also found to be more for BSM61 than mutants BSM3 and BSM5, possibly due to more clumping and aggregation of the bacteria. Thus the confocal microscopic revelations go well in agreement with the quantitative estimations of biofilms.

The scanning electron microscopy also showed that the biofilms formed by the parent strain have aggregated and clumped bacteria with a cloud of exopolysaccharide surrounding the cells. The apparent biofilm formed by mutant BSM3 has very few cells individually scattered along the surface. The cells were arranged in short chains with absence of exopolysaccharide matrix. The biofilm formed by the mutant BSM61 was very much patchy. The aggregates of cell clumps were separated by large voids, indicating that original biofilm forming ability as of the parent strain was disrupted to some extent by mutation **(Figure 2)**.

The two-dimensional protein electrophoresis of total protein from parent strain and mutants BSM3, BSM5 and BSM61 was carried out to compare the expression of total proteins. The protein profile of parent strain was called as group1 while the protein profile of mutants BSM3, BSM5 and BSM61 was collectively called as group 2. The ratio of group 2 to group 1 was used to identify the differentially expressed proteins between the parent strain and the mutants. The proteins were identified on the basis of their MW and pI data obtained from www.stdgen.lanl.gov/oralgen/bacteria/smut/. (**Table 1 see Supplementary material)**.
shows the identification of proteins that are at least five-fold over- or under-expressed in the parent strain and the mutants. The number of these proteins was 57 whereas the total identified spots were 401. Of these 57 proteins, 13 are expressed more in parent strain than in the mutants. 10 amongst 13 of these proteins could not be identified. Rex A which modulates transcription in response to changes in cellular NADH/NAD(+) redox state is expressed 5.5 folds more in parent strain while amongst group 2, it is more expressed in BSM 61 and least in BSM 3. Two other under expressed proteins in group 2 are ribonucleotide reductase and a conserved hypothetical protein **(Table 1 see Supplementary material)**.

Table 1 shows that 44 proteins are expressed more in group 2 than in group1. Apart from the 3 unidentified proteins, most of these proteins are associated with basic metabolism that takes place in the cell. This can be

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accounted as the planktonic cells have more active metabolism than the biofilm cells. Heat shock protein Dna K (spot id 26) was expressed 6.65 fold more in the mutants than in the parent strain. An up regulation of Dna K by 5.6 folds after radiation had also been reported earlier **[21]**. Rec A shows a 9.74 folds up regulation in the mutants while it shows 2 fold up regulation after radiation **[21]**. It might be due to the exposure of cells to chemical mutagen results in constitutive over expression of heat shock protein and Rec A. These proteins are also over expressed in biofilm phase, salt and oxidation stress **[22]**. 11 proteins are identified to show quite low expression in the parent strain (mean percent volume nearly 0). Cap A, a membrane associated protein, possibly a capsule biosynthesis protein shows a high expression in mutants but insignificant expression in parent strain.

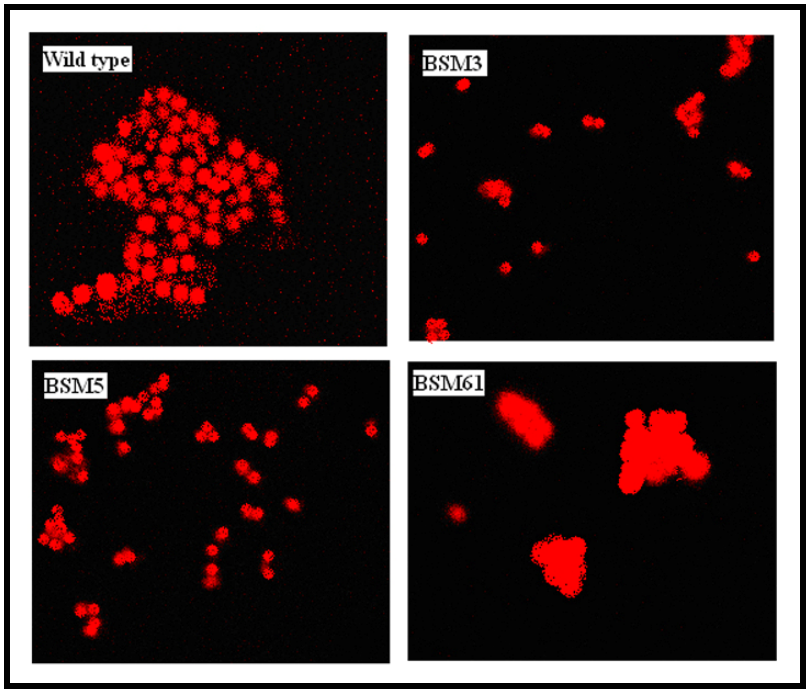

Figure 1: Confocal Laser Scanning microscopy of the parent strain and the sucrose independent mutants; top left panel is control (wild type), top right panel is BSM3 (mutant), bottom left panel is BSM5 (mutant), bottom right panel is BSM61 (mutant). All mutant are showing scattered pattern of cells as compared with wild type.

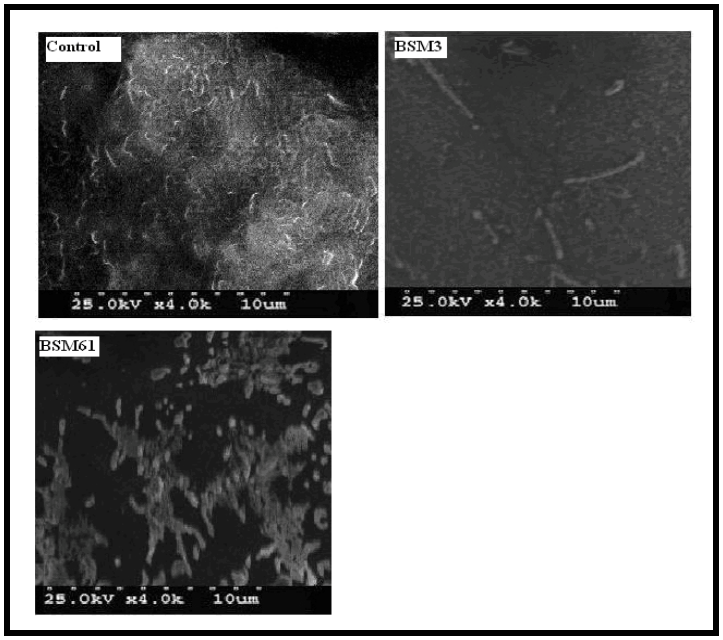

Figure 2: Scanning electron micrographs (4000X) of parent strain and sucrose independent mutants of *S. mutans*; top left panel is control cell (wild type) with strong biofilm, top right panel is biofilm mutant cells (BSM3), bottom left panel is again biofilm defective mutant (BSM61); these mutant showing reduced biofilm.

Conclusion:
We describe the concerted involvement of several proteins in the formation of Streptococcus mutans biofilm. These mutants show low ex-pression of cell surface adhesion proteins. Some of the differentially expressed proteins are basic metabolic proteins, redox proteins and heat shock proteins.

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Supplementary material:

Table 1: Identification of at least five folds differentially expressed proteins in parent strain (P) and mutants BSM3, BSM5 and BSM61 (mean of the mutants M)

Spot ID	pI	MW(Da)	Ratio(P/M)	Gene ID	Protein identified
1	5.35	80608	-9.79	Not identified	
2	4.5	80291	-9.54	Not identified	
3	5.19	81285	-8.35	Not identified	
4	5.28	81034	-7.96	Not identified	
5	5.49	81430	-7.96	SMu0610	nrdE ribonucleotide reductase, large subunit
6	5.72	80537	-7.67	Not identified	
7	4.95	80784	-6.30	Not identified	
8	4.86	80537	-6.22	Not identified	
9	4.14	77912	-5.90	Not identified	
10	5.54	10945	-5.48	Smu1499	Rex A transcriptional regulator
11	5.30	21017	-5.28	Smu1446	conserved hypothetical protein
12	4.00	77875	-5.25	Not identified	
13	4.34	1784	-5.04	Not identified	
14	4.70	41825	5.12	Smu0240	agmatine depimerase
15	4.77	3100	5.47	Not identified	
16	4.71	14667	5.65	Smu0819	conserved hypothetical protein
17	6.04	29697	5.67	Smu1377c	hypothetical protein
18	4.32	41903	5.75	Smu1538	Glucose-1-phosphate adenylyl transferase
19	6.34	41658	5.84	Smu0780	aroC chorismate synthase
20	5.47	43565	5.91	Smu0537	trpB
21	5.29	42413	6.04	Smu1505	D-3 phosphoglycerate dehydrogenase
22	5.87	15160	6.42	Smu0443	General stress protein
23	6.13	43399	6.43	Smu1311	UDP-N-acetyl glucosamine-2-epimerase
24	4.61	11087	6.50	Smu1432	hypothetical protein
25	4.85	13601	6.64	Smu0743	large conductance mechanosensitive channel
26	4.59	65280	6.65	Smu004	Chaperone protein Dna K
27	5.05	31065	7.19	Smu0422	NAD(+) 11C-component synthetase
28	4.69	41074	7.33	Smu0004	uncharacterized GTP-binding protein
29	6.04	45464	7.38	Smu0226	nifS
30	5.40	43994	8.30	Smu1128	Phosphopentomutase
31	5.50	39717	9.19	Smu0107	Alcohol dehydrogenase class III
32	4.55	59637	9.67	Smu0208	conserved hypothetical protein
33	5.10	43900	9.74	Smu0300	arginosuccinate synthase
34	5.61	42251	9.86	Not identified	
35	5.10	29734	10.64	Smu0407	Gamma glutamyl kinase
36	5.92	29978	11.06	Smu1183	glutathione -S- transferase
37	6.20	44705	14.36	Smu1429	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
38	4.82	43674	15.35	Smu1097	30S ribosomal protein S1
39	6.41	18396	17.06	Smu08852	2-amino-4-hydroxy-6-hydroymethyldihydropteridine pyrophosphokinase
40	5.21	10989	18.11	Smu0357	conserved hypothetical protein
41	6.43	45033	20.44	Smu0766	thiamine biosynthesis
42	4.82	40111	20.87	Smu0454	hypothetical protein
43	5.49	41245	25.48	Not identified	
44	6.15	40192	28.93	Smu0783	carbamoyl-phosphate synthase
45	5.30	41689	38.63	Smu0286	hippurate hydrolase-peptidase aminocyclase
46	4.71	23800	86.57	Smu0447	transaldolase family protein
47	5.72	43480	241.81	Smu1293	coproporphyrinogen III oxidase
48	5.60	41130	540.52	Smu0608	N-acetylornithine aminotransferase
49	4.49	30335	α	Smu0388	hypothetical protein
50	5.12	41389	α	Smu1892	rec A
51	6.23	44270	α	Smu0767	CapA
52	5.81	42703	α	Smu0394	N-acetylglucosamine 6-phosphate deacetylase
53	5.61	45219	α	Smu1109	Dehydrooratase
54	5.12	41665	α	Smu1945	tRNA (5-methylaminmethyl-2-thiouridylate)-methyltransferase
55	5.67	41373	α	Smu0118	aminoacid aminohydrolase-hippurate aminohydrolase
56	6.00	42325	α	Smu1398	glucogen biosynthesis protein
57	5.92	45574	α	Smu1386	UDP-N-acetylglucosamine 1-carboxyvinyltransferase

Table 2: Initial adherence of the sucrose-independent mutants

Candidate mutants	Percent initial adherence
BSM3	25.4±0.2
BSM10	32.6±0.1
BSM5	31.2±0.3
BSM29	29.8±0.3
BSM13	34.2±0.3
BSM23	39.6±0.4
BSM61	46.2±0.3
BSM20	43.3±0.2
BSM9	38.1±0.3
BSM11	41.8±0.1
BSM16	35.4±0.1
BSM33	27.8±0.2

The OD630nm of the cells was read after release of the crystal violet dye using 7% acetic acid. The OD630nm of the control regarded as 100% was subsequently used to calculate the percent initial adherence

Table 3: Percent biofilm formation for the parent strain and the sucrose-independent mutants

Candidate mutants	Percent biofilm formation			
	6 h	12 h	20 h	24 h
Parent strain	22.89	86.74	95.18	100
BSM3	10.22	25.61	29.98	32.34
BSM5	12.26	33.15	37.23	39.17
BSM9	15.23	38.51	41.8	44.21
BSM10	12.99	34.25	38.39	43.33
BSM13	13.65	35.55	40.26	42.98
BSM16	14.54	37.12	40.43	42.55
BSM11	16.72	42.12	46.67	48.92
BSM20	16.95	43.73	48.72	51.34
BSM23	15.97	40.24	44.13	46.65
BSM29	11.52	27.29	32.24	35.49
BSM33	10.98	27.65	30.88	33.16
BSM61	17.09	45.25	49.08	53.27

The maximum biofilm formation by the parent control strain at 24 h was assumed to be 100 to calculate the percent biofilm formation at different phases by the parent as well as mutant strain.

Table 4: Average percent biofilm formation by the parent strain (PS), sucrose-independent adherence defective mutants (SI) and sucrose-dependent adherence defective mutant strains (SD) at varied phases of growth

Incubation time (h)	Percent biofilm formation		
	PS	SI*	SD**
6	22.89	14.01	22.54
12	86.74	35.87	52.17
20	95.18	39.98	57.31
24	100	42.78	58.31

*represents the average result of 12 sucrose-independent mutants
**represents the average result of 18 sucrose-dependent mutants

Table 5: Thickness of the biofilm viewed by CLSM

Name of the mutant	Thickness of the biofilm (μm)
Parent strain	24.5±0.4
BSM3	12.9±0.3
BSM5	14.7±0.5
BSM61	17.5±0.6