Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2012, 2 (4):1087-1094

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Isolation, cloning and transformation studies of Glucan binding protein (GBP) from *Streptococcus mutans*

Chandrashekhar G. Unakal[1]*; V. S. Goudar[2]; A. Naregal[2]; H.V Pavan[2]; R.M. Yendigery[2]; Gizachew Yismaw[1]; Mengistu Endris[1]

[1]*Department of Microbiology, School of Biomedical and Laboratory Sciences, University of Gondar, ETHIOPIA*
[2]*Department of Microbiology and Biotechnology, Karnataka University Dharwad, INDIA*

ABSTRACT

Many species of bacteria synthesize a polymers and glucan-binding proteins. Less attention has been given to the biological roles of glucans in commensal oral bacteria. The ability of oral streptococci to exploit glucan-binding properties of extracellular and cell-surface proteins to facilitate colonization and survival in the oral environment may have ecological implications for determining the bacterial composition of dental plaque. The transformation studies of gbp gene was done using PUC 18 vector followed by the Sequence analysis. The sequence obtained by PCR (1225 bp) was analyzed by nBLAST at NCBI. The DNA was isolated from the fresh culture, found to have molecular weight more than 24 kb. The sequence analysis confirmed that, the gbp gene as of S. mutans isolate SJ-32 gbp gene. The phylogenetic tree obtained and sequence was further translated to protein to know coding sequences by ORF finder. The virtual protein was further analyzed with pBLAST which confirmed the protein as glucan binding protein of S. mutans. The amplified gene product was transformed into E. coli BL-21 strain. The ability to synthesize extracellular glucans is generally believed to be one of the virulence properties of Streptococcus mutans which contributes to plaque formation and to the subsequent development of dental caries.

Key words: *S. mutans*, *gbp*, dental plaque.

INTRODUCTION

Many species of bacteria synthesize a polymers and glucan-binding proteins. Common forms of glucan include glycogen, a storage form of glucose [1], and the beta-linked-D-glucans that modulate osmolarity within the periplasm of Gram-negative bacteria [2]. Glucan binding proteins may include the enzymes that catalyze the synthesis of the glucans, as well as enzymes capable of hydrolyzing glucans, including starch and cellulose, that can act as substrates for microbial growth [3]. For many oral streptococci, glucans comprise an extracellular slime layer produced in the presence of sucrose that promotes adhesion and the formation of a dental plaque biofilm [4]). These glucans are synthesized from sucrose by the enzymatic action of one or more glucosyltransferases (GTFs) and can be water-insoluble or soluble. Research into dental plaque development and the etiology of dental caries established the central role of glucan in sucrose dependent adhesion and the correlation between sucrose consumption and increased caries rates (5). For these reasons, many of the initial investigations into the virulence of the mutans

streptococci (MS; including 3 serotypes of *S. mutans*, 2 serotypes of *S. sobrinus*, *S. criceti*, *S. ratti*, and *S. downei*) focused on the synthesis of glucan and its effects on promoting the adhesion, aggregation, and accumulation of MS onto smooth tooth surfaces.

However, glucan-binding proteins are also found among the commensal oral streptococci. Extracellular polysaccharides are synthesized by one or more GTF enzymes in *S. salivarius*, *S. gordonii*, *S. sanguis*, *S. oralis*, and *S. cristatus* (6). Less attention has been paid to the biological roles of glucans in commensal oral bacteria. The ability of oral streptococci to exploit glucan-binding properties of extracellular and cell-surface proteins to facilitate colonization and survival in the oral environment may have ecological implications for determining the bacterial composition of dental plaque.

MATERIALS AND METHODS

All the chemicals used in this study were of analytical grade supplied from Himedia, SRL and Rankem, Mumbai, India.

Culture

The *Streptococcus mutans* (MTCC- 890) was obtained from the culture collection of BioGenics, Hubli. The culture was revived on Brain heart infusion agar plates at 37^0C for 24 h.

DNA Isolation

For the isolation of DNA, the culture of *S.mutans* was first centrifuged at 10000 rpm for 3 min. The pellet obtained was then dissolved in 500 µl of lysis buffer (100 mM tris-HCl, pH 8.2, 50 mM EDTA and 10 mM lysozyme). It was then incubated at room temperature for 10 min. 500µl of saturated phenol was then added, mixed well and incubated at 55˚C for 10 min. This was again centrifuged at 10,000 rpm for 10 min. The upper layer was carefully separated and an equal volume of chloroform-isoamyl alcohol was added. To this $1/20^{th}$ the volume of sodium acetate (3M) was added and again centrifuged at 10,000 rpm for 6 min. The upper layer was carefully separated and three volume of chilled alcohol was added.

Agarose gel electrophoresis:

Electrophoresis is technique to separate DNA molecule based on their molecular weight. 30 ml of TAE with 1% dissolved agarose was poured in the electrophoretic cassette. The comb was placed in the cassette to create the wells. It was left to solidify. Formed gel was transferred to electrophoretic unit, which contains TAE (100mM tris-HCl, pH 8.2 adjusted with glacial acetic acid, 50 mM EDTA) buffer. The samples were mixed with loading dye (TE buffer with 1% bromophenol blue and 6% glycerol) and loaded on the specific wells. Electric current was maintained at 100 volts for 10 minutes and later it was reduced to 50 V, till the sample moves up to $3/4^{th}$ of the gel. The gel was taken out from the electrophoretic unit and subjected to UV-trans-illuminator (UVP, Germany) to visualize the bands.

Purification of DNA

After visualization of bands on agraose gel, the DNA was subjected to purification. The RNA was removed by treating the sample with RNase-H. This was done at 37^0C for 1 h followed by the incubation at 60^0C for 10 min, for the denaturation of RNase. The treated sample was run on agarose gel again to confirm the removal of RNA.

Primer designing

The gene sequences of *gbp* gene were selected for the primer designing. Those sequences were pasted on note pad in the FASTA format. This FASTA format contained ">", name of organism/microorganism or enzymes followed by gene sequence. These sequences were uploaded into the Clustal-X, which is helpful in the multiple sequence alignment and the identification of conserved sequences. After the completion of multiple sequence alignment the conserved sequences were identified by identifying the **"*"** symbol on the top of the sequences. The conserved sequences were selected for the designing of the primers. These conserved sequences were then submitted to the online software Primer3. The parameters were set like, the amplified product length of 100-1500 bp, primer GC more than 45%, Tm difference of not more than 0.5^0C. The software selected many primers and the one pair, more suitable for the amplification of the present gene was selected. These designed primers were synthesized at Sigma-Aldrich, Bangalore. The primers' sequences were gbp 1F 5'ctggcttttctcttctttca, gbp1R 5'- gcaattg tctcctgattagc, gbp 2F 5'- acaggcagcagcaaataataa and gbp 2R 5'- ttgtttgaa gataaagcaaacaat

Polymerase Chain Reaction (PCR)

The DNA isolated from the above procedure was then used for the amplification of the DNA through PCR. To amplify DNA through PCR, the PCR mix is to be prepared. It was prepared by adding 1µl of Taq assay buffer, 4 µl of the template, 0.2 µl of each of the primers, 0.4µl of the dNTP mix, 0.3 µl of Taq polymerase and 4.3µl of HPLC grade water. The PCR mix was then used for amplifying the DNA. The denaturation was done at a temperature of 94˚C for 1 min. The annealing of the primers to the DNA took place at 50.8˚C. The time set for annealing is 1 min. The chain elongation step was done at 72˚ C for 1 min. Thus, each cycle takes 3 min and an overall of 35 cycles were carried to obtain the amplified DNA. The amplified DNA thus obtained was then analysed by Agarose gel electrophoresis. The amplified DNA is mixed with an equal volume of bromo phenol blue stain and mixed well. 20µl of this is then loaded on wells carefully. It was then observed for band development.

Transformation studies of *gbp* gene

Restriction Digestion

The PUC 18 vector was used for the experiment (Fig. 1). The vector was linearized using Hind III enzyme. The reaction mixture contained 1ml of assay buffer (10X), 0.3µl of PUC-18, 0.032 µl of HindIII, 7.668 µl HPLC water. The tube was incubated in water bath at 37^0C for 1 hr.

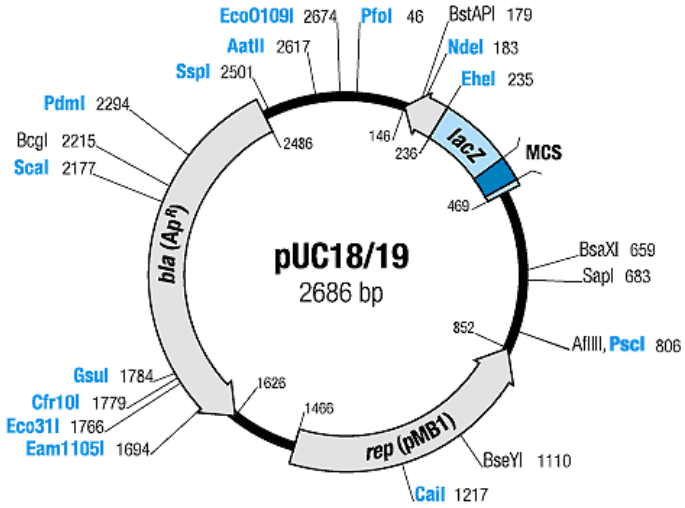

Fig. 1 Vector pUC 18

Ligation

The digested vector was used to insert the amplified product in multiple cloning sites. The reaction mixture contained 7µl of digested product, 2µl of ligation assay buffer, 0.132 µl of T4 DNA ligase and 20µl of amplified product this mixture was incubated at 16^0C overnight.

Preparation of Competent cells

1ml of overnight culture was inoculated into 100 ml of LB medium and incubated at 37˚C for 2-3 hours. The culture flask was then chilled on ice for 20 min. The culture was then aseptically centrifuged at 6000 rpm for 8 min, at room temperature. The supernatant was then discarded and to the pellet 15µl of ice cold 0.1 M $CaCl_2$ aseptically. The pellet was suspended gently into the solution by keeping in ice. The tubes were then placed on ice for 30 min, and centrifuged at 6000 rpm for 8 min at room temperature. The supernatant was then discarded and re-suspended in 200µl of cold 0.1 M $CaCl_2$ solution. Aseptically 100µl of competent cells were transferred into pre-chilled vial without removing the centrifuged tubes from ice. The competent cells prepared in this way were then used for transformation studies.

Transformation
To the 200 µl of competent cells 15 µl of plasmid DNA was added and incubated on ice for 20 min. The cells were given a heat shock by placing the vials in 42˚C water bath for 2 min. The vials were then placed on ice to chill for 5 min. 1ml of Luria-bertani broth was added aseptically to the vials and incubated at 37˚C for 1 hour. (This was to allow bacteria to recover and express the antibiotic resistance.). Then to the three labeled LB-Amp plates with X-Gal (50 µl, 2% in DMF) and IPTG (10µl, 100mM) as a, b, and c. to each of these plates 100µl of LB broth was pipetted and then added with 25, 50 and 100µl of transformed cells to plates a, b, and c respectively and spread thoroughly using a spreader. 100µl of competent cells that has not been transformed was also plated and used as control plate. The plates were incubated overnight at 37˚C. The plates were then observed for blue (non- recombinants) and white (recombinants) colonies.

RESULTS

The research on antigenic proteins has intensified. The present study was an approach to study such a protein (gbp) which causes dental plaque.

Source:
The culture of *S. mutans* was used for the present study.

Isolation of DNA:
The DNA was isolated from the fresh culture. After adding the absolute alcohol, the fibrous DNA could be seen. The DNA was obtained by centrifuging and dissolving in TE. The DNA was later run on 1% agarose gel and visualized under UV. The bands of DNA and RNA were seen. The DNA was found to have molecular weight more than 24 kb. The thick, fluffy bands of RNA were seen towards the end of the gel.

Purification of DNA
Removal of RNA:
After visualization of bands on agarose gel, the DNA was subjected to purification. The RNA was removed by treating the sample with RNase-A. The RNase treated DNA was run on the gel and observed under UV to confirm the absence of fluffy band towards the end of the gel.

Primer Designing
The primer was designed by multiple alignments of the sequence and using Primer3 online software. The alignment and primer results are presented in following page along with phylogenetic tree (Fig. 2).

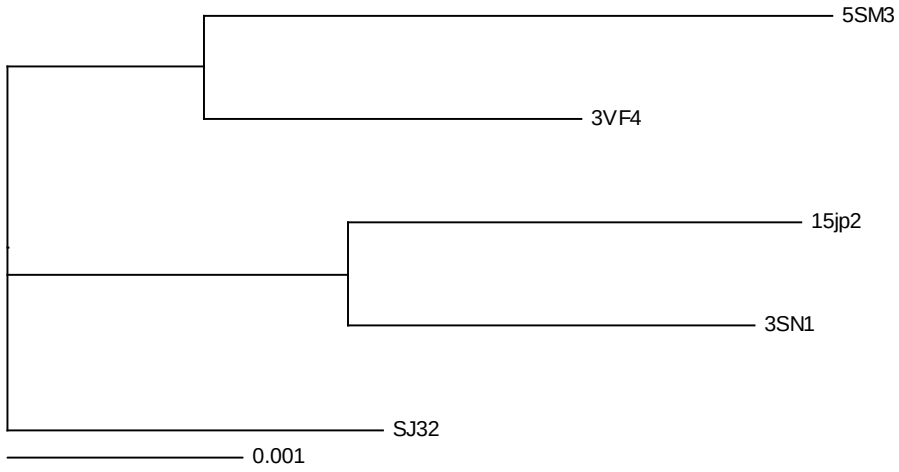

Fig.2 The phylogenetic tree obtained by the alignment of *gbp* gene sequences

Polymerase chain reaction (PCR)
PCR is one of the simple and rapid tools to amplify the length of target DNA into thousands of copies. The specific *gbp* primers were used for the study of genome.

Optimization of PCR

Initially, the annealing was set for 55^{0}C, but could not amplify. The optimization was to be done to successfully amplify. The parameters like annealing temperature, template concentration were changed accordingly to optimize. The results of final optimized PCR conditions are presented below. Programme of 40 cycles including denaturation at 94^{0}C for 1 min, annealing at 58.0^{0}C for 1 minute and extension at 72^{0}C for 1 min. The cycles were concluded by adding final extension at 72^{0}C for 10 min. The amplified product was run on 1.0% gel and observed that the product is around 800 bp.

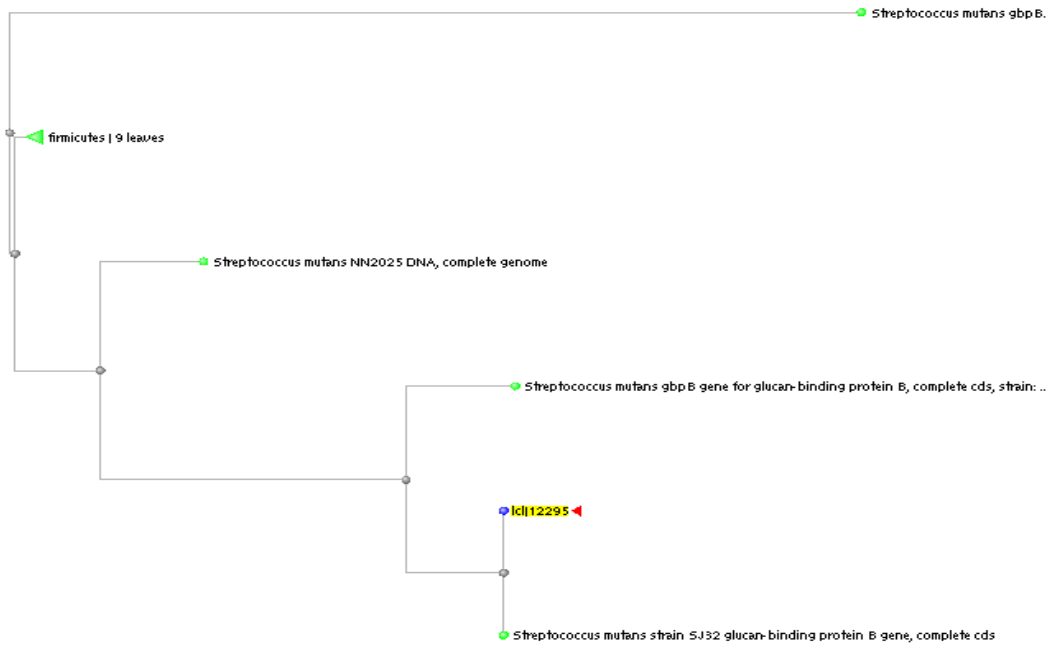

Fig. 3 Dendrogram obtained by BLAST analysis of the *gbp* gene sequence

Sequence analysis

The sequence obtained by PCR (1225 bp) was analyzed by nBLAST at NCBI. The analysis confirmed it as the *gbp* gene as it matched with the same sequence with accession no AY046410.1 of *S. mutans* isolate SJ-32 gbp gene. The phylogenetic tree obtained is presented in Fig. 3. The sequence was further translated to protein to know coding sequences by ORF finder (www.bioinformatics.org/sms/orf_find.html). The gene contained one CDs encoding protein of 401 amino acids. The virtual protein was further analyzed with pBLAST which confirmed the protein as glucan binding protein of *S. mutans.* The dendrogram obtained is presented in Fig. 4.

gcgcagattgcgtcacaagattctaaaatcaacaacttgaccgcacaacagcaagcagcacaagcacaagttaatacgattcaaggacaagtaagtgctttacagacaca
acaagctgaattacaagctgaaaatcaaagacttgaagctcagtctgctactttgggtcaacaaattcaaacactttcaagcaaaattgttgcacgtaatgaatctttgaagca
acaagctcgtagtgctcaaaaaagtaacgcagctaccagctatattaatgctatcattaattcaaaatcagtttctgatgctattaatcgtgtttcggctattcgtgaagttgtatc
tgctaatgaaaaaatgcttcaacaacaagagcaagataaagcagctgttgagcaaaagcaacaagaaaatcaagcagcaattaatactgttgcagctaatcaggagaca
attgctcaaaatacaaatgctttaaatacacagcaagctcaattagaagcagcacaactaaacttgcaagctgaattgactactgcacaagatcaaaaagctactttagttgc
tcaaaaagcggcagcagaggaagctgcacgccaagcagcagcggcacaagcggcagcagaagctaaggccgcagcagaagcaaaagctttacaagaacaagca
gcgcaagcacaggtagcagcaaataataatactcaagctacagatgcttctgaccaacaagcagcggcagctgataacactcaagcagcacaaacaggtgattcaact
gagcagtcagcagcgcaagcagtaaataattctgatcaagaaagtactacagcaacagaagcacaaccatcagcttctagtgcttcgacagcggctgtagcagctaata
cttcttctgctaatacatatccagcagggcaatgtacttggggtgttaaatcattagctccttgggtaggaaactactggggtaatggtggacaatgggcagcaagtgcagc
agcggcaggatatagagttggttctacaccttcagctggagctgtagctgtatggaatgatggcggttatggacacgttgcttatgttacaggtgttcaaggtggccaaattc
aagttcaagaagctaactatgcaggtaaccaatctattggtaactaccgtggttggtttaatccaggtagtgtaagctatatctatccaaactaatttaatattataaaaaggtaa
g

***gbp gene* sequence**

AQIASQDSKINNLTAQQQAAQAQVNTIQGQVSALQTQQAELQAENQRLEAQSATLGQQIQTLSSKIVARNE
SLKQQARSAQKSNAATSYINAIINSKSVSDAINRVSAIREVVSANEKMLQQQEQDKAAVEQKQQENQAAIN

TVAANQETIAQNTNALNTQQAQLEAAQLNLQAELTTAQDQKATLVAQKAAAEEAARQAAAAQAAAEAK
AAAEAKALQEQAAQAQVAANNNTQATDASDQQAAAADNTQAAQTGDSTEQSAAQAVNNSDQESTTATE
AQPSASSASTAAVAANTSSANTYPAGQCTWGVKSLAPWVGNYWGNGGQWAASAAAAGYRVGSTPSAG
AVAVWNDGGYGHVAYVTGVQGGQIQVQEANYAGNQSIGNYRGWFNPGSVSYIYPN

***gbp* virtual protein sequence**

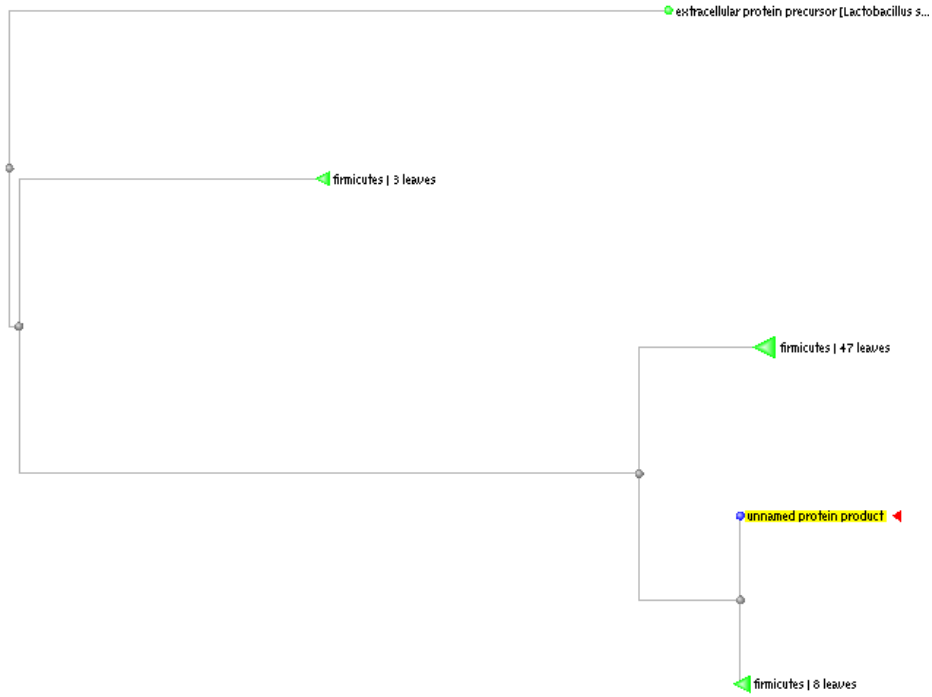

Fig. 4 Dendrogram obtained by BLAST analysis of the *gbp* protein sequence

Transformation

The amplified gene product was transformed into *E. coli* BL-21 strain. For that, the recombinant plasmid was prepared by restriction digesting the pUC 18 vector with EcoRI at multiple cloning site. The digested vector was mixed with the amplified product and ligated. The rDNA was transformed into the competent host cells by $CaCl_2$ method. The initial effort to transform *gbp* was failed. In the later efforts, the success was achieved as greenish and white colonies were observed (Fig.5).

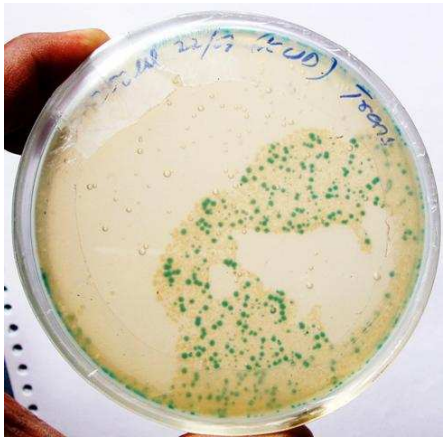

Fig. 5 Colonies of gbp transformed *E. coli*

DISCUSSION

The GBPs of *S. mutans* were designated in the order of their discovery. GbpA was originally isolated by Russell (1979) and designated GBP. Several years later, Smith *et al*. (1994) reported the isolation of GbpB. Unlike GbpA and GbpB, which were isolated primarily by affinity chromatography, GbpC was identified by Sato *et al*. (1997) following mutagenesis to pinpoint the genetic locus responsible for conferring dextran-dependent aggregation when the bacteria were grown under certain defined stress conditions (9,10,11). Shah and Russell (2002) analyzed the *S. mutans* genome sequence for amino acid repeats common to the carboxyl terminus regions of GTFs and GbpA to locate *gbpD* (12). Functionally, the *S. mutans* GbpC and the GBL/GBP-4 from *S. sobrinus* or *S. criceti* are involved in dextran-dependent aggregation, though the conditions for aggregation of *S. mutans* are more limited than for *S. sobrinus* or *S. criceti*. Sato *et al*. (1997) reported that the high alanine and proline content of GbpC is similar to that reported for GBL/GBP-4 (8, 11). Little is known about the regulation of GBPs. Yoshida *et al*. (2002) have recently reported that the disruption of the *luxS* gene in *S. mutans* disrupts biofilm formation, perhaps due to enhanced auto-aggregation. The authors speculate that the expression of GTFs and GBPs may be regulated by auto-inducer- 2 (AI-2) (13), the signaling molecule for a quorum-sensing system that is conserved among many Gram-positive and Gram negative bacterial species (14). Similarly, there is also a recent report that differential display PCR indicated that inactivation of *luxS* in *S. gordonii* resulted in a down regulation of several genes, including gtfG (15).

The ability to synthesize extracellular glucans is generally believed to be one of the virulence properties of *Streptococcus mutans* which contributes to plaque formation and to the subsequent development of dental caries. The initiatory component of plaque produced by *S. mutans* is an insoluble glucan called mutan, which is synthesized by the glucosyltransferase (GTF) enzymes. *S. mutans* produces an extracellular protein designated GBP which becomes associated with the cell in the presence of sucrose and forms dental plaques. In the present study, the *gbp* gene was isolated by amplifying the genome of *S. mutans*. The gene specific primers were designed using ClustalX and primer3 software and used for PCR. The amplifed product was sequenced and analyzed by BLAST and virtually translated by ORF finder. The gene and protein sequences were further analzyed for conserved domains and phylogeny. The gene was also transformed into *E.coli* BL-21 strain with pUC 18 vector with amp^r and *lacZ* markers successfully. The dental caries has become a major threat for the oral hygiene and health which is caused mainly because of the glucan binding proteins. A further work of induced expression of gene isolated in the present study should lead to the production and purification of monoclonal antibodies which may be used further for the protection of teeth.

The composition of microbial community could, in turn, affect the state of oral health or disease. Many of the early investigations utilized strains of *S. mutans* that later became classified as *S. sobrinus*. After the taxonomic split, it was apparent that *S. sobrinus* strains more readily aggregated than did strains of *S. mutans*, and a major GBP from *S. sobrinus* was designated the glucan-binding lectin (GBL) (7). Subsequently, Ma *et al*. (1996) defined GBLs as GBPs that confer upon bacteria the property of aggregation in the presence of exogenous alpha-1, 6 glucan (8). According to this definition, all GTFs and GBLs are glucan-binding proteins, but not all glucan-binding proteins are GTFs or GBLs. Measurement of transcription from the *gbpA* promoter with use of a *cat* reporter gene provided evidence for constitutive expression in the presence and absence of sucrose (16). An *S. mutans* strain with streptococcal GTP-binding protein (SGP) synthesis suppressed by antisense RNA displayed significantly greater levels of GbpA as detected by Western immunoblotting (17). It is likely that, in the near future, several microarray analyses will be undertaken and the basis for the regulation of GTF and GBP expression will be known in much greater detail, both at the level of the individual gene and at the level of signaling networks. Measurement of transcription from the *gbpA* promoter with use of a *cat* reporter gene provided evidence for constitutive expression in the presence and absence of sucrose (16). An *S. mutans* strain with streptococcal GTP-binding protein (SGP) synthesis suppressed by antisense RNA displayed significantly greater levels of GbpA as detected by Western immunoblotting (17). It is likely that, in the near future, several microarray analyses will be undertaken and the basis for the regulation of GTF and GBP expression will be known in much greater detail, both at the level of the individual gene and at the level of signaling networks.

Acknowledgement
The authors are grateful to the Bio-Genics. Hubli, INDIA for providing the necessary facilities.

REFERENCES

[1] Preiss J, Romeo T. *Adv Microb Physiol.*, **1989**, 30,183-238.
[2] Bohin J-P. *FEMS Microbiol Lett.*, **2000**, 186,11-19.
[3] Warren RAJ. *Annu Rev Microbiol.*, **1996**, 50,183-212.
[4] Gibbons RJ, van Houte J. *J Periodontol.*, **1973,** 6,347-360.
[5] Loesche WJ. *Microbiol Rev.*, **1986,** 50:353-380.
[6] Whiley RA, Beighton D. *Oral Microbiol Immunol.*, **1998,** 13,195-216.
[7] Drake D, Taylor KG, Bleiweis AS, Doyle RJ. *Infect Immun.*, **1988**, 56,1864-1872.
[8] Ma Y, Lassiter MO, Banas JA, Galperin MY, Taylor KG, Doyle RJ. *J Bacteriol.*, **1996**, 178,1572-1577.
[9] Douglas CWI, Russell RRB. *Arch Oral Biol.*, **1982,** 27,1039-1045.
[10] Smith DJ, Akita H, King WF, Taubman MA. *Infect Immun.*, **1994**, 62,2545-2552.
[11] Sato Y, Yamamoto Y, Kizaki H. *Infect Immun.*, **1997,** 65,668-675.
[12] Shah DS, Russell RRB. Streptococcal genetics. 6th ed. Asheville, NC: American Society for Microbiology, **2002**
[13] Yoshida A, Merritt J, Kuramitsu HK. Role of the Streptococcus mutans luxS gene in biofilm formation. American Society for Microbiology, **2002**.
[14] Schauder S, Shokat K, Surette MG, Bassler BL. *Molec Microbiol.*, **2001,** 41,463-476.
[15] McNab R, Ford S, El-Sabaeny A, Barbieri B, Lamont RJ. A luxS-based signalling system in Streptococcus gordonii. In: ASM Conference on Streptococcal Genetics. Asheville, NC:American Society for Microbiology, **2002**
[16] Banas JA, Potvin HC, Singh RN. *FEMS Microbiol Lett.*, **1997**, 154:289-292.
[17] Baev D, England R, Kuramitsu HK. *Infect Immun.*, **1999**, 67, 4510-4516.