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Comparative Proteomic Analysis of *Streptococcus agalactiae* (GBS) in Biofilms and Planktonic Growth Conditions

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ABSTRACT

Several studies have been carried out in bacteria for the identification of gene(s) or protein(s) involved in biofilm formation for the development of anti-biofilm targets but with limited success. However, information about the involvement of proteins during biofilm formation in GBS is poorly understood. Therefore, in the present study, by using mass spectrometry, a comparative proteomics study was performed for the GBS cultured during planktonic and biofilm conditions to identify proteins expressed exclusively during the biofilm development. Two hundred and four proteins were found commonly present in both the planktonic and the biofilm stages. However, 192 and 98 proteins were exclusively detected in planktonic and biofilm stages, respectively. Out of these, 98 proteins present in the biofilm stage were associated with cellular metabolism, translations, cell wall and cell membrane synthesis machinery. Moreover, only 20 proteins (out of 98) have been characterized so far in GBS for their role in pathogenesis and therefore, further characterization of the remaining 78 proteins in biofilm formation and pathogenesis may open avenues for designing new-age drugs to combat GBS burden world-wide.

Key words: *Streptococcus agalactiae*, biofilm, antibiotic resistance, ESI-LC-MS/MS, proteomics

INTRODUCTION

Group B streptococcus is the leading cause of neonatal morbidity and mortality, as well as causes serious infections in immune-compromised adults (Raabe and Shane, 2019). To prevent GBS infection, intrapartum antibiotic prophylaxis (IAP) regime is being used by some developed countries to prevent the transmission of GBS from mother to neonate during delivery. However, this is not available to all women around the world. As well as increased resistance to first- and second-line antibiotics used for GBS treatment, emphasizing the need for the development of GBS vaccine. Extensive use of antibiotics over the year has resulted in the emergence and spread of antibiotics resistance bacteria turning as a global concern (Akova, 2016). According to the National Institutes of Health (NIH), USA, biofilms account for up to 80% of bacterial infections in humans. Biofilm protects the bacteria from antimicrobial agents and thus contributes to increased resistance to antimicrobial treatments. Bacterial biofilms could become several fold-more resistant to antimicrobials than their planktonic counterparts (Sharma *et al.,* 2019). Therefore, biofilm development should be viewed as a core mechanism of resistance. Streptococci strains are known to produce biofilm which protect them from antibiotics, disinfectants and host defence. Bacteria inside biofilm are far more resistant to antimicrobial agents than planktonic forms.

Several *in vitro* investigations have shown that GBS can form biofilm-like structures, which is similar to other gram-positive pathogens. The ability of GBS to form biofilms attained significant attention for its possible role in survival and pathogenesis and antibiotic resistance (Kaminska *et al.,* 2020). Upon infection, GBS expresses several virulence factors that help the bacteria to adapt to the host's challenging environments and survival strategies including biofilm formation that facilitates the disease manifestation. In this regard, GBS expresses a diverse array of surface-associated and secretory proteins that mediate specific host-cell interactions and helps bacteria to survive against host innate immune clearance (Yadav *et al.,* 2020). Some adhesion and invasion factors were identified from both epithelial and endothelial tissues and characterized. GBS surface proteins called adhesins enable the bacterium to make

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persistent and intimate contact with the host cells (Shabayek and Spellerberg, 2018). Several studies have demonstrated the application of proteomics in the investigation of bacterial biofilms from both gram-positive and gram-negative strains, leading to a better understanding of the dynamic functional state of a genome at the protein level and learn more about the proteins that may be involved in the emergence of a given observed phenotype.

However, despite the strong evidence of biofilm formation in GBS, relatively little is known about the genes and proteins that contribute to the development of the biofilm phenotype. Till to date no proteomic analyses of GBS biofilm have been attempted. Therefore, in this study, the differences in the whole-cell protein expressions of GBS cultivated under biofilm versus planktonic conditions were investigated.

MATERIALS AND METHODS

A clinical isolate of GBS that produces moderate biofilm was used. GBS culture was streaked on 5% sheep blood agar plate (BAP) and incubated at 37 °C for 24-30 h. Todd Hewitt Broth medium (THB; Himedia Laboratories, India) supplemented with 1% glucose was inoculated with single isolated colony from the BAP and incubated at 37°C until their OD_{600} reached between 0.4-0.6. To produce biofilms, GBS culture (OD_{600} = 0.5) was dispensed in 60 mm diameter, tissue culture-treated petri dishes and incubated at 37°C for 48 h under static conditions. After incubation, the supernatant was removed followed by two times washing with phosphate buffer saline (PBS). Adhered GBS biofilms were harvested by scraping the plates with the help of a sterile scraper followed by centrifugation (6000 rpm; 10 min; 4°C), and the obtained pellet was washed twice with PBS (pH 7.5) before cell lysis. For the planktonic culture, GBS was cultured in THB with 1% glucose and incubated at 37°C (150 rpm) until OD_{600} reached 0.5 which corresponded to the logarithmic phase. After reaching the desired absorbance, planktonic cells were then harvested by centrifugation (6000 rpm; 10 min; 4°C), and the obtained pellet was washed twice with PBS before cell lysis.

Protein was extracted from GBS cells as described previously with some modifications. Briefly, GBS cell pellets from biofilm and planktonic cultures were suspended in lysis buffer (10 mM Tris-HCl pH 8.0, 1% sodium dodecyl sulphate) containing a protease inhibitor cocktail (Roche). An equal volume of 0.1 mm glass beads was added and vigorously stirred for 20 sec followed by cooling on ice for one minute. This procedure was repeated five times to ensure complete cell lysis. The cell debris was removed by centrifugation at 12000 rpm at 4°C for 20 min. The proteins in the supernatant were collected and transferred into separate sterile tubes for further quantification and SDS-PAGE analysis.

Protein measurements were performed with the BCA protein assay kit (Thermo Fisher Scientific) followed by their separation using SDS-PAGE. Fifteen micrograms of proteins from each sample were separated on 10% poly-acrylamide gels. Gels were fixed for 30 min in a solution containing 50% ethanol and 10% glacial acetic acid, washed twice with deionized water and protein bands were visualized by staining with colloidal Coomassie brilliant blue (R-250). The protein gel lane, containing the same amount of protein from both planktonic and biofilm was cut into three gel fractions (based on the abundance or occurrence of unique bands in the gel) and digested with trypsin (Sequencing grade modified, Promega, India). The gel slices were further minced into smaller pieces and de-stained at room temperature using 50 mM ammonium bicarbonate solution in 50% acetonitrile for 30 min followed by dehydration in 100% ACN for 5 min. After de-staining, each fraction was subjected to in-gel reduction, alkylation, and trypsin digestion. For trypsin digestion, protein samples were reduced using 10 mM DTT in 50 mM ammonium bicarbonate for 1 h at 60°C and alkylated for 30 min at room temperature in dark with 55 mM Iodo-acetamide in 50 mM ammonium bicarbonate. ACN was removed and gel pieces were allowed to air dry for 5-10 min. Ten µl of trypsin solution (0.2 µg/µl) was added in tubes which contained gel pieces in dry state. Tubes were placed on ice for 10 min to swell and absorb the trypsin solution. Gel fractions were washed and dehydrated using 100 µl of ACN, overnight at 30°C at 50 rpm shaking. After overnight incubation, 10 µl of extraction solution (1% formic acid) was added and samples were incubated for 5 min and this step was repeated once more for efficient

peptide recovery. Samples were centrifuged and collected supernatant was concentrated in the speed-vac and sent to National Centre for Plant Genomic Research (NIPGR), JNU campus, New Delhi for the Liquid Chromatography Mass Spectrometry (LC-MS) quadru-pole time of flight mass spectrometry (TOF-MS) (Eksigent Nano LC 400-TripleTOF® 6600 quadrupole time-of-flight (QTOF).

The MS/MS spectra were extracted and searched in FASTA (C:/AB SCIEX/ProteinPilot Data/SearchDatabases/Streptococcagalactiace_NCBI.fasta) using ProteinPilot™ software (version 1.0, 5.0.2.0, 5346). The following parameters were used for searching: trypsin as a digestion enzyme, iodoacetic acid as cysteine alkylation, and identification as sample type. Other parameters, such as tryptic cleavage specificity, precursor ion mass accuracy and fragment ion mass accuracy were built-in functions of ProteinPilot™ software, and the Paragon method was adopted. The raw peptide identification results from the Paragon Algorithm (version 5.0.2.0, 5174) were further processed by the ProGroup Algorithm within the ProteinPilot™ software before the final display. The following criteria were set to consider a protein for further statistical analysis: unused ProtScore ≥2 with at least two peptides with 95% confidence per repetition; mean, standard deviation, and *p* values to estimate the statistical significance of the protein changes calculated by ProGroup. The candidate proteins were carefully examined in the Protein ID of the ProteinPilot™ software. The relative expression of a peptide was considered and observed by mass/charge ratio (m/z). The analysis of protein was done by UniprotKB beta and Clusters of Orthologous Genes (COG) database.

RESULTS AND DISCUSSION

The SDS-PAGE analysis of GBS proteins isolated from planktonic and biofilm stages revealed considerable variations in their expression. In biofilm samples, the expression of several proteins was either reduced or induced as compared to planktonic samples. Moreover, at least four protein bands were detected exclusively in biofilm samples (Fig. 1). These data demonstrated that under biofilm conditions, GBS may use different cellular reprogramming machinery for its growth and survival.

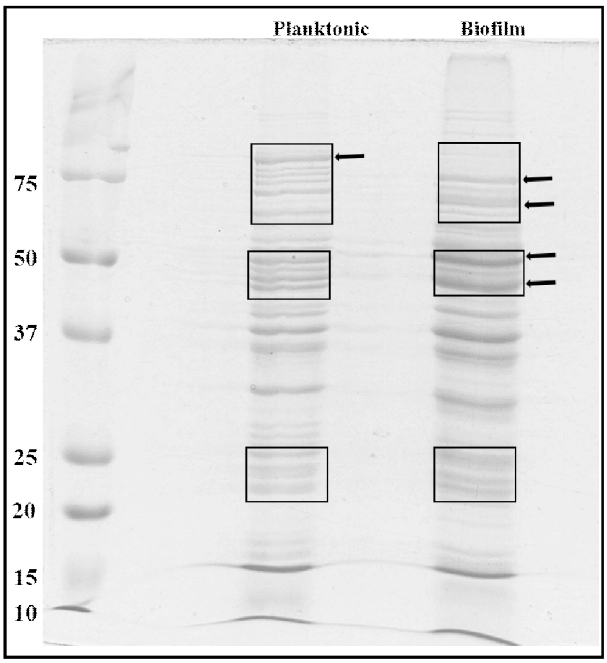

Fig. 1. Protein profiles of group *B streptococcus* grown from planktonic (lane 2) and biofilm stages (lane 3). A molecular marker with standard molecular weight proteins is shown in the left lane. Arrows indicate the proteins expressed exclusively or abundantly during biofilm stage.

To identify proteins exclusively expressed during the biofilm stage, a comparative proteomic analysis was conducted by applying liquid chromatography-tandem mass spectrometry (LC-MS/MS). This identified 396 proteins in planktonic GBS and 302 proteins in biofilm conditions. A total of 204 proteins were found to be commonly expressed in both planktonic and biofilm conditions, whereas 98 and 192 proteins were exclusively expressed during biofilm and planktonic conditions, respectively (Fig. 2). Further *in silico* analysis, these 98 proteins were carried out for their annotations and role in biological systems (Table 1). Out of 98 proteins expressed during the biofilm conditions, interestigly, only 20 proteins have been studied so far for their role in biofilm formation in other bacteria (Table 2). For instance, the expression of the LCP family protein BrpA in biofilm samples was observed. The BrpA has been shown to contribute to biofilm phenotype in GBS as a BrpA defective mutant exhibited increase in chain length and biofilm defect (Patras *et al,* 2018). Similarly, DUF402 domain-containing

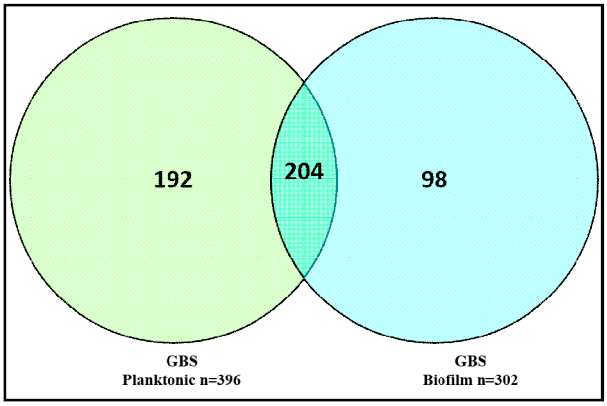

Fig. 2. Venn plot illustrating protein identification overlaps between planktonic and biofilm.

proteins was expressed in GBS during the biofilm stage. The DUF402 family has been identified in archaea including *Thermococci* and *Halobacteria* as well as *Firmicutes* and *Actinobacteria.* In *Entercoccus faecalis*, DUF402 domain-containing protein (hypothetical protein) was required for biofilm formation and other phenotypes of diverse bacteria (Willett *et al,* 2019). Other identified proteins were the Trk system potassium transporter TrkA (Binepal *et al.,* 2016) and glycosyl-transferase family 4 (Gtf4) protein (Rainey *et al.,* 2019), which have been characterized to play a role during biofilm formation in other streptococcal species. Hence, these studies support our data and the occurrence of these proteins during biofilm formation in GBS.

Next, the identified proteins were further classified according to the sub-cellular localization by UniProtKB. From the localization screening data, maximum proteins were located in the cytoplasm, membrane and ribosome of the cells i.e. presence in different locations/regions. However, the sub-cellular localization of only 48 proteins (out of 98 from biofilm samples) was available on UniProt. Thus, 33 were cytoplasmic predicted proteins, 11 were cell membrane and cell wall associated, and four were located in ribosomes, while the remaining 50 were of unknown sub-cellular localized (Fig. 3).

To determine the functions of identified proteins, they were classified into functional COG categories. Most of the protein differentially expressed in biofilm conditions belonged to cellular metabolism and protein synthesis while proteins associated with cell cycle and DNA replication were not detected in these samples. Out of the 98 different proteins identified during the biofilm conditions, 23 proteins (23% of the total proteins) were associated with translation, ribosome structure and biogenesis. This was followed by proteins belonging to the category of unknown function (11%). Other categories were the one related to cell wall/membrane/ biogenesis (9%), amino acid transport (8%), signal transduction (7%), transcription (7%), energy production and conversion (6%), carbohydrate transport and metabolism (5%). Lower percentages were for the categories cell cycle control and cell division (1%), intracellular trafficking, secretion and vesicular transport (1%; Fig. 4).

These findings demonstrate that in biofilm conditions, GBS does not proliferate as compared to planktonic conditions. This study demonstrated that GBS modifies its proteome response differentially depending on the lifestyle stage. As planktonic and biofilm proteomic analyses are much scarcer in GBS, therefore, in the present study, proteins were identified which may play a role during biofilm formation in GBS.

In conclusion, it is clear that biofilms have different protein expression patterns that differ from those of planktonic bacteria. However, more studies are required to understand the role of these proteins and their genetic background in biofilm formation. The growing knowledge of the bacterial features in biofilm indicates that the growth on the substratum involves significant modifications in gene transcription, including the establishment of new genetic traits.

FUTURE DIRECTIONS

In the current study, differential proteomic profile was performed of a GBS isolate cultured under biofilm and planktonic conditions. The overall aim of this study was to identify some protein(s) of unknown function and target them as anti-biofilm with therapeutic purposes. The current work reveals the expression of 98 proteins in the biofilm stage in GBS. However, only 20 proteins are known to involve in biofilm and pathogenesis in bacteria other than GBS, while the role of 78 proteins is yet to be identified. Future research on these 78 proteins may help scientists to understand the GBS pathogenesis at molecular levels and

Table 1. Information of 98 proteins expressed exclusively in the biofilm conditions

Accession No.	Protein name	Location	Functional categories	Family
WP_000818341.1	RNA polymerase sigma factor RpoD	Cytoplasm	Transcription	Sigma-70 factor family
WP_000578325.1	DUF4479 and tRNA-binding domain-containing protein	Cytoplasm	General function prediction only	
WP_000159519.1	Ribosome recycling factor	Cytoplasm	Translation, ribosomal structure and biogenesis	RRF family
WP_000357891.1	Response regulator transcription factor	Cytoplasm	Transcription	CheY-like_superfamily
WP_001008627.1	Asp-tRNA(Asn)/Glu-tRNA(Gln) amidotransferase subunit GatB		Translation, ribosomal structure and biogenesis	GatB/GatE family
WP_000688108.1	Pyridoxal phosphate-dependent aminotransferase		Amino acid transport and metabolism	
WP_057486563.1	Thiol peroxidase	Cell membrane	Inorganic ion transport and metabolism	
WP_000086620.1	50S ribosomal protein L6	Ribosome	Translation, ribosomal structure and biogenesis	Ribosomal protein uL6 family
WP_000003861.1	Guanylate kinase	Cytoplasm	Nucleotide transport and metabolism	Guanylate kinase family
WP_000118223.1	Beta-ketoacyl-[acyl-carrier-protein] synthase family protein		Lipid transport and metabolism	Beta-ketoacyl-ACP synthases family
WP_001002578.1	DUF2130 domain-containing protein		General function prediction only	
WP_000083755.1	Methylenetetrahydrofolate--tRNA-(uracil(54)-C(5))-methyltransferase (FADH(2)-oxidizing) TrmFO	Cytoplasm	Amino acid transport and metabolism, Translation, ribosomal structure and biogenesis	MnmG family
WP_000056607.1	Preprotein translocase subunit YajC	Cell membrane	Intracellular trafficking, secretion, and vesicular transport	YajC family
WP_000024418.1	50S ribosomal protein L4	Ribosome	Translation, ribosomal structure and biogenesis	Ribosomal protein uL4 family
WP_000752455.1	C69 family dipeptidase	Cytoplasm	General function prediction only	
WP_011324939.1	Peptide chain release factor 2		Translation, ribosomal structure and biogenesis	
WP_000073158.1	U32 family peptidase		Translation, ribosomal structure and biogenesis	U32 family
WP_000715592.1	dTMP kinase	Cytoplasm	Nucleotide transport and metabolism	Thymidylate kinase family
WP_000221011.1	Glutamate-5-semialdehyde dehydrogenase		Coenzyme transport and metabolism	
WP_001244947.1	Hydroxymethylglutaryl-CoA synthase	Cytoplasm	Lipid transport and metabolism	HMG-CoA synthase family
WP_001867156.1	50S ribosomal protein L13	Ribosome	Translation, ribosomal structure and biogenesis	Ribosomal protein uL13 family
WP_001287287.1	50S ribosomal protein L10	Ribosome	Translation, ribosomal structure and biogenesis	
WP_000628277.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--L-lysine ligase		Cell wall/membrane/ envelope biogenesis	

WP_060914826.1	Cysteine--tRNA ligase		General function prediction only	
WP_000772246.1	Phosphoribosylamine--glycine ligase		Nucleotide transport and metabolism	
WP_000244022.1	Ribosome biogenesis GTPase Der	Cytoplasm	Translation, ribosomal structure and biogenesis	EngA (Der) GTPase family
WP_000759712.1	FAD-containing oxidoreductase		Energy production and conversion, Coenzyme transport and metabolism	
WP_001220335.1	Trk system potassium transporter TrkA	Cytoplasm	Signal transduction mechanisms, Inorganic ion transport and metabolism	
WP_001286946.1	Exodeoxyribonuclease VII large subunit	Cytoplasm	Replication, recombination and repair	XseA family.
WP_000043874.1	Glutathione-disulfide reductase		Energy production and conversion	Class-I pyridine nucleotide-disulfide oxidoreductase family
WP_000573351.1	GTPase HflX	Cytoplasm	Translation, ribosomal structure and biogenesis	HflX GTPase family
WP_000229966.1	sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC		Lipid transport and metabolism	
WP_000220675.1	Amidophosphoribosyltransferase		Nucleotide transport and metabolism	
WP_000869689.1	Aspartate kinase		Amino acid transport and metabolism	
WP_000008542.1	DEAD/DEAH box helicase		Replication, recombination and repair	
WP_001019853.1	Tyrosine--tRNA ligase	Cytoplasm	Translation, ribosomal structure and biogenesis	TyrS type 1 subfamily
WP_000061657.1	GTPase ObgE	Cytoplasm	Translation, ribosomal structure and biogenesis	OBG GTPase family
WP_000048107.1	UDP-N-acetylmuramate--L-alanine ligase	Cytoplasm	Cell wall/membrane/ envelope biogenesis	MurCDEF family
WP_000777522.1	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	Cytoplasm	Cell wall/membrane/ envelope biogenesis	MurCDEF family
WP_000119582.1	Peptidase T	Cytoplasm	General function prediction only	
WP_000537055.1	LemA family protein	Cell membrane	Cell wall/membrane/ envelope biogenesis	LemA family
WP_001033073.1	Wzz/FepE/Etk N-terminal domain-containing protein	Cell membrane	Cell wall/membrane/ envelope biogenesis	CpsC/CapA family
WP_000217762.1	Insulinase family protein		Posttranslational modification, protein turnover, chaperones	
WP_000453281.1	Dihydroxyacetone kinase subunit L		Carbohydrate transport and metabolism	
WP_000197731.1	Glycogen/starch/alpha-glucan family phosphorylase		Carbohydrate transport and metabolism	Glycogen phosphorylase family
WP_001028826.1	Peptide chain release factor 1	Cytoplasm	Translation, ribosomal structure and biogenesis	Prokaryotic/mitochondrial release factor family
WP_001185383.1	Aminoacyltransferase	Cytoplasm	Amino acid transport and metabolism	FemABX family
WP_000042577.1	HAMP domain-containing histidine kinase	Cell membrane	Signal transduction mechanisms	

WP_000818772.1	Transcription elongation factor GreA		Transcription	GreA/GreB family
WP_000599671.1	TIGR01440 family protein		Amino acid transport and metabolism	UPF0340 family
WP_000272489.1	Holliday junction branch migration protein RuvA		Translation, ribosomal structure and biogenesis	
WP_000410289.1	ABC transporter ATP-binding protein	Cytoplasm	General function prediction only	
WP_000130587.1	Uracil-DNA glycosylase family protein		Translation, ribosomal structure and biogenesis	
WP_001034427.1	Arginine repressor	Cytoplasm	Transcription	ArgR family
WP_000675286.1	Histidine phosphatase family protein		Signal transduction mechanisms	
WP_001029971.1	ABC transporter ATP-binding protein	Cytoplasm	General function prediction only	
WP_000681579.1	L-serine ammonia-lyase, iron-sulfur-dependent subunit beta		Amino acid transport and metabolism	Iron-sulfur dependent L-serine dehydratase family
WP_001065465.1	Cell wall metabolism sensor histidine kinase	VicK	Cell membrane	Signal transduction mechanisms
WP_001200106.1	tRNA 4-thiouridine(8) synthase ThiI		Translation, ribosomal structure and biogenesis	
WP_000745456.1	4-alpha-glucanotransferase		Carbohydrate transport and metabolism	
WP_000699093.1	Response regulator transcription factor	Cytoplasm	Transcription	CheY-like_superfamily
WP_000770391.1	Xanthine phosphoribosyltransferase	Cell membrane	Coenzyme transport and metabolism	
WP_001016380.1	Crp/Fnr family transcriptional regulator		Transcription	
WP_000766628.1	NAD(P)H-dependent oxidoreductase		Energy production and	
WP_000064984.1	LCP family protein	Cell membrane	Cell wall/membrane/envelope biogenesis conversion	LytR/CpsA/Psr (LCP) family
WP_001239184.1	DUF402 domain-containing protein		General function prediction only	UPF0374 family
WP_001094117.1	NAD(P)H-dependent oxidoreductase		Energy production and conversion	Azoreductase type 1 family
WP_000750933.1	FAD/NAD(P)-binding protein		General function prediction only	
WP_060914836.1	Nitroreductase family protein		Energy production and conversion	
WP_000028887.1	tRNA uridine-5-carboxymethylaminomethyl (34) synthesis GTPase MnmE		Translation, ribosomal structure and biogenesis	
WP_001869051.1	Helix-turn-helix transcriptional regulator		Transcription	
WP_000895125.1	GDSL-type esterase/lipase family protein		Lipid transport and metabolism	
WP_000173347.1	Cysteine desulfurase	Cytoplasm	Amino acid transport and metabolism	Class-V pyridoxal-phosphate-dependent aminotransferase family
WP_000240199.1	Aminoacyl-tRNA hydrolase	Cytoplasm	Translation, ribosomal structure and biogenesis	PTH family
WP_000323651.1	NAD-dependent succinate-semialdehyde dehydrogenase		Energy production and conversion	Aldehyde dehydrogenase family
WP_000902654.1	23S rRNA (uracil(1939)-C(5))-methyltransferase RlmD		Translation, ribosomal structure and biogenesis	RNA M5U methy-ltransferase family
WP_000934868.1	Hypothetical protein		General function prediction only	
WP_000455620.1	Ribosome maturation factor RimM	Cytoplasm	Translation, ribosomal structure and biogenesis	RimM family
WP_000323510.1	ABC transporter ATP-binding protein/permease	Cell membrane	Defense mechanisms	
WP_000357880.1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Cytoplasm	Cell wall/membrane/envelope biogenesis	MurA subfamily

WP_000138202.1	Chromosomal replication initiator protein DnaA	Cytoplasm	Replication, recombination and repair	DnaA family
WP_000707048.1	Type I 3-dehydroquinate dehydratase		Amino acid transport and metabolism	Type-I 3-dehydroquinase family
WP_000603277.1	Copper homeostasis protein CutC	Cytoplasm	Inorganic ion transport and metabolism	CutC family
WP_000031277.1	Fe-S cluster assembly protein SufD		Posttranslational modification, protein turnover, chaperones	UPF0051 (ycf24) family
WP_001219475.1	Glycosyltransferase family 4 protein		Cell wall/membrane/envelope biogenesis	
WP_000699857.1	Glycogen synthase GlgA		Carbohydrate transport and metabolism	Glycosyltransferase 1 family
WP_000960182.1	DNA recombination protein RmuC		Replication, recombination and repair	
WP_000594351.1	FtsX-like permease family protein	Cell membrane	Cell cycle control, cell division, chromosome partitioning	
WP_001238954.1	CCA tRNA nucleotidyltransferase		Translation, ribosomal structure and biogenesis	tRNA nucleotidyltransferase/ poly(A) polymerase family
WP_000613483.1	ATP-dependent Clp protease proteolytic subunit	Cytoplasm	Posttranslational modification, protein turnover, chaperones	Peptidase S14 family
WP_001285395.1	Toxic anion resistance protein		Cell wall/membrane/envelope biogenesis	TelA family
WP_000787243.1	Glucose-1-phosphate adenylyltransferase		Carbohydrate transport and metabolism	
WP_001200982.1	Competence/damage-inducible protein A		Replication, recombination and repair	
WP_001003542.1	HD domain-containing protein		Signal transduction mechanisms	
WP_000974717.1	Superoxide dismutase SodA	Cytoplasm	Inorganic ion transport and metabolism	Iron/manganese superoxide dismutase family
WP_000149079.1	CYTH domain-containing protein		Signal transduction mechanisms	
WP_000714456.1	Cell wall-active antibiotics response protein LiaF	Cell membrane	Signal transduction mechanisms	
WP_000892188.1	Hypoxanthine phosphoribosyltransferase	Cytoplasm	Nucleotide transport and metabolism	Purine/pyrimidine phosphoribosyltransferase family

Table 2. List of proteins which were identified exclusively during biofilm formation and have been reported to play a role during biofilm formation in other microorganisms

Accession No.	Protein identified in GBS	Protein's role in biofilm	Bacteria name
WP_057486563.1	Thiol peroxidase	Oxygen limitations and spatial physiological heterogeneity have been observed between biofilms. Peroxide stress increases production of extracellular polysaccharide that enhances biofilm formation.	*Escherichia coli O157:H7* and *Acinetobacter oleivorans*
WP_001220335.1@	Trk system potassium transporter TrkA	Trk system is the main virulence attribute of *S. mutans*, responsible for K^+ transportation: No K^+ abolished biofilm formation	*Streptococcus mutans*
WP_000043874.1#	Glutathione-disulfide reductase	Clinical *P. aeruginosa* strain mature and immature biofilms were disrupted by the addition of GSH.	*Pseudomonas aeruginosa*
WP_001185383.1	Aminoacyltransferase	By incorporating L-amino acids into the inter-chain cross-bridge of peptidoglycan chains, aminoacyltransferase (*femX*) contributes to the synthesis of peptidoglycans and upregulates the establishment of biofilms.	*Staphylococcus aureus*
WP_000272489.1	Holliday junction branch migration protein RuvA	The proteins RuvA is structurally related to the eDNA lattice present in biofilm. RuvA helps in structural and mechanical integrity of biofilm.	*NTHI, and Staphylococcus epidermidis*
WP_000745456.1@	4-α-glucanotransferase	4,6-α-glucanotransferase produce the α-glucan exopolymers which contribute in the biofilm matrix as this protein enhances biofilm formation.	*Streptococcus oralis*
WP_001016380.1	Crp/Fnr family transcriptional regulator	Crp/Fnr is transcription factor of *lmo0753* in *L. monocytogenes* and the deletion of *lmo0753* resulted less biofilm generation.	*L. monocytogenes*
WP_000064984.1@	LCP family protein	A key factor in the formation of biofilms is the LytR-CpsAPsr (LCP) family of proteins, which are significant mediators of cell wall integrity and maintenance. On the basis of similarities in sequence and phenotype *with Streptococcus mutans* BrpA, it has been determined that the GBS LCP protein is "biofilm regulatory protein A," encoded by *brpA*. Biofilm formation was dramatically reduced in GBSΔ*brpA* compared to wild type.	*Streptococcus mutans and Streptococcus agalactiae*
WP_000750933.1@	FAD/NAD(P)-binding protein	As the absence of FAD-I led to an increase in *radD* expression, the FAD-I lipoprotein influences RadD at the transcriptional level. In addition to increasing the aggregation of Δ*fad-I F*, higher expression of *radD* also markedly promoted the formation of dual-species biofilms in comparison to wild-type.	*Fusobacterium nudeatum derivatives with Streptococcus gordonii*
WP_060914836.1	Nitroreductase family protein	It is expressed under oxidative stress, stress leads to biofilm formation.	*Thermotoga maritima*

Contd.

Table 2 *contd.*

WP_001869051.1	Helix-turn-helix transcriptional regulator	A novel helix-turn-helix repressor called BigR (biofilm growth-associated repressor) regulates the transcription of an operon relevant to biofilm growth.	*Bartonella henselae*
WP_000895125.1	GDSL-type esterase/lipase family protein	Polysaccharide alginate of the extracellular biofilm from *P. aeruginosa* interacts with LipA (lipase family protein). Electrostatic interactions imply that enzyme development and its immobilization within biofilms.	*Pseudomonas aeruginosa*
WP_001219475.1@	Glycosyltransferase family 4 protein	Glycosyltransferases, enzymes responsible for the glucan matrix components of the *S. mutans* biofilm. More Glycosyltransferase synthesize more glucans which help in biofilm formation.	*Streptococcus mutans*
WP_000699857.1	Glycogen synthase GlgA	The GglA produces and accumulates glycogen in biofilm matrix but it is only function when GglP is express as normal.	*Enterobacteriaceae*
WP_000594351.1	FtsX-like permease family protein	FtsX protein is homologous to cell division protein. Biofilm formation is altered when cell division is blocked.	*Fusobacterium nucleatum*
WP_000613483.1#	ATP-dependent Clp protease proteolytic subunit	ClpP inhibits biofilm formation because it enhances *Agr* and cell wall hydrolase Sle1. *clpP* mutant increases biofilm formation.	*Staphylococcus aureus*
WP_000787243.1	Glucose-1-phosphate adenylyltransferase	It is responsible for glycogen synthesis, acomponent of biofilm. Activation of the protein enhance the biofilm formation by synthesizing glycogen. Inhibition of the protein in stationary phase reduces biofilm formation but in exponential phase there is no effect on biofilm formation.	*CandidatusBrocadiasinica*
WP_001200982.1#	Competence/damage-inducible protein A	Competence inducible protein A is a protein that helps in natural competencies, for uptake DNA, significant for molecular evolution. It may reduce biofilm formation.	*Bacillus subtilis*
WP_001003542.1#	HD domain-containing protein	HD-Y domain protein, negatively regulate the biofilm formation because the Δ*rpfG* mutant produced twice of biofilm as the wild type strain.	*Xanthomonas oryzae*pv. *Oryzicola*
WP_000974717.1	Superoxide dismutase SodA	SodA is expressed when oxidative stress occurs and the stress leading to biofilm formation. The protein is found in biofilm biomass which arises due to H_2O_2 stress.	*Klebsiella pneumoniae, Escherichia coli* O2 Strain E058

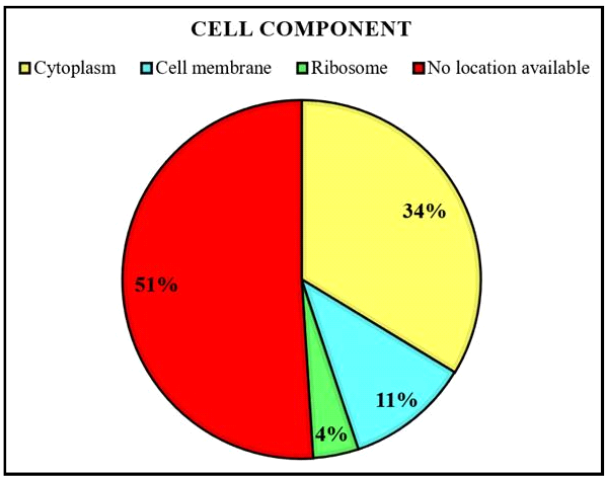

Fig. 3. Gene ontology (GO) annotation of identified proteins at the cell component level.

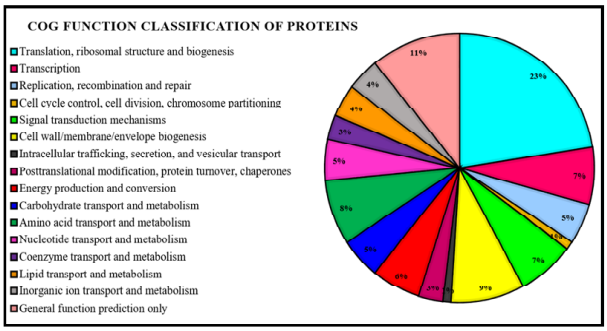

Fig. 4. COG functional annotation analysis based on molecular function.

could open avenues to design new drugs to inhibit biofilm formation. If so, it would ease the GBS disease burden worldwide.

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REFERENCES

Akova, M. (2016). Epidemiology of antimicrobial resistance in bloodstream infections. *Virulence* **7**: 252-266.

Binepal, G., Gill, K., Crowley, P., Cordova, M., Brady, L. J., Senadheera, D. B. and Cvitkovitch, D. G. (2016). Trk2 potassium transport system in *Streptococcus mutans* and its role in potassium homeostasis, biofilm formation, and stress tolerance. *J. Bacteriol.* **198**: 1087-1100.

Kaminska, D., Ratajczak, M., Szumala-Kakol, A., Dlugaszewska, J., Nowak-Malczewska, D. M. and Gajecka, M. (2020). Increasing resistance and changes in distribution of serotypes of Streptococcus agalactiae in Poland. *Pathogens* **9**: 526. *doi: 10.3390/pathogens9070526.*

Patras, K. A., Derieux, J., Al-Bassam, M. M., Adiletta, N., Vrbanac, A., Lapek, J. D., Zengler, K., Gonzalez, D. J. and Nizet, V. (2018). Group B streptococcus biofilm regulatory protein A contributes to bacterial physiology and innate immune resistance. *J. Infect. Dis.* **218**: 1641-1652.

Raabe, V. N. and Shane, A. L. (2019). Group B streptococcus (*Streptococcus agalactiae*). *Microbiol. Spectr. 7. https://doi: 10.1128/microbiolspec.GPP3-0007-2018.*

Rainey, K., Michalek, S. M., Wen, Z. T. and Wu, H. (2019). Glycosyltransferase-mediated biofilm matrix dynamics and virulence of *Streptococcus mutans*. *Appl. Environ. Microbiol.* **85**. *https://doi.org/10.1128/AEM.02247-18.*

Shabayek, S. and Spellerberg, B. (2018). Group B streptococcal colonization, molecular characteristics and epidemiology. *Front. Microbiol.* **9**: 01-14.

Sharma, D., Misba, L. and Khan, A. U. (2019). Antibiotics versus biofilm: An emerging battle ground in microbial communities. *Antimicrob. Resist. Infect. Control.* **8**: 01-10.

Willett, J. L., Ji, M. M. and Dunny, G. M. (2019). Exploiting biofilm phenotypes for functional characterization of hypothetical genes in *Enterococcus faecalis*. *npj Biofilms. Microbiomes* **5**: 23. *https://doi.org/10.1038/s41522-019-0099-0.*

Yadav, P., Verma, S., Bauer, R., Kumari, M., Dua, M., Johri, A. K., Yadav, V. and Spellerberg, B. (2020). Deciphering Streptococcal biofilms. *Microorganisms* **8**: 01-31.