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Identification of exopolysaccharide synthesizing genes in bacteria isolated from Bonny River in Rivers State, Nigeria

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Abstract

Microbial polysaccharide is the most important biopolymer secreted either by bacteria, fungi, or yeast as natural non-toxic, biodegradable and renewable sugar monomers. Microbial EPS owing to their interesting physicochemical and rheological properties has a wide range of industrial application such as in pharmaceuticals, cosmetics, food and Microbial enhanced oil recovery. This study was aimed to identify exopolysaccharide synthesizing genes in three bacterial isolates from Bonny River in Rivers State, Nigeria. The bacterial isolates were identified using the 16S rRNA gene sequence of the strains which carefully studied by making reference to the GenBank database using a BLAST search as *Bacillus xiamensis, Bacillus safensis* and *Exiguobacterium aurantiacum* using 16S rRNA PCR technique. Two priming Gylcosyltranferase genes (pGT) rfbp and cpsD were probed in the genome of the isolates after identification of the isolates. Two isolates *Bacillus safensis* and *Exiguobacterium aurantiacum* had both rfbp and cpsD genes while *Bacillus xiamensis* had only rfbp gene but no cpsD gene. The three isolates were examined for the Exopolysaccharide production on a minimal medium that contained g/l: KH_2PO_4 0.2, K_2HPO_4 1.5, $MgSO_4.7H_2O$ 0.2, $CaSO_4.2H_2O$ 0.1, $FeCl_3$ 0.002, yeast extract 0.5 and sucrose 20 and all three isolates produced EPS. At Optimum biomass of 0.15g/10 ml, the highest EPS yield of 10.20 g/l was obtained from *Bacillus safenis*. *Exiguobacterium aurantiacum* had optimum biomass of 0.11g/10ml with an EPS yield of 6.15g/l. The least biomass of 0.08g/10ml and EPS yield of 3.62 g/10ml was observed in *Bacillus xiamensis*. The biomass production was determined at Optical Density (OD) of 600nm while the EPS production was quantified by the Phenol Sulphuric acid method using glucose as a standard.

Keywords: Exopolysaccharide; Priming Glycosyltransferase; Riboflavin-binding protein (RFBP); Capsular Polysaccharide (CPSD)

1. Introduction

Microbial exopolysaccharides (EPS) are the polymers that consists principally carbohydrates and are excreted by some bacteria and fungi outside of their cell wall. EPS is occur in two forms depending on their location, i.e. capsular polysaccharide (capsule) where the polymer is closely associated with the cell surface and as the slime polysaccharides that are loosely associated with the cell surface(1). Extracellular polysaccharides (EPS) are produced by a great variety of bacteria, including environmental bacteria, pathogens, and food bacteria. These polymers may be assembled as capsular polysaccharides (CPS) tightly associated with the cell surface, or they may be liberated into the growth medium (2). Bacterial exopolysaccharides (EPSs) represent a large group of carbohydrate polymers which can be either covalently associated with the cell surface, forming a capsule, or loosely attached or even as homo- or heteropolysaccharides, depending on whether they are composed, respectively, of one or more than one type of sugar. EPSs are widely distributed among bacteria. They can have a protective function in the natural environment against phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds, and osmotic stress. Moreover, EPSs also have a role in cell recognition, adhesion to surfaces, and biofilm formation (4). Over the past few decades, the number of known exopolysaccharide (EPS) produced by microbial fermentation has gradually increased. Microbial

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biopolymers have many application in biotechnology fields including pharmaceutical, tissue engineering, cosmetics, food, textile, oil recovery, metal mining and metal recovery(5). Bacterial EPS synthesis is initiated by a priming glycosyltransferase that transfers a phosphosugar onto a polyprenol phosphate substrate (6). The membrane-bound product serves as a substrate for additional glycosyltransferases which catalyze the stepwise addition of monomeric carbohydrate units. The formed EPS repeating subunit is then flipped across the inner bacterial membrane. Finally, the EPS repeating subunit is polymerized and exported (7). Polysaccharides produced by microbes can be generally classified by their biological functions into intracellular storage polysaccharides (glycogen), capsular polysaccharides which are closely linked to the cell surface (e.g., K30 O-Antigen) and extracellular bacterial polysaccharides (for example, xanthan, sphingan, alginate, cellulose, etc.) that are important for biofilm formation and pathogenicity (8). At present four general mechanisms are known for the production of these carbohydrate polymers in bacteria:

- The Wzx/Wzy-dependent pathway;
- The ATP-binding (ABC) transporter-dependent pathway;
- The synthase-dependent pathway and
- The extracellular synthesis by use of a single sucrase protein (8).

The precursor molecules, which are necessary for the stepwise elongation of the polymer strands, are realized by various enzymatic transformations inside the cell, and follow in principle the same concept of producing activated sugars/sugar acids in the first three cases of different biosynthesis pathways. For the extracellular production, the polymer strand is elongated by direct addition of monosaccharides obtained by cleavage of di- or trisaccharides. In the Wzx/Wzy dependent pathway individual repeating units, which are linked to an undecaprenol diphosphate anchor (C55) at the inner membrane, are assembled by several glycosyltransferases (GT's) and translocated across the cytoplasmic membrane by a Wzx protein (so called flippase). In a next step their polymerization occurs at the periplasmic space by the Wzy protein before they will be exported to the cell surface (9). Transport of the polymerized repeat units from the periplasm to the cell surface has been shown to be dependent upon additional protein(s) assigned to the polysaccharide co-polymerase (PCP) and the outer membrane polysaccharide export (OPX; formerly OMA) families (10). All polysaccharides assembled by the Wzx/Wzy pathway have a highly diverse sugar pattern (up to four or five types of sugar within their chemical structure are common) and are therefore classified as heteropolymers (e.g., xanthan). All strains using this pathway carry the genes for the flippase (Wzx) and the polymerase (Wzy) within their extracellular polysaccharide operons. The second pathway of bacterial exopolysaccharide biosynthesis is the ABC transporter dependent pathway which is mainly present in capsular polysaccharide (CPS) biosynthesis (11). These polysaccharides do not really represent EPS, since they are still linked to the cell surface. Like the Wzx/Wzy dependent EPS, the CPS synthesized via the ABC-transporter dependent pathway are assembled by the action of GT's at the cytoplasmic face of the inner membrane, resulting in homopolymers when only a single GT-containing operon is involved, or in heteropolymers when multiple GT's are used for the assembly process (11). The export across the inner membrane and translocation to the cell surface, however, is different as it is realized by a tripartite efflux pump like complex. The complex is composed of ABC transporters spanning the inner membrane, and periplasmatic proteins of the PCP and OPX family (9). These proteins are closely related to OPX and PCP proteins involved in secretion process of the Wzx/Wzy pathway. CPSs produced via this pathway all carry a conserved glycolipid at the reducing terminus composed of phosphatidylglycerol and a poly-2-keto-3-deoxyoctulosonic acid (Kdo) linker. This represents one of the main differences of the Wzx/Wzy and the ABC dependent pathways. Just recently novel insights into the early steps in CPS biosynthesis were provided by new discoveries of the structure of this conserved lipid terminus (12). The third pathway is the synthase dependent pathway, which secretes complete polymer strands across the membranes and the cell wall, and is independent of a flippase for translocating repeat units. The polymerization as well as the translocation process is performed by a single synthase protein, which in some cases (alginate, cellulose) is a subunit of an envelope-spanning multiprotein complex (13). Synthase dependent pathways are often utilized for the assembly of homopolymers requiring only one type of sugar precursor as observed in curdlan biosynthesis. The biosynthesis of hyaluronic acid (HA) is catalyzed by a single enzyme (hyaluron synthase), which performs both polymerization and secretion. Most of the enzymatic steps for exopolysaccharide precursor biosynthesis appear inside the cell while polymerization/secretion is localized in the cell envelope. But there also exist some examples of extracellular synthesized polysaccharides, such as, e.g., dextran or levan. The biosynthesis of these occurs via GT's, which are secreted and covalently linked to the cell surface. In alignment with the various EPS biosynthesis pathways, the chemical structure and material properties of the final polymers are quite variable. The genes involved in the different biosynthesis pathways encode various types of GT's, polymerizing and branching enzymes, as well as enzymes responsible for addition of substituents or modifications of sugar moieties. Not all steps in the various pathways are currently understood, and sometimes the differences between the pathways become less defined. The genes encoding these enzymes can be found in most of the EPS producing microbes clustered within the genome or on large plasmids (13). In this study, we isolated three organisms namely *Bacillus xiamensi, Bacillus safenis* and *Exiguobacterium aurantiacum* from Bonny River, examined their exopolysaccharide production potential and determine the gene

fragments that code for priming Glycocyltranferase (pGT) which catalyzes the first step in exopolysaccharide (EPS) synthesis using PCR technique. The two priming Glycocyltranferase genes necessary for EPS synthesis that were probed are rfbp gene which is responsible for transferring galactose-1-phophate to the lipid precursor undecaprenol phosphate in the first step of O-polysaccaharide biosynthesis as well cpsD gene which is synthesized in the cytoplasm.

2. Material and methods

2.1. Sample collection

Water sample were collected from Bonny River in Rivers state Nigeria. To isolate EPS producing bacteria, the seawater sample was serially diluted in normal saline and plated on nutrient agar (NA) as well as Zubell Marine Agar (ZMA) and incubated for 48hrs. Plate counts of bacterial isolates were done while identification of likely EPS producing microorganisms were initially done to physical identification of isolated that had mucoid morphology. Mucoid colonies were then sub-cultured in to a nutrient media for further identification process.

2.2. Phenotypic and biochemical analysis

The bacterial isolates were biochemically analyzed with the oxidase, catalase, methyl red Voges Proskauer (MR-VP) test, motility, indole production and citrate utilization according to guidelines outlined by Bergey's Manual of Systematic Bacteriology (14).

2.3. Identification of bacterial isolate by 16S rRNA gene and sequencing techniques

Zymo Quick DNA Fungal/Bacterial Kit was used for the DNA extraction. Bacterial 16S rRNA gene was amplified from the extracted genomic DNA using universal primers, forward 27F (5' AGAGTTTGATCMTGGCTCAG 3') and reverse primer 1525R (5'AAGGAGGTGWTCCARCCGCA 3'). PCR was performed with 2.5µl of 10x PCR buffer,1µl of 25mM MgCl2, 1µl each of forward primer and reverse primer, 1µl of DMSO, 2µl of 2.5mMDNTPs, 0.1µl of 5µ/µl Taq DNA polymerase, and 3µl of 10ng/µl DNA. The total reaction volume was made up to 25µl using 13.4µl Nuclease free water. The PCR program has initial denaturation at 94 ℃ for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45sec. This is then followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10 ˚C forever. Amplified fragments were visualized on Safe view-stained 1.5% agarose electrophoresis gels. The size of the amplicon is about 1500bp and the DNA ladder used is Hyperladder from Bioline.

2.4. Screening for EPS Production

The ability of the isolates to produce exopolysaccharide was examined by growing the isolates on a synthetic media containing g/l: KH_2PO_4 0.2, K_2HPO_4 1.5, $MgSO_4.7H_2O$ 0.2, $CaSO_4.2H_2O$ 0.1, $FeCl_3$ 0.002, yeast extract 0.5 and sucrose 20 (15). The cell-free broths after centrifuging at 10,000 rpm for 10 minutes were mixed with two volumes of cold ethanol and stored at 4ºC overnight. The precipitates were collected centrifuging at 10,000 rpm for 20 minutes, dried, weighed and dissolved in distilled water (16). They were quantified by phenol sulphuric acid method using glucose as standard (17).

2.5. Identification of EPS producing genes from the isolates

A PCR- based method was used to probe for putative exopolysaccharide producers from the isolates using two Priming Glycocyltranferase genes (GTs) namely rfbp (undecaprenyl-phophate sugar phosphor-transferase) and cpsD (galactoyl-tranferase). Four primers were used in the gene probing to screen for the potential of the isolates to produce Exopolysaccharide (EPS). Primer sets (CPSD-F - TTCTCYGTGCGCATGGAATC; CPSD -R - CCCATAATSGACCAGTTCTGCAC) and Primer sets (RFBP-F - GATTCYGAGACCATGCGTAC; RFBP-R - GCATARTCCGACTGTTCCTGAG).

3. Results

3.1. Isolation and identification of bacterial isolates from marine samples

A total of thirteen marine bacterial isolates were isolated from Bonny River, River state, Nigeria. Only three of these were found to be EPS producers. The ESP-producing isolates were differentiated and identified by a 16S rRNA PCR technique as two isolates belonged to the genus Bacillus and one belonged to Exiguobacterium. Identification of the isolates was achieved by studying their morphological characters and biochemical features, and confirmed genetically by using 16S rRNA PCR sequence techniques. Molecular characterization of the selected isolate was achieved by performing PCR for the 16S ribosomal RNA gene, followed by alignment of the sequence produced by the PCR with the

GenBank database, which also showed high similarity Bacillus and Exiguobacterium as shown on the phylogenetic tree in Figure 1.

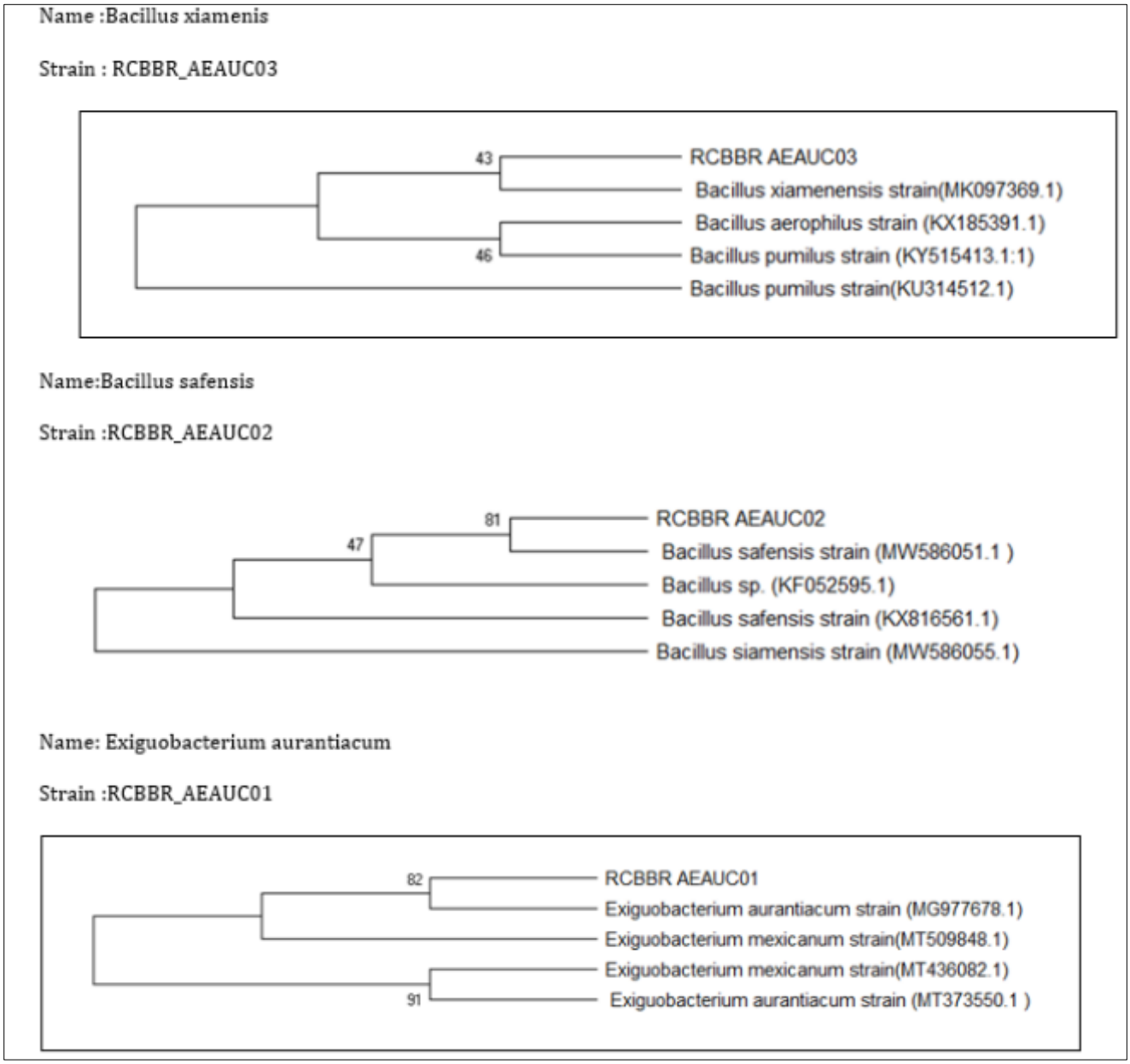

Figure 1 Phylogenetic tree of bacterial isolates

3.2. Selecting the isolate with the highest EPS production

All three bacterial isolates exhibiting a shiny mucoid appearance were examined for their ability to produce EPSs. The isolate produced EPS with different productivity rate. At Optimum biomass of 0.15g/10ml, the highest EPS yield of 10.20 g/l was obtained from *Bacillus safenis*. *Exiguobacterium aurantiacum* had optimum biomass of 0.11g/10ml with an EPS yield of 6.15g/l. The least biomass of 0.08g/10ml and EPS yield of 3.62 g/10ml was observed in *Bacillus xiamensis*. After morphological and biochemical identification of all isolates and confirmation of their identity by 16S rRNA PCR analysis, the screened isolates were used for the rest of the study.

3.3. Identification of the exopolysaccharide producing genes from the bacterial isolates

An insilico analysis of the genome of the three isolates allowed for the detection of rfbp, cpsD or both genes in the genome. Two isolates *Bacillus safensis* and *Exiguobacterium aurantiacum* had both rfbp and cpsD genes while *Bacillus xiamensis* had only rfbp gene but no cpsD gene as shown in figure 2 and 3.

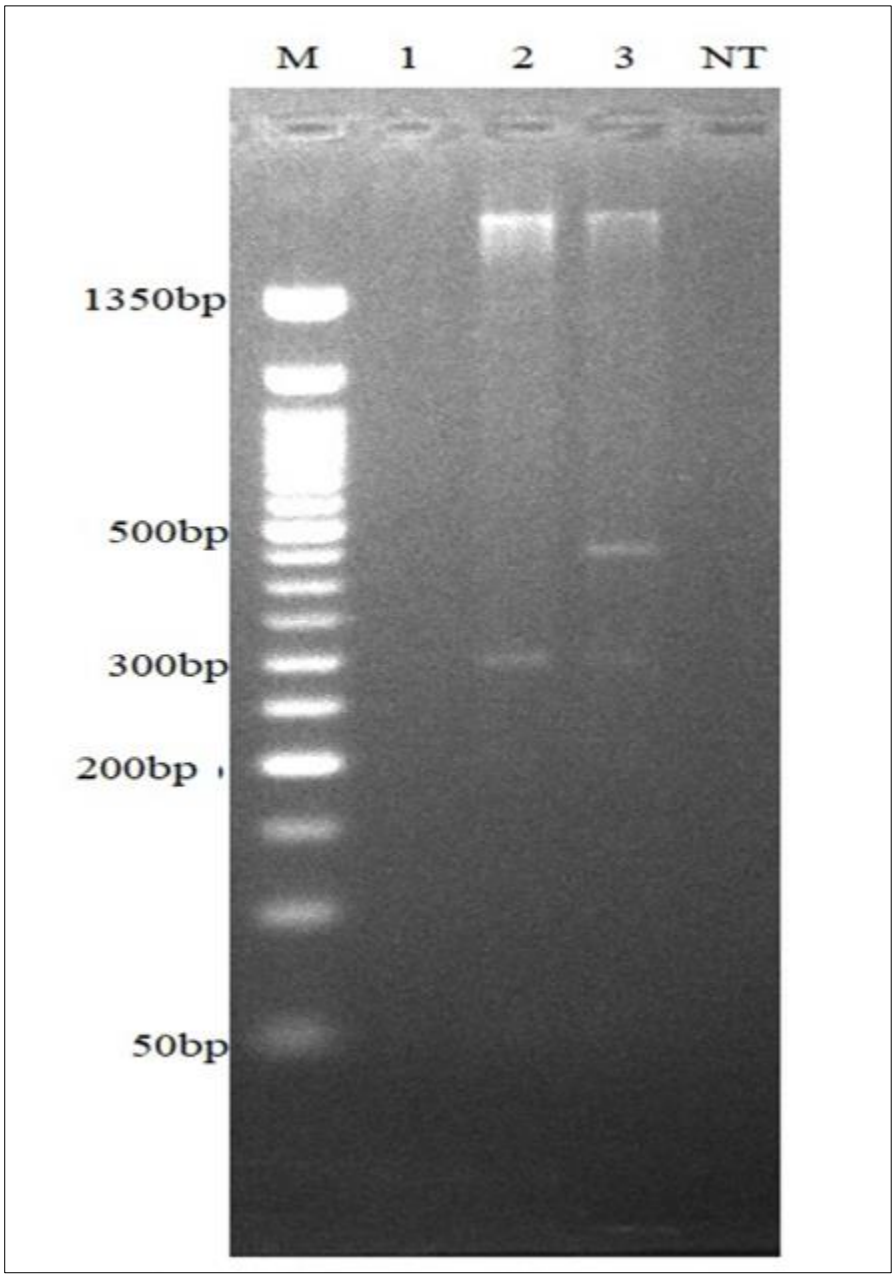

Figure 2 Gel image of amplification of CPSD primer. 1 = *B. xiamensis;* 2 = *B. safensis*; 3 = *E. aurantiacum*; NT = no template control; M = 50bp DNA latter

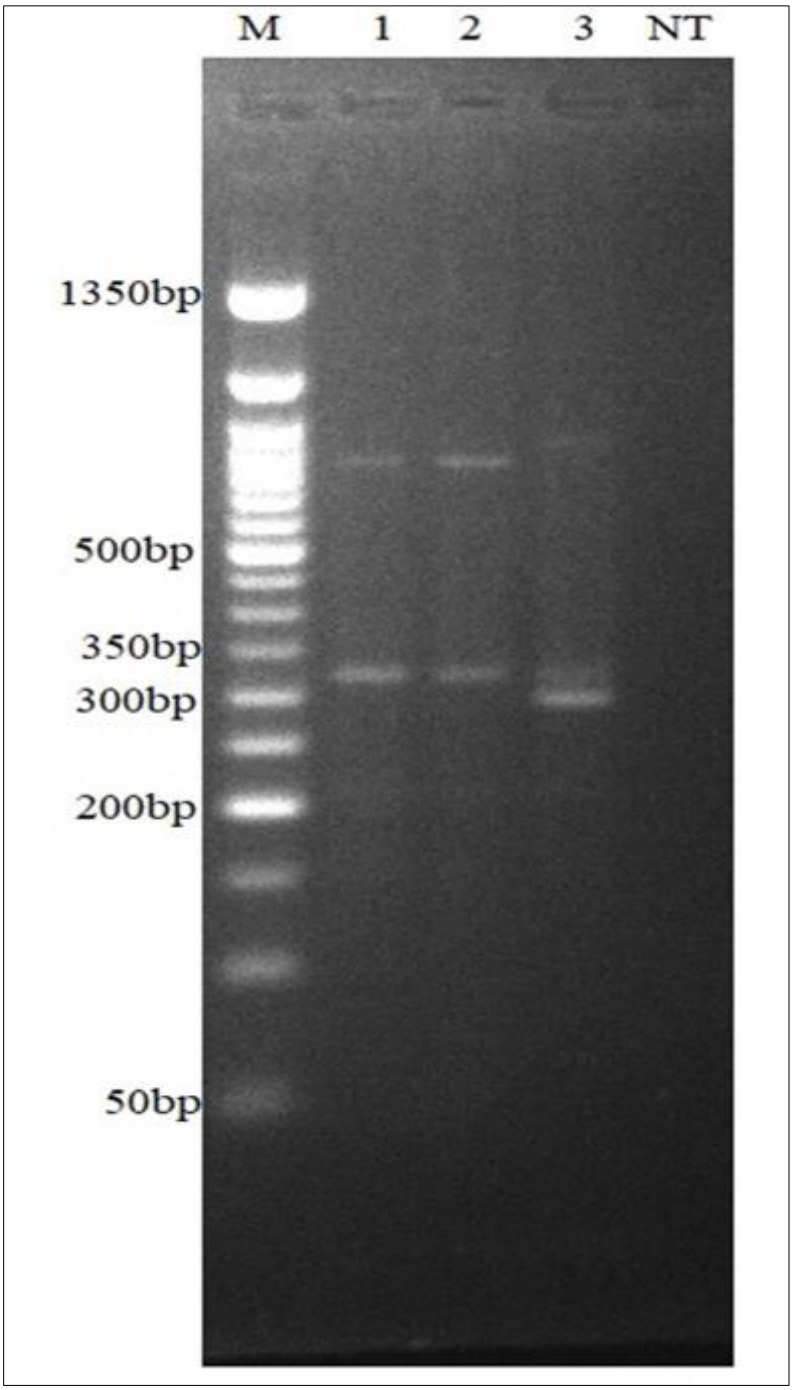

Figure 3 Gel image of amplification of RFBP primer. 1 = *B. xiamensis;*; 2 = *B. safensis*; 3 = *E. aurantiacum*; NT = no template control; M = 50bp DNA latter

4. Discussion

The three bacteria isolates used for this study were *Bacillus xiamensis, Bacillus safensis* and *Exiguobacterium aurantiacum.* Microorganisms belonging to *Bacilli* have been known to produce exopolysaccharides. *Exiguobacterium aurantiacum* is an alkaliphilic extremophile belonging to the *Bacilli* class(18). Extremophiles can be used in various applications due to the presence of rare enzymatic activities which enable them to produce exopolysaccharides (19). It has been reported that the production of EPS is a response to the nutrient composition of the growth medium. This work is reporting for the first time the identification of exopolysaccharide synthesizing genes from different organisms isolated from Bonny River. The initial step in EPS synthesis is the pGT-catalysed addition of a sugar-1-phosphate to a lipid carrier molecule anchored to the cell membrane. Additional sugar moieties are linked to the initial monosaccharide

by forming glucosidic bonds, catalyzed by glycosyltransferases (GTs), to form repeating units (20). The sugars used to form repeating units are in an activated form (nucleoside di- or monophosphorylated sugars), and the structures of different repeating units are dependent upon the GTs involved in their syntheses. Previous studies have demonstrated that the production of EPS was altered or interrupted after inactivation of the pGT gene, indicating that pGTs are necessary for the biosynthesis of EPS (21). Most genes coding for GTs are dissimilar to each other; however, the pGT genes in various bacteria are relatively similar and defined conserved amino acid sequences in a specific group of pGTs, particularly in the carboxy terminus (22). The relatively high homology may be related to the presence of domains that interact with lipid carrier (23). The genome of *Bacillus safensis* and *Exiguobacterium aurantiacum* contains two pGT genes coding for galactosyltransferase (cpsD) and undecaprenyl-phosphate sugar phosphotransferase (rfbP). However, in *Bacillus xiamensis* only one of the two genes undecaprenyl-phosphate sugar phosphotransferase (rfbP) was found. Earlier report have showed that the two genes were identified in *Bifidobacterium longum* NCC2705 strain while one or either of the two gene were identified in other strains of *B. longum.* EPS production from the isolates showed that the organisms produced exopolysaccharide. This finding is consistent with earlier works reported on *Exiguobacterium aurantiacum* by (24) after fermentation for 6days as well. Other extensive research on EPS production by Bacillus species have been well reported as in the case of (15, 25), these reports are in tandem with the findings of this research where EPS production has been reported from the three different bacterial isolates.

5. Conclusion

In conclusion, this work has further buttressed the fact that priming Gylcosyltranferase genes are responsible for exopolysaccharide biosynthesis. PCR was used to detect gene fragments coding for pGT, which catalyzes the first step of EPS production. This is supported with the result of EPS produced by the three bacterial isolates where it revealed that EPS production was high on isolates that had both rfbp and cpsD genes in their genome in the case of *Bacillus safensis* and *Exiguobacterium aurantiacum.* The reason that can be easily deduced by the low EPS production in *Bacillus xiamensis* in this work can be attributed to lack of rfbp gene which is responsible for transferring galactose-1-phosphate to the lipid precursor undecaprenol phosphate in the first step of O-polysaccharide biosynthesis.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no conflict of interest exist in the work.

References

[1] Ates O. (2015). Systems biology of microbial exopolysaccharideproduction. *Front Bioeng Biotechnol*. (3):200-207.

[2] Freitas F, Alves V.D., Reis M.A., Crespo J.G., Coelhoso I.M.(2014). Microbial polysaccharide-based membranes: current and future applications. *J Appl Polym Sci*. (6):131-133

[3] Nikolic M, López P, Strahinic I, Suárez A, Kojic M, Fernández-García M, Topisirovic L, Golic N, Ruas-Madiedo P. (2012). Characterisation of the exopolysaccharide (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics. *Int J Food Microbiol* **158:**155–162.

[4] Sutherland, I.W.(2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147:**3–9.

[5] Sandip P. Patil and Leena P. Shirsath (2015). Production of exopolysaccharide by an osmotolerant, thermostable and metal resistant *Bacillus subtilis. Int.J.Curr.Microbiol.App.Sci.* 4(2): 965-971.

[6] Xu, C.; Ruan, H.; Cai,W.;Staehelin, C.; Dai,W.(2021). Identification of an Exopolysaccharide Biosynthesis Gene in Bradyrhizobium diazoefficiens USDA110. *Microorganisms* 9:249.

[7] Acosta-Jurado, S.; Fuentes-Romero, F.; Ruiz-Sainz, J.-E.; Janczarek, M.; Vinardell, J.-M.(2021). Rhizobial Exopolysaccharides: Genetic Regulation of Their Synthesis and Relevance in Symbiosis with Legumes. *Int. J. Mol. Sci.* 22:6233.

[8] Brenner, D. J. , & Farmer, J., III (2015). Enterobacteriaceae. *Bergey's Manual of Systematics of Archaea and Bacteria* , 1–24 .

[9] Islam, S. T., and Lam, J. S. (2014). Synthesis of bacterial polysaccharides via the Wzx/Wzy-dependent pathway. *Can. J. Microbiol.* 60, 697–716.

[10] Morona, R., Purins, L., Tocilj, A., Matte, A., and Cygler, M. (2009). Sequencestructure relationships in polysaccharide co-polymerase (PCP) proteins. *Trends Biochem. Sci.* 34:78-84.

[11] Whitney, J. C., and Howell, P. L. (2013). Synthase-dependent exopolysaccharide secretion in Gram-negative bacteria. *Trends Microbiol.* 21, 63–72.

[12] Willis, L. M., and Whitfield, C. (2013). Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. *Carbohydr. Res.* 378, 35–44.

[13] Rehm, B. H. (2010). Bacterial polymers: biosynthesis, modifications and applications. *Nat. Rev. Microbiol.* 8, 578–592.

[14] Schmid J., Sieber V. and Rehm B. (2015) Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Front. Microbiol. 6:496-504

[15] Moghannem S.A.M., Farag M.M.S., Shehab A.M. and Azab M.S.(2018). Exopolysaccharide production from *Bacillus velezensis* KY471306 using statistical experimental design. *Brazillian Journal of Microbiology.*

[16] Hassan, S.W.M. and Ibrahim H.A.H., (2017). Production, characterization and valuable applications of exopolysaccharide from marine *Bacillus subtilis* SHI. *Polish Journal of Microbiology*. 66(4):449-461.

[17] Dubois M, Gilles K.A., Hamilton J.K., Rebers P.A. and Smith F., (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. 28:350-356.

[18] Collins, M.D., Lund, B.M., Farrow, J.A., Schleifer, K.H. (1993). "Chemotaxonomic study of an alkalihilic bacterium *Exiguobacterium aurantiacum* gen-nov sp-nov", *Journal of General Microbiology*, 129. 2037-2042.

[19] Kalin, M., wheeler, W.N., Meinrath, G. (2005). "The removal of uranium from mining waste water using algal/microbial biomass*", Journal of Environmental radioactivity*, 78. 151-177.

[20] C. Hidalgo-Cantabrana, B. Sanchez, C. Milani, M. Ventura, A. Margolles and P. Ruas-Madiedo, Appl. Environ. Microbiol., 2014, 80, 9–18.

[21] Morona, L.K., Morona, R. and Paton, J.C. (1999). Tyrosine phosphorylation of cpsD negatively regulates capsular polysaccharide biosynthesis in *Streptococcus pneumonia. J. Bacteriol.,*181, 5355–5364

[22] Wang, L., Liu, D. and Reeves, P.R.J., (1996). *Bacteriol.,* 178:2598–2604.

[23] Provencher, C., LaPointe, G., Sirois, S., Van Calsteren, M.R. and D. Roy, (2003). Consensus-degenerate hybrid oligonucleotide primers for amplification of priming glycosyltransferae gene of the exopolysaccharide locus in strains of the *Lactobacillus casei* group. *Appl. Environ. Microbiol.,* 69:3299–3307.

[24] Y. D. Dah Dossounon, K. Lee, K. Belghmi, F. Benzha, and M. Blaghen,"Exopolysaccharide (EPS) Production by *Exiguobacterium aurantiacum* isolated from Marchica Lagoon Ecosystem in Morocco." *American Journal of Microbiological Research*, vol. 4, no. 5 (2016): 147-152.

[25] Sahar S. M., Shaimaa K. A., Manal S. S., and Hala M. R., (2018). Characterization and applications of exopolysaccharide produced by marine Bacillus altitudinis MSH2014 from Ras Mohamed, Sinai, Egypt. *Egyptian Journal of Basic and Applied Sciences.* 5:204–209.

[26] Ruas-Madiedo P., Abraham A., Mozzi F. and de los Reyes-Gavilán C.G. **(**2008). Functionality of exopolysaccharides produced by lactic acid bacteria, p137–166. In Mayo B, López P, Pérez-MartínezG(ed), Molecular aspects of lactic acid bacteria for traditional and new applications. *Research Signpost,Kerala, India*