

## Identification of *Streptococcus sanguinis* Genes Producing Biofilm from Gingivitis

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### ABSTRACT

*Streptococcus sanguinis* is a teeth commensal frontier colonizer and among the most common species in the oral biofilm. Dental plaque, caries, and gingivitis/periodontitis are caused by dysbiosis of oral flora. A biofilm assay was developed to investigate biofilm formation in *S. sanguinis* using the microtiter plate, tube, and Congo red agar methods in order to identify causing bacteria and determine responsible genes. Three genes, including pur B, thr B, and pyre E, are suspected of playing a role in forming in vivo biofilms in *S. sanguinis*. The present study shows these genes to be responsible for increased biofilm formation in gingivitis patients.

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### Introduction

*Streptococcus sanguinis*, a native gram-positive bacterium, is a commensal microorganism essential for oral health but may become pathogenic if it gets the opportunity. Environmental factors such as a moist, warm niche for colonization by dozens of various bacterial species (usually harmless or beneficial to human oral health) can affect microbial communities in the oral cavity. The *Streptococcus* genus has the highest abundance of such bacterial species. *S. sanguinis*, a primary colonizer of the tooth surface in this environment, benefits the human host by protecting against the negative impacts of some other microorganisms (1,2). When the microbiome's homeostasis is disrupted, oral streptococci cause certain oral diseases. The most widespread oral illnesses are dental caries, gingivitis, and periodontitis (3).

*S. sanguinis* has been identified as a pioneer colonizer, assisting in the attachment of succeeding organisms and playing an important role in plaque biofilm development (4). In nature, biofilms are formed by well-organized and cooperative microorganism communities that cover biotic or abiotic surfaces (5). *S. sanguinis* binds to pellicle molecules of the film, adsorbed to tooth surfaces, primarily composed of salivary glycoproteins and microbial components. Adhesion begins with the surface of *S. sanguinis* adhering to pellicle components via hydrophobic and electrostatic interactions (6,7). Following that, receptors interact with pellicle ligands such as  $\alpha$ -amylase/secretory

IgA (SIgA) complexes and proline-rich proteins (7,8). Pellicle-adherent cells create new binding points and encourage local changes such as H<sub>2</sub>O<sub>2</sub> production, extracellular polysaccharide production, and DNA release. Such environmental changes could encourage or inhibit potential microbial descendants of the complex biofilm community, affecting their pathogenicity (9). Microbial cells are also exposed to spontaneous mutations as a consequence of enhanced endogenous oxidative stress within biofilms. Genetic variations then increase physiologically heterogeneous microbial subpopulations that impact biofilm formation capacity (10). H<sub>2</sub>O<sub>2</sub> production impacts *S. sanguinis* cell wall homeostasis through unknown mechanisms, promoting genomic DNA release, which appears to function as a structural component of biofilm extracellular matrix and a source of genes conferring competitive edge (11).

Enhanced antibiotic resistance or tolerance is a common issue in biofilms. It could be owing to microbial growth conditions, or it could be associated with the exchange or mutations of antibiotic resistance genes (12). Infections are difficult to eradicate despite proper antibiotic therapy as biofilms adapt to survive such treatments (10).

Biofilms have been recognized as key infectious disease determinants. Therefore, the association between biofilm formation and tolerance/resistance to antibiotics in gingivitis/periodontitis patients remains uncertain. To define whether antibiotic resistance and increased biofilm formation are associated with particular genes, three new

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genetic loci required for biofilm formation in *S. sanguinis* are determined here.

**Materials and Methods**

**Isolation and Detection of Bacteria**

Dental plaque samples were obtained from 50 patients aged 28 to 57 years who attended the Department of Periodontics at the College of Dentistry/Hawler Medical University from August 2021 to December 2021. They were diagnosed clinically as having gingivitis/periodontitis and representing both genders. Samples were taken by the dentist by curette; all the patients showed clinical signs of gingival inflammation. Then, the samples were put into the transport medium for examination in the laboratory. On blood agar, all samples were cultured for 24 hs at 37 °C and subcultured on nutrient agar. Bacterial plates of an isolated colony were sent to the laboratory for identification by employing the Vitek 2 Compact System, followed by antibiotic tests for all identified samples.

**Antimicrobial Susceptibility Test**

Mueller-Hinton agar was utilized as a growth medium to investigate the impact of various antimicrobials on *S. sanguinis* isolates. The final concentration of antibiotics was added to the media and poured into sterile Petri dishes after sterilizing and cooling at 45 °C. The streaking technique was used to inoculate the plates with *S. sanguinis*, and then they were incubated at 37 °C for 24 h. The results were recorded the following day (13).

**Biofilm Formation Assay**

The Congo red agar (CRA), tube, and microtiter plate methods were used to test *S. sanguinis* bacteria for biofilm production. *S. sanguinis* bacteria were tested and inoculated onto the surface of the CRA medium and incubated at 37 °C for 24 hrs (14). In addition, the tube method was performed by taking a loop full of each tested bacterial isolate; they were then inoculated into sterile test tubes containing brain heart infusion broth and incubated at 37 °C for 24 hs. Next, the supernatants were wasted, and the tube was stained with safranin (15). Ultimately, the microtiter plate technique's bacterial isolates were cultivated with 0.5% glucose in Tryptic Soy Broth (TSB) (Merck, Germany) and incubated at 37 °C overnight. Cultures with 0.5% glucose and 1:40 in fresh TSB were diluted (Sigma, USA); 200 L of the diluted solution was added to microtiter plate wells and incubated at 37 °C for 48 h. The negative control wells contained merely 200 µL of TSB–0.5% glucose with no bacterial suspension. Wells were delicately rinsed 3 times with phosphate buffer saline (PBS) (pH 7.2), fixed with methanol for 20 min, dried at room temperature, and stained with 0.1% crystal violet. 1 mL of 95% ethanol per well was used to dissolve the dye attached to the adhering cells. Ultimately, optical density (OD) at 570

nm (A570) was determined for each well using an ELISA reader (BioTek ELx800, US). For the optical density cut-off, the average OD of negative controls + Standard Deviation (SD) was calculated (ODc). Biofilms generated by distinct strains were examined and classified based on the absorbance of crystal violet stain (16) connected to adherent cells (Table 1).

**Molecular Study**

*S. sanguinis* genomic DNA was prepared from a typical cultivated colony in 1 ml TSB for 24 hs at 37°C. QIAGEN DNA Minikit (Fermentas, Germany) was used as directed by the manufacturer. The NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized to measure DNA concentration and purity. Biofilm genes were identified using previously announced specialized primers, shown in Table 2. PCR products were routinely sequenced by Sanger sequencing and analyzed by agarose gel electrophoresis (Table 3).

**Partial Sequence of pyre E and thr B gene**

The DNA was sequenced separately by the ABI 3130X genetic analyzer (Applied Biosystems) using both a forward primer (ACATCCGCACCTTCTCGTTT) and a reverse primer (TTAACATGGGCGTCAGGCA) for pyre E and a forward primer (TTTGACTCCGTTGGGGTAGC) and a reverse primer (TCGTGCTCAATCACCCACTC) for thr B gene. The PCR products of the eight samples were used as a source of DNA template for sequence-specific PCR amplification (Fig. 1).

**Sequence Alignment and Submission**

For the Basic Local Alignment Search Tool (BLAST),

**Table 1.** Categorization of biofilm formation abilities by the Microtiter Plate Technique.

Cut-off values	Mean of OD570 values	Biofilm formation abilities
OD>4*ODc	OD>0.557	Strong
2*ODc<OD≤4*ODc	0.278<OD≤0.557	Moderate
ODc<OD≤2*ODc	0.139<OD<0.278	Weak
OD≤0.139	OD≤0.139	None

**Table 2.** *S. sanguinis* biofilm genes.

Genes	Sequence of primer	Amplicon size (bp)
<i>Pyre E</i>	ACATCCGCACCTTCTCGTTT	362
	TTTACATGGGCGTCAGGCAT	
<i>pur B</i>	AACTTTGCCAACATTCCGCC	231
	GCTGCCCTTTTGACCTTTGG	
<i>Thr B</i>	TTTGACTCCGTTGGGGTAGC	83
	TCGTGCTCAATCACCCACTC	

**Table 3.** PCR amplification parameters with minor modifications and conditions.

Genes	Initial denaturation	No. of cycles	Stages			
			Denaturation	Annealing	Extension	Final extension
<i>pyreE</i>	95°C for 5 min	35	95°C for 30 sec	45°C for 30 sec	72°C for 120 sec	72°C for 4 min
<i>purB</i>						
<i>thrB</i>						

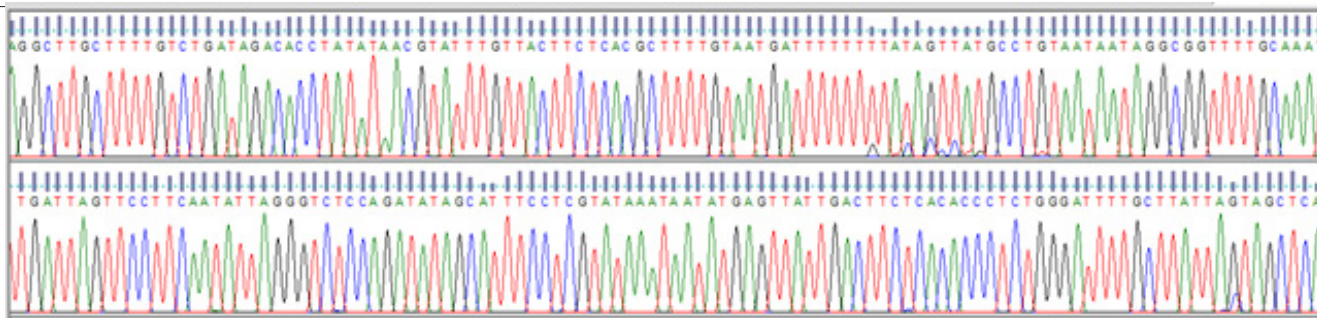


Figure 1. The chromatography sequence result of *pyre E* gene.

the *pyre E* gene sequences were used. BLAST is a search tool that applies the sequence alignment method (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), accessible at the NCBI (National Center for Biotechnology Information) website, for comparing laboratory or query sequences with other biological sequences to find out more similarity with other targets.

## Results

### Detection and Isolation

Fifty dental plaque samples were taken from gingivitis patients in the age range 28 to 57 among both genders (22 males, 28 females); from all samples collected, 22 (44%) isolates were of *S. sanguinis*. The patient samples were taken by specialized dentists from the dental plaque by a curette. The isolates were diagnosed by cultured and confirmed by the Vitek2 compact system, which identified the isolated bacteria as *S. sanguinis*.

### Antibiotic Sensitivity Test

Twenty-two *S. sanguinis* isolates were screened for their susceptibility to nine widely used antibiotics, including Amoxicillin, Amikacin, Ceftriaxone, Erythromycin, Ampicillin, Ciprofloxacin, Tetracycline, Imipenem, Vancomycin, Clindamycin, Chloramphenicol, and Cefotaxime. All isolates vary in their response to antimicrobial agents and have the highest sensitivity percentage, as presented in Table 4.

### Biofilm Production

As shown in Table 5, *S. sanguinis* produced slime on CRA. Twenty (90.9%) isolates were positive for biofilm production. Incubated plates showed a positive result for biofilm production by the colony morphology. Twenty isolates created black colonies on CRA, and two formed red colonies, which was a negative result.

The tube method results showed that 19 (86.3 %) isolates were biofilm positive, and 3 (13.7 %) isolates were biofilm negative, as shown in Table 6. The presence of vi-

Table 5. CRA for biofilm formation detection.

Number of isolates	Number of positive (%)	Number of negatives (%)
22	20 (90.9%)	2 (9.1%)

Table 6. Tube technique test for biofilm formation detection.

Number of isolates	Number of positive (%)	Number of negatives (%)
22	19 (86.3%)	3 (13.7%)

Table 7. Microtiter plate method for biofilm formation detection.

Number of isolates	No. (%)	No. (%)	No. (%)
	Strong	Moderate	week
22	4 (18.1%)	8 (36.3%)	10 (45.6%)

sible film lined on the walls and bottom of the tubes determined the positive outcome.

Other methods, such as the microtiter plate method, showed that each bacterial isolate had a different potential to form biofilm under similar conditions. Furthermore, as shown in Table 7, 4 (18.1 %) of the isolates were strong biofilm producers, 8 (36.3 %) were moderate biofilm producers, and 10 (45.6 %) were weak biofilm producers.

### Detection of Biofilm Genes *thr B*, *pur B*, and *pyre E*

A molecular-based method was performed to detect the three biofilm genes of isolated *S. sanguinis* bacteria. DNA was extracted, and the PCR technique was used for 22 isolates to detect three biofilm genes of *thr B*, *pur B*, and *pyre E* of *S. sanguinis*. The results showed that the products were positive for *thr B*, *pur B*, and *pyre E* genes as 19 (86.3%), 17 (77.2%), and 13 (59%), respectively, as presented in Table 8.

### Molecular Identification of Genus and Species of Bacteria

The eight COI sequence samples with a size of 250–350

Table 4. Antibiotic sociability test for *S. sanguinis*.

Antimicrobial agent	No. of Sensitive (%)	Antimicrobial agent	No. of Sensitive (%)
Vancomycin	22 (100%)	Amikacin	3 (13.6%)
Ciprofloxacin	20 (90.9%)	Ceftriaxone	3 (13.6%)
Imipenem	19 (86.3%)	Clindamycin	0 (0%)
Amoxicillin	19 (86.3%)	Erythromycin	0 (0%)
Ampicillin	17 (77.2%)	Tetracycline	0 (0%)
Cefotaxime	17 (77.2%)		
Chloramphenicol	8 (36.3%)		

**Table 8.** Detection of *S. sanguinis* biofilm genes.

Biofilm gene	No of isolates	No (%) positive
<i>Thr B</i>	22	19 (86.3%)
<i>Pur B</i>	22	17 (77.2%)
<i>Pyre E</i>	22	13 (59%)

were alimented using the BLAST program from the Gene bank (<http://blast.ncbi.nlm.nih.gov/>). It was utilized to compare our amplified sequences with other stored species of *pyre E* and *thr B* gene sequences. According to BLAST results, the highest identity number query sequence was 100%, and the lowest identity number query sequence was 95%, as shown in Fig. 2. These aliments indicated submission of our query sequences inside the NCBI Gene bank, given accession numbers as MW894651, MW894652, MW894653, MW894654, MW894655, MW894656, MW894657, and MW894658.

**Phylogenetic Inferences**

The distribution percentage of *S. sanguinis* according to BLAST of Gene bank NCBI of partial *pyre E* and *thr B* genes. Depending on the eight species sequence divergence similarity data and phylogeny made; species belonged to respective genera closely related to each other. MW894654, MW894655, and MW894656 had high similarity to BLAST gene bank accession number CP054570 of *S. sanguinis* from the USA, with the identic number as 100% MW894652, MW894653, and MW894658 located in the second group of the same branch for the *pyre E* gene with a distance of 0.2 %. In contrast, MW894651 and MW894657 genes of *thr B* were located in the second branch at a distance of 0.8 %, with the *S. sanguinis* cluster of BLAST Gene bank accession number LS48346 from the UK was showed in (Fig.3 and 4), and Table 9.

**Protein Alignment of *pyre E* and *thr B* genes**

According to phylogenetic inferences, the *pyre E* gene of *S. sanguinis* (MW894652, MW894653, MW894654, MW894655, MW894656, and MW894658) is located in the first group, while the genes of *thr B* *S. sanguinis* (MW894651 and MW894657) are located in the second group of the phylogenetic tree. After protein alignment for both genes, they appeared to be located on chromosome one.

The location of the chromosome and alignment in its correct place according to query serious sequences of PCR product amplified as clarified in Table 10 and (Fig. 5).

**Table 9.** BLAST analysis to identify observed samples.

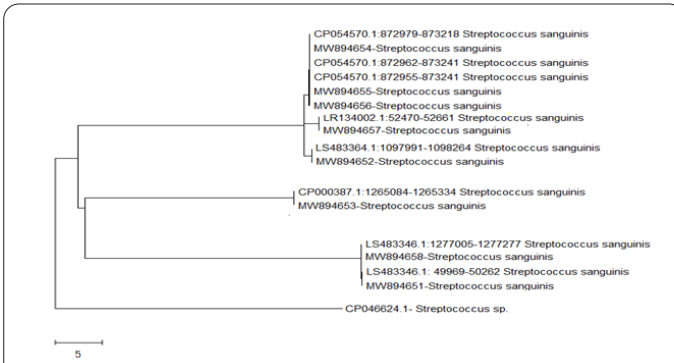
Bacterial accession number samples	Query Cover %	Identic Number %	Genbank Accession Number	Genbank Insect Species Identification	Country Identification
MW894651	100	100	LS483346.1	<i>Streptococcus sanguinis</i>	United Kingdom
MW894652	100	100	LS483364.1	<i>Streptococcus sanguinis</i>	United Kingdom
MW894653	100	94	CP000387.1	<i>Streptococcus sanguinis</i>	USA
MW894654	100	100	CP054570.1	<i>Streptococcus sanguinis</i>	USA
MW894655	100	100	CP054570.1	<i>Streptococcus sanguinis</i>	USA
MW894656	100	100	CP054570.1	<i>Streptococcus sanguinis</i>	USA
MW894657	100	100	LR134002.1	<i>Streptococcus sanguinis</i>	United Kingdom
MW894658	100	100	LS483346.1	<i>Streptococcus sanguinis</i>	United Kingdome



**Figure 2.** Biofilm genes product of *S. sanguinis* isolates, *thr B* (86.3%), *pyre E* (77.2%), and *pur B* (59%).

Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
<a href="#">Streptococcus sanguinis strain FAHRCOS T70 chromosome, complete genome</a>	506	506	100%	2e-143	100.00%	CP054570.1
<a href="#">Streptococcus sanguinis strain CDM1001 chromosome, complete genome</a>	470	470	100%	1e-132	97.14%	CP040739.1
<a href="#">Streptococcus sanguinis strain NCTC11085 genome assembly, chromosome 1</a>	456	456	100%	3e-128	96.07%	LS483346.1
<a href="#">Streptococcus sanguinis strain CDM1010 chromosome, complete genome</a>	456	456	100%	3e-128	96.07%	CP040739.1
<a href="#">Streptococcus sanguinis strain SK16, complete genome</a>	447	447	100%	2e-125	95.36%	CP000387.1
<a href="#">Streptococcus sanguinis strain NCTC1084 genome assembly, chromosome 1</a>	443	443	100%	2e-124	95.00%	LR134002.1

**Figure 3.** NCBI blasting pairwise alignment of *S. sanguinis* query sequence of *pyre E* and *thr B* genes.



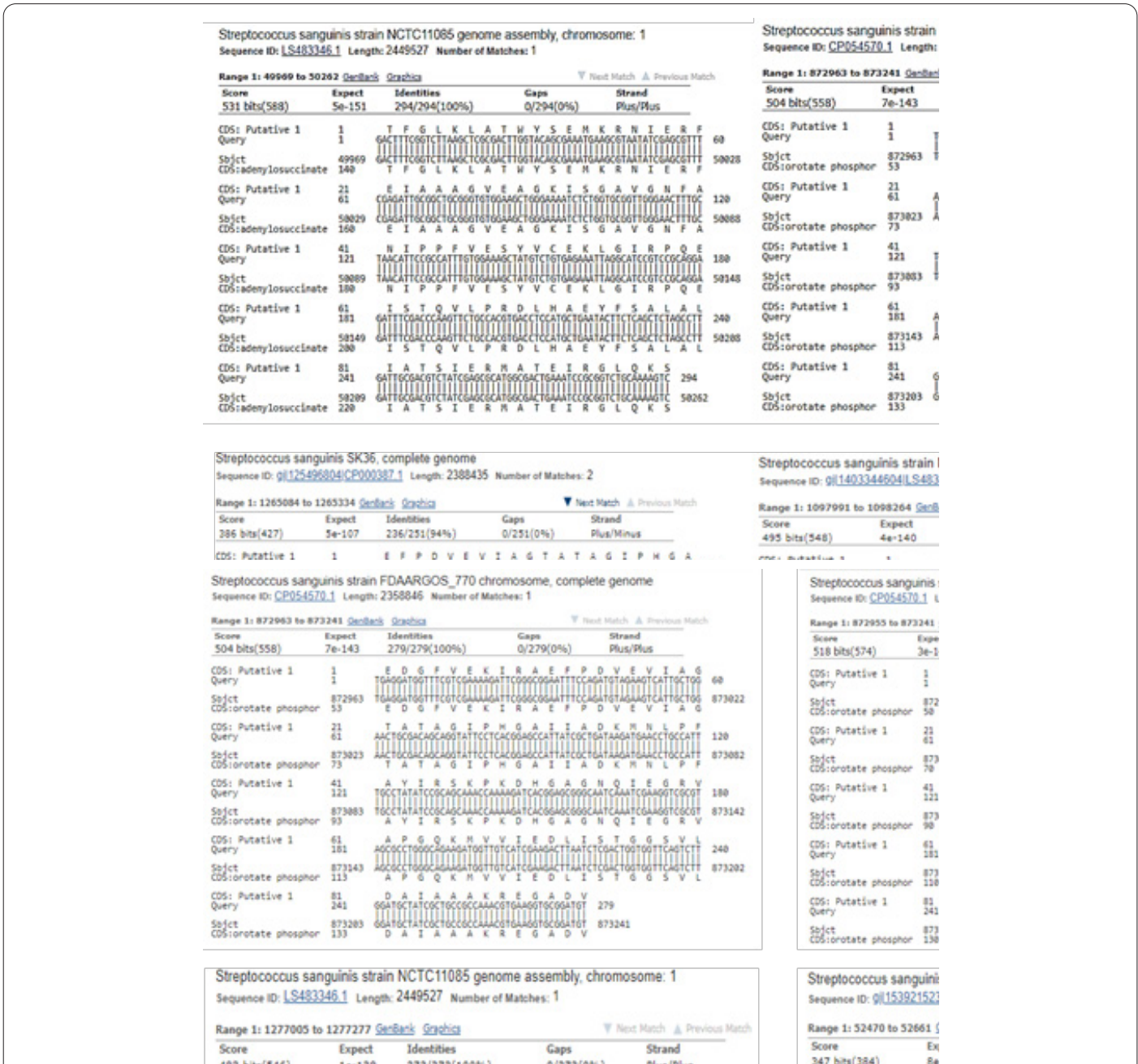
**Figure 4.** Molecular phylogenetic analysis using the maximum likelihood technique: This technique was used to infer the evolutionary history based on the Tamura-Nei model.

**Discussion**

*Streptococcus sanguinis* has long been regarded as a pioneering colonizer due to its remarkable capacity to inhabit tooth surfaces, which may be associated with specific cell wall/surface characteristics; further, it is an important participant in plaque biofilm development (4,17). Compared to other streptococcal species in the human microbiome, such as *S. pneumoniae* and *S. mutans*, the ge-

**Table 10.** Range and location of nucleotides amplified according to the high similarity BLAST database.

Seq. ID	Chromosome length	Alignment range	Number of amino acids compared	gene detected	Gene bank accession number	The similarity in amino acid
MW894651	2449527	49969-50262	98	Thr-B	LS483346	none
MW894652	2331387	1097991-1098264	92	pyre	LS483364	none
MW894653	2388435	1265084 - 1265334	83	pyre	CP000387	none
MW894654	2358846	872963-873241	93	pyre	CP054570	none
MW894655	2358846	872955-873241	96	pyre	CP054570	none
MW894656	2358846	872979-873218	80	pyre	CP054570	none
MW894657	2360223	52470 - 52661	64	Thr-B	LR134002	none
MW894658	2449527	1277005-1277277	90	Pyre	LS483346	none



**Figure 5.** The protein alignments of eight separate sequences of pyre E and thr B genes of *S. sanguinis* for detecting amino acid mutations.

nome of *S. sanguinis* comprises a plethora of genes encoding cell wall-attached proteins and lipoproteins (18,19); bacterial biofilm formation is considered part of their survival mechanism. Medical microbiologists are interested in microorganisms' biofilm lifestyle, linking them to a

variety of clinical infections (20). The bacteria within the biofilm cluster adapt to environmental stress and nutrient limitation by exhibiting altered metabolism, gene expression, and protein production, which could also result in a decreased metabolic and cell division (20,21). The above

adaptations make the bacteria more resistant to antimicrobial therapy. Innate and acquired host immune responses can both be activated simultaneously, neither of which can eliminate the biofilm pathogen but instead promotes collateral tissue damage.

Initially, the culture of dental plaque samples showed growth of *S. sanguinis*, confirmed by the Vitek2 compact system. *S. sanguinis* is the most abundant and significantly associated with dental plaque biofilm (22,23). All isolates were verified for their response to antimicrobial agents, and their responses varied. A previous study showed that biofilm formation is affected by the growth medium composition (24). In this study, experiments were performed to investigate biofilm formation using different methods. Highly selective and differential media was used to analyze and approve *S. sanguinis*'s role in forming biofilms.

According to the present study, *S. sanguinis* showed almost the same results in biofilm formation with the CRA media and tube method, while the microtiter plate assay showed a varied concentration of biofilm producers of the isolates. These outcomes are in agreement with the more recent research (25).

Many infections have distinct metabolic pathways connected with the expression of virulence genes that coordinately regulate illness progression in vivo investigations (26,27). Thus, we were heartened to investigate the possible role of *thr B*, *pur B* and *pyre E* genes in the *S. sanguinis* biofilm formation. Several investigations have demonstrated that genes, including *pur B*, *purL*, *pyrE*, *thrB*, *adcA*, *spi*, *sptR*, and *sptS*, influence biofilm formation in *S. sanguinis* via unknown pathways (19,28,29). According to prior studies, genes involved in nucleotide biosynthesis (*pur B*, *pur L*, and *pyr E*) are implicated in the biofilm formation network. Certain nucleotides, including cyclic di-GMP, cyclic di-AMP, cAMP, and (p) ppGpp, have been well explored as tiny molecular signals for influencing biofilm formation in other bacteria (30); however, this has not been established in *S. sanguinis*. More research is needed to determine whether these nucleotides influence biofilm development in *S. sanguinis*.

The present study aimed to characterize more details of the *S. sanguinis* biofilm genes: *thr B*, *pur B* and *pyre E*. Despite their historical link with the illness, oral streptococci virulence factors that contribute to the pathogenic process remain unknown, and many suspected virulence genes have not been tested experimentally. We believe that investigating the genes will eventually assist in improving clinical outcomes, particularly in areas with limited diagnostic and therapeutic choices. In this report, the PCR technique was used to detect biofilm genes *thr B* and *pyre E* of *S. sanguinis* isolates. Eight candidate extract fragments were analyzed by comparing the sequence file with the data available on the NCBI webpage. According to the results, *S. sanguinis* strains had strong similarities to the identical species genomes.

This research is the first to describe the function of the *thr B*, *pur B*, and *pyre E* genes in the development of *S. sanguinis* biofilms, to the best of the researcher's knowledge. They may therefore be utilized to create therapeutic targets to recover dental biofilm homeostasis and to establish a bacterial model for further research into the genetic matrix involved in biofilm construction and function.

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