UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Health Sciences,

School of Medicine

Master's degree in medical biotechnology

Thesis

Designing novel genetic tools to control the biofilm-forming bacterium Aggregatibacter

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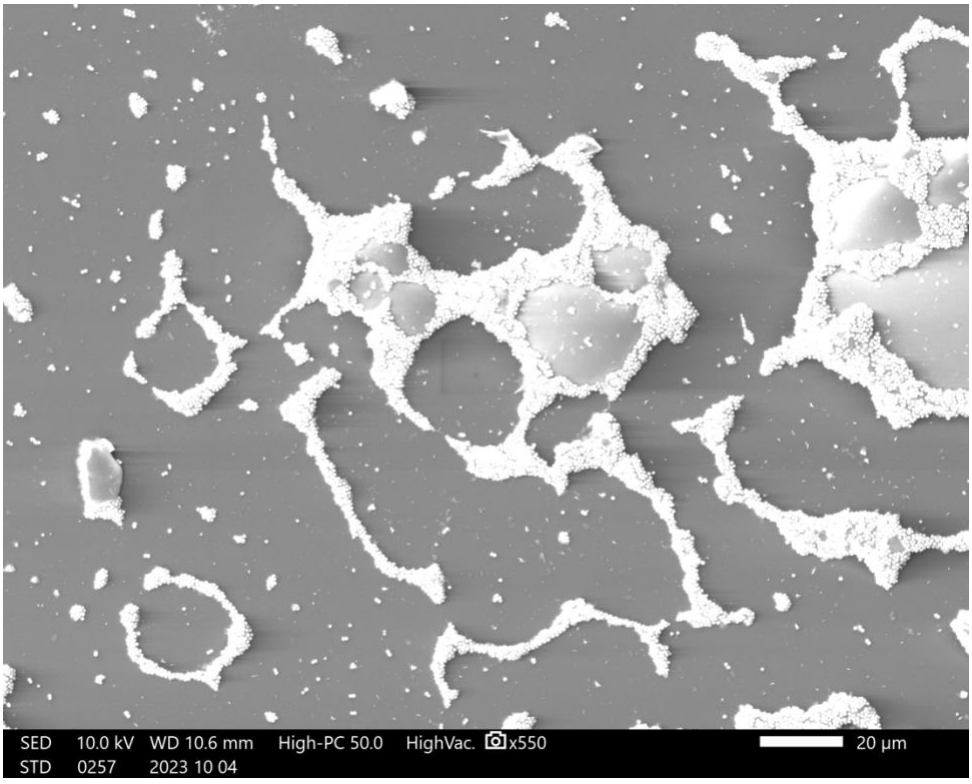
1. INTRODUCTION

Actinobacillus actinomycetemcomitans, reclassified by Nørskov-Lauritsen and Kilian (2006) in *Aggregatibacter actinomycetemcomitans*, is a non-motile, gram-negative, capnophilic, fermentative coccobacillus, described by Klinger (1912). It closely resembles several Haemophilus species but does not require X or V growth factors. Microscopically, cultures appear predominantly bacillary with a few coccal forms. The organism has been categorized into 10 biotypes based on the variable fermentation of dextrin, maltose, mannitol, and xylose, and into 3 serotypes based on the heat-stable cell surface antigens (Zambon JJ, 1985).



A. actinomycetemcomitans is known to be associated with juvenile and chronic adult periodontitis (Slots et al, 1980). *A. actinomycetemcomitans* is commonly part of the normal

flora of the mouth of humans, especially the gingival and supragingival crevices. Periodontal pathogens can persist and adhere to oral surfaces, thanks to adherence mechanisms driven by the production of polymeric substances and lipopolysaccharides. Facilitated by forming aggregates or biofilms, these bacteria release virulence factors, such as leukotoxin, lipopolysaccharides, immunosuppressive factors, and adhesins, that contribute to the stability and development of the infection. Bacterial biofilms are made up of microcolonies organized into functionally heterogeneous communities. Forming biofilms protects bacteria from the immune system and keeps them in constant contact with nutrients, allowing their survival (Haase EM et al, 2006). One species can commonly co-aggregate across multiple partners, producing a dense plaque, immersed in a complex microenvironment.

The genetic diversity of the *A. actinomycetemcomitans* strains is associated with different expression levels of these virulence factors, challenging the association between factor production and the pathophysiology of diseases. Moreover, bacterial and host factors are also critical in extraoral infections, such as endocarditis (Nakano et al, 2007), brain abscesses (Stepanovic et al, 2005), and rheumatoid arthritis (Gómez-Bañuelos E. et al, 2019).

1.1. Clinical significance

Periodontal diseases are highly prevalent and can affect up to 90% of the worldwide population. Gingivitis, the mildest form, is caused by biofilm-forming bacteria. However, gingivitis does not result in losing the supporting structure of teeth adjacent to the gingiva (gum). Periodontitis, of which we know different types, was described for the first time in 1923 by Dr. Bernhard Gottlieb, a Viennese physician, who described a soldier condition who were afflicted by a mild form of periodontal disease, marked by minimal inflammation and advanced bone loss around molar teeth (Fine DH et al, 2015). Actinobacillus (*A. actinomycetemcomitans*) was associated with the disease, not before 1975. A key feature of aggressive periodontitis, indeed, is that the patients exhibit loss of connective tissue and bone support, leading to periodontal attachment loss at multiple teeth and a bilateral loss of tissue (Pihlstrom BL et al, 2005). A. actinomycetemcomitans serotype b is often designed as the prior cause of juvenile and chronic periodontitis. Thanks to the production of host tissue destruction virulence factors, *A. actinomycetemcomitans* induces bone resorption, inhibits host tissue repair, and modulates the

promotion, colonization, and inflammation. (Henderson B. et al, 2002) (P.M. Fives-Taylor et al, 2000). The interaction between virulence factors and the host immune system's response is crucial for the disease development. Phagocyte death, lymphocytic activity impairment and antibody production interference are biological mechanisms largely used. Examples of factors that drive *A. actinomycetemcomitans* colonization includes adhesins (outer membrane proteins vesicles, frimbriae or amorphous material), bacteriocins, invasins and antibiotic resistance. It produces actinobacillin, an antibiotic active against streptococci and Actinomyces. Resistance to tetracyclines, the gold standard in the treatment of periodontal disease, is a distinct tract that evidences the infection's gravity. *A. actinomycetemcomitans* utilize a remarkable mechanism to migrate into deeper tissues, eliciting its uptake into epithelial cells and spreading to adjacent cells by usurping normal epithelial cell function. it is of considerable interest to know that *A. actinomycetemcomitans* possesses so many virulence factors but unfortunate that only a few have been extensively studied.

A. actinomycetemcomitans can be also isolated from a variety of non-oral infections such as bacteremia, septicemia, endocarditis, atherosclerosis, pneumonia, skin infections, osteomyielitis, infectious arthritis, urinary tract infections, and abscesses. Curiously, common forms of periodontal diseases have been associated with cardiovascular disease, stroke, pulmonary disease, and diabetes, but the causal-effect relation has not been established yet.

1.2. Protein production and secretome

It is known that biofilms evade antimicrobial challenges by multiple mechanisms, and it is important to study protein expression by comparing bacteria biofilms with free-floating (planktonic) cells. Previous bioinformatic analysis showed that there are differences in the planktonic and biofilm protein production of *A. actinomycetemcomitans* (Llama-Palacios A. et al, 2017), which comprises more than 20 proteins involved in the general metabolism, metabolism of Purines, Transcription, Amino Acid Biosynthesis, cellular processes, and detoxification. Furthermore, it has been shown that, depending on the growing conditions, even the generation of virulence factors produces varying quantities and qualities.

The secretome of *A. actinomycetemcomitans* is presumed to represent a key element to elucidate its implication in periodontal and systemic disease. However, a complete secretome of this species is largely unexplored, for example, the set of secretion types of machinery used to deliver proteins to the extracellular component is unknown. Demuth et al (2003), demonstrated that *A. actinomycetemcomitans* delivered some virulence factors using the outer membrane vesicles (OMV's) system, or independently. Analyzing mass spectrometry data (LC-MS/MS) and in silico approach, has elucidated a potential overview of the active secretome pathways and molecular basis of *A. actinomycetemcomitans* pathogenic potential (Zijnge V. et al, 2012). Different studies identified ltxA and CDT exotoxins as important virulence factors in the D7S strain during biofilm growth, but also other proteins such as Chaperonin GroL, Flp pilus assembly protein TadD, TadE, and G (involved in intracellular trafficking), Hemolysin A and Macrophage infectivity protein (chaperone functions).

1.3. Biofilm Environment

Periodontal microorganisms form biofilms to survive against harsh environmental conditions. Biofilms are complex and arranged microenvironments composed of an extracellular polymeric substance (EPS) enriched with a wide range of living microorganisms (Gupta P, et al, 2016). Quorum sensing regulates biofilm formation, according to nutrient availability, hydrodynamic conditions, hostile environment, and cell-to-cell communication. This intricate organization drives changes in genetic expression, altering microbial phenotype and leading to the acquisition of antibiotic resistance. It has been shown how altered metabolisms, decreased growth rate, and production of virulence factors reinforce bacteria survival (Rather MA. et al, 2021).

The biofilm formation process involves the adhesion of microorganisms, either irreversibly or reversibly, resulting in the production of planktonic bacteria or microcolonies. Microcolonies develop into mature entities with distinct compositions, shapes, and architectural patterns. A mature biofilm is a heterogeneous mixture of water channels, several signaling and stabilizing molecules such as acyl-homoserine lactones (AHL), lipids, polysaccharides, proteins, and extracellular DNA (eDNA), as well as planktonic, sessile, persistent, and dead cells (Chandki R et al, 2011). The table below shows an overview of the workflow of biofilm formation, starting from a preliminary adhesion to surfaces (reversible phases), followed by a maturation

phase (Irreversible), characterized by a stable attachment and the formation of a complex matrix. The dispersion and propagation phase allows bacteria to survive and begin a new cycle.

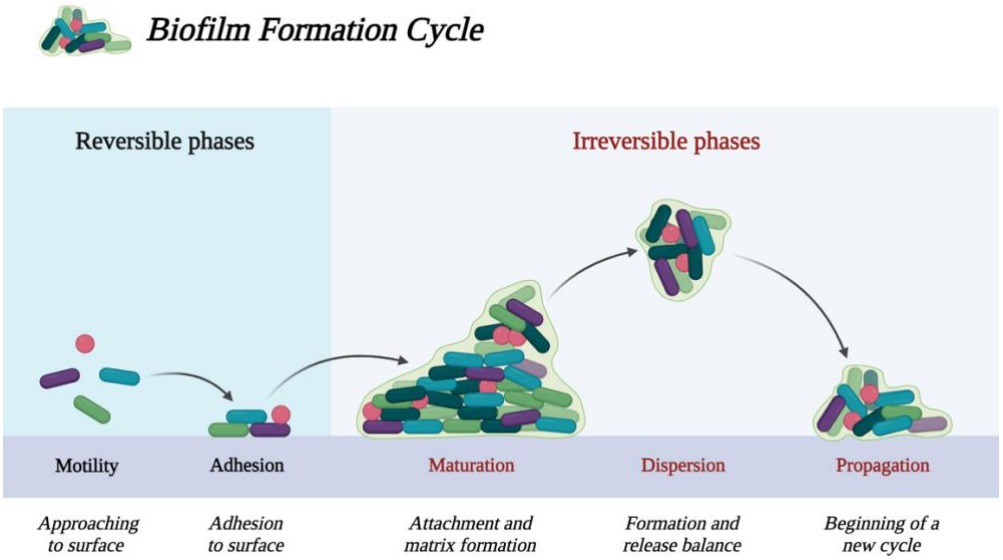


Figure: Biofilm formation phases

1.4. Leucotoxin A (LtxA)

As mentioned, *A. actinomycetemcomitans* produces the RTX (repeats-in-toxin) leukotoxin (LtxA). This large pore-forming protein consists of 1055 amino acids encoded by the ltxA gene in the leukotoxin operon (Johansson A., 2011). It acts as a virulence factor, enabling immune system evasion by binding β_2 integrine lymphocyte function-associated antigen 1 (LFA-1; heterodimer CD11a/CD18) on blood cells (WBCs) (Vega B.A. et al, 2019). LtxA activates neutrophil degranulation, freeing lysosomal enzymes and metalloproteinases (MMPs), and driving apoptosis in leukocytes. Additionally, LtxA activates the inflammasome complex in monocytes/macrophages, activating protein caspase-1, leading to the secretion of pro-inflammatory cytokines IL-1β and IL-18 (Belibasakis G.N. et al, 2019). The expression of secreted protein LtxA is due to the presence of fermentable sugars and oxygen levels. Notably, the anaerobic condition enhances the production of LtxA, which reflects the oral cavity environment.

1.5. Cytolethal distending toxin (CDT)

A novel exotoxin, cytolethal distending toxin (CDT), has been recently identified as the cause of host cell death by blocking their proliferation. CDT is likely to be involved in the elicitation of DNA damage upon the intoxicated host cells. It consists of three different subunits, CdtA, B, and C; Subunits A and C mediate CdtB translocation into the nucleus, hence it causes DNA damage, apoptosis, and arrest of the cell cycle (Belibasakis G.N. et al, 2019). CDT causes gingival epithelium damage, as demonstrated by in vivo experimental models on rat gingival tissue (Ohara M. et al, 2011). Among involvement in the impairment of local immunity, CDT stimulates, as LtxA, the production of pro-inflammatory cytokines such as interferon (IFN)-γ, Interleukin (IL)-1β, IL-6, and IL-8. CDT is also involved in RANKL activation, a crucial molecule that stimulates osteoclast differentiation, driving bone reabsorption.

1.6. Role of biofilm microenvironment on the production of exotoxins

Bacteria thrive and multiply in neo-formed periodontal pockets, due to the detachment of gums from the teeth, establishing a chronic inflammation and gradual damage of bones. According to the pathophysiology of periodontitis and other diseases, the surrounding environment is driven to change dramatically. Bleeding shows the presence of iron-sequestering molecules like lactoferrin and transferrin. Oxygen level is depleted, and CO_2 levels increase, favoring the growth of anaerobic bacterial cells. following the infection in vivo, bacteria experience different conditions ranging from abundant resources to scarcity. The adaptability of bacteria leads to the expression of certain genes that help them to survive in the prevailing conditions (Fletcher JM. Et al, 2001). These results indicate that *A. actinomycetemcomitans* could alter its morphological features to efficiently adapt to a naïve environment, resulting in a niche complex to study and evaluate.

Mono-carbohydrates such as D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, L-arabinose, N-acetyl-D-glucose amine, and others are components of exopolysaccharides (Lempre P. et al, 2012), progressively catabolized by bacteria metabolism, are considered essential for the control of expression of lethal and virulence genes. As previously mentioned, the amount of nutrients available directly affects the expression of exotoxins ltrA and CDT and other virulence factors, varying feeding requirements considering biofilm's phases.

It is essential to gain a deeper understanding of the pathophysiology and to identify new virulence factor genes. This knowledge could be utilized to enhance clinical assessment and to develop a more comprehensive biofilm biology profile. Furthermore, in the future, the assessment of virulence factors could be used to predict specific clinical outcomes.

A. actinomycetemcomitans is considered an opportunistic pathogen. Its transition from a commensal organism to a pathogen depends on cues from the host, other members of the oral commensal microflora, or a combination of both.

1.7. Construction of GFP-expressing strain

Therefore, enabling the analysis of infection dynamics, we considered constructing a GFP-expressing strain of *A. actinomycetemcomitans* useful. According to a DEG analysis, it has been selected a promoter recognized by host cells located upstream of sfGFP to ensure adequate transcription, during the biofilm formation. As previously mentioned, biofilm formation is directly correlated to the survival of A. actinomycetemcomitans and the infection maintenance, it has been selected as a promoter that belongs to a gene upregulated in the biofilm phase, lsrR promoter.

1.8. AIMS AND RATIONALE OF THE STUDY

Progression in molecular analysis of *A. actinomycetemcomitans* virulence factors has continued in recent years, due to the design of experimental systems for genetic transfer, transposon mutagenesis, and gene complementation. The lack of animal models available for an accurate study of the virulence mechanisms of LtxA and other virulence factors is a persistent certainty, because of the high specificity of toxins against human immune system cells. The study aimed to design a vector system based on the customization of a marker gene-expressing strain of *A. actinomycetemcomitans* that would enable following and evaluating the

pathological dynamics of this species in experimental models. By doing so, the *A. actinomycetemcomitans* strain ATCC® 29523™ has been transformed by electroporation with pJT4 plasmid developed by Professor Donald R. Demuth and his team at the University of Louisville (USA), which has inserted the reporter gene "superfolder" Green Fluorescent Protein (sfGFP). The DNA fragment containing the reporter gene sfGFP was excited from the commercially available plasmid *pBAD-sfGFP* (Addgene), purified by gel electrophoresis, and inserted downstream of methylenetetrahydrofolate reductase (MetF) gene promoter, which seems to be upregulated in the biofilm formation phase, confirmed by RT-qPCR and further DEG analysis. This electroporation system allowed the introduction of a stable shuttle plasmid into *A. actinomycetemcomitans* strain.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Media, and Growth Conditions

A commercially available strain of *Aggregatibacter actinomycetemcomitans* ATCC 29523) was purchased from the American Type Culture Collection (ATCC, MA, USA) and cultivated following the manufacturer's instructions. Bacteria were grown from stock in BD Difco™ Bacto™ Brain Heart Infusion (BHI, cod# 10462498) containing 0,1% Agar, and Fildes enrichment of 6.5% sodium chloride. Solid medium was supplemented with 15 g of agar per liter. All cultures were incubated at 37°C in an orbital shaker under aerobic conditions. After plasmid transformation, *A. actinomycetemcomitans* was grown in the same medium containing 50 µg/ml Kanamycin. *Escherichia coli* strain DH5α was grown aerobically in 2x Yeast Extract Tryptone medium (2xYT, Sigma-Aldrich, cod# Y2377) supplemented with Kanamycin 50 µg/ml when appropriate, at 37°C.

2.2. Biofilm formation

To enter the logarithmic growth phase, a subculture of bacteria was grown by incubating 50 ml tubes on an orbital shaker at 37°C for 3 hours. After achieving an optical density (OD_{600}) of

0.001 (corresponding to approximately 1×10^5 cells/ml), 1 ml of bacteria was plated on a polystyrene 6/w tissue culture plate with a glass coverslip on the bottom of each well and placed on a revolving shaker (120 rpm) at 37°C for 90 minutes. The supernatant was collected to remove the floating (planktonic) cells. Non-adherent bacteria were rinsed with 1x phosphate-buffered saline (PBS, 136.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.46 mM KH_2PO_4, 0.46 mM $MgCl_2$, pH 7.4), and additional BHI broth was added. Biofilm samples were collected at multiple time checkpoints (24 -48 -72 hours, respectively).

To collect the bacteria from the biofilm, the coverslips were transferred in a 15 ml tube with the addition of PBS 1x. Biofilm has been detached using the sonication/vortexing-based method (sonicate for 7 minutes and vortex for 40 seconds, at full speed). Finally, the PBS solution containing the biofilm was transferred into 1.5 ml tubes. For the correct storage, the tubes containing planktonic and biofilm bacteria were centrifugated at 18.000 g for 10 minutes. After removing the supernatant, the pellet was stored at -80°C.

2.3. Strain confermation

To confirm the genotype of *A. actinomycetemcomitans* ATCC 29523 strain was performed a real-time PCR (qPCR), using designed primers for the 16S ribosome subunit of *A. actinomycetemcomitans, Staphylococcus Aureus, Staphylococcus Epidermidis and Escherichia coli* was used as positive control.

Oligonucleotides	**Sequence (5'- 3')**	**Tm**
S.A_16SV1V3_qPCR_F	CGGGAAACCGGAGCTAATAC	63
S.A_16SV1V3_qPCR_R	TGATTAGGTACCGTCAAGATGTG	63
S.E_16SV1V3_qPCR_F	GGGTGAGTAACACGTGGATAAC	63.5
S.E_16SV1V3_qPCR_R	GTACCGTCAAGACGTGCATAG	63.5
A.A_16SV4_qPCR_F	GGACCTTTAAGTGAGGTGTGAA	63
A.A_16SV4_qPCR_R	ACCAGGGTATCTAATCCTGTTTG	63

2.4. RNA extraction

A. actinomycetemcomitans planktonic and biofilm RNA has been extracted using RNeasy Kits for RNA purification (QIAGEN), following manufacturer's instructions, at 24, and 72-hour time points in both conditions (planktonic and biofilm). Bacterial lysis consists of treatment with 15 mg/ml lysozyme for 10 min, contained in TE buffer (30 mM Tris·Cl, 1 mM EDTA, pH 8.0). 10–20 µl QIAGEN Proteinase K was added to the appropriate volume of TE buffer containing lysozyme into the pellet. After adding ethanol, a precipitate may form.

2.5. Next-generation sequencing (NGS)

RNA-Seq Transcriptomic analysis of log-phase bacteria was performed in 2 experimental conditions, each with three biological replicates. These include the growth of planktonic strain ATCC 29523 in BHI medium, the same with growth-biofilm-forming strain, following specific time points of 24 and 48 hours. The starter bacterial cultures were prepared by transferring 10–15 bacteria colonies from agar into 5 ml of BHI and incubated overnight at 37 ◦C in a humidified incubator. The colony forming unit/ml was estimated based on optical density (OD600). An aliquot (0.2–0.4 mL) of the bacterial culture was transferred into each well of a polystyrene 6-well tissue culture plate (SARSTEDT, TC-Platte 6 well, Suspension), and 3 mL of fresh mBHI was added to each well.

The plate was then incubated for 24 and 72 hrs respectively. For biofilm-forming ATCC 29523, the culture supernatant was removed, and the biofilm attached to the bottom of the well was gently rinsed with warm fresh medium once, and then 2 mL of fresh mBHI.
The bacterial cells were then collected with the aid of a cell scraper (Greiner Bio-One, cat #10516762, Fisher Scientific), pelleted by centrifugation at 10,000 rpm for 2 min, kept at 4 ◦C for one hour, and then stored at −80 ◦C until used.
To collect the planktonic phase, a 24-72 hours' time point was followed, as previously mentioned. 2 ml of the culture was substituted with pre-warmed mBHI and incubated. OD600 was measured to ensure bacteria were still in the log phase.

2.6. Differentially Gene Expression (DGE) analysis

We aimed to study the differential expression of *A. actinomycetemcomitans* protein-coding genes, when growing in biofilms and planktonic state, by using Differential gene expression (DEG) analysis. As mentioned before, RNA was extracted from biofilm and planktonic state following different time points, scheduled as planktonic 24 hours-72 hours and the same with biofilm samples. Consequently, a DEG analysis was outsourced to a transcriptomics laboratory in UPO-CAAD (Center for Autoimmune and Allergic Diseases, AOU).

2.7. Validation of DGE analysis by quantitative PCR (RT-qPCR)

The extracted RNA was previously treated with a DNAse I (Sigma-Aldrich, Catalog number AMPD1). For 500 ng, 1 µl of DNAse has been added (amplification grade 1 Unit/µl) into a final volume of 10 µl. Reverse transcriptase reaction has been performed using iScript cDNA Synthesis Kit (BIO-RAD, cat. #1708890). SYBER Selected Master Mix for CFX (applied biosystem by life technologies, cat. # 4474515) was the choice for Real-Time PCR Assay. 16S v4 *A. actinomycetemcomitans* isoform was selected as the housekeeping gene; MetF was tested using different time point conditions for biofilm and planktonic form (Planktonic 24h vs Biofilm 24h; Planktonic 24h vs Biofilm 72h; Planktonic 72h vs Biofilm 72h).

To validate the expression levels obtained by RNA-Seq, quantitative real-time PCR (qRT-PCR) was also performed on selected genes, such as metF, and hypothetical proteins GIBDFADM_00611, GIBDFADM_00734, GIBDFADM_00204, and GIBDFADM_01265, respectively. According to DEG analysis, two Housekeeping genes were used to validate the expression levels of upregulated genes, such as GyrA and 16S v3 isoform.

Oligonucleotides	**Sequence (5'- 3')**	**Tm**
A.A_metF_qPCR_F	ACATGGTGCGTGTACTGTC	63
A.A_metF_qPCR_R	AACTCGGACGAATGCCTAAC	63

A.A_gyrA_qPCR_F	CGGTGACGTGATCGGTAAAT	63
A.A_gyrA_qPCR_R	GCCCGTCCACCAACATATAA	63
A.A_GIBDFADM_00734_qPCR_F	ACCGAGCAGAATTTGGGTATC	63
A.A_GIBDFADM_00734_qPCR_R	TACGCCATTAAGCCCTCTTTC	63
A.A_GIBDFADM_00611_qPCR_F	CACATGGGATTATTGGCAACAG	63
A.A_GIBDFADM_00611_qPCR_R	CTGATTTATCGGCGGGAGTT	63
A.A_GIBDFADM_00204_qPCR_F	TGCCCAAGCCCAACAATTA	63
A.A_GIBDFADM_00204_qPCR_R	GCGTGGTGCTTTGTTCAATC	63
A.A_GIBDFADM_01265_qPCR_F	GGTTGGTGTTGAGCGATTTG	63
A.A_GIBDFADM_01265_qPCR_R	GCAAAGCGTAAGGAGCAATG	63
A.A_glgX_qPCR_F	CCTTGGGACATCGGACATTAT	63
A.A_glgX_qPCR_R	ACCAGAAACGGCACATATCA	63
A.A_16SV4_qPCR_F	GGACCTTTAAGTGAGGTGTGAA	63
A.A_16SV4_qPCR_R	ACCAGGGTATCTAATCCTGTTTG	63

2.8. Plasmid Customization and Primers

pJT4 plasmid was a kind gift of Prof. Donald R. Demuth (the University of Louisville School of Dentistry, Louisville, KY, USA). It contains the putative origin of replication, two ORFs, and the cluster of genes involved in the mobility of pYGK-LacZ, such as *MobA*, *MobB*, and *MobC*. Additionally, it contains *aph(3')Ia*, a transposon-encoded aminoglycoside phosphotransferase in E. coli and S. enterica and *strB*, streptomycin resistance protein. Starting from pJT4, other two plasmids were constructed, whose contain respectively *MetF* and *lsrR* promoter upstream of the *sfGFP* sequence, amplified by *pBad-sfGFP*, a gift from Ryan Mehl (Addgene plasmid # 85482; http://n2t.net/addgene:85482; RRID: Addgene_85482). *BamHI*

and *XbaI* restriction enzyme sites were used to insert the previously amplified constructs from *A. actinomycetemcomitans* gDNA, resulting in the transcriptional/translational reporter plasmids. Primers used to amplify MetF and lsrR promoter, sfGFP are evidenced in the table below.

Oligonucleotides	**Sequence (5'- 3')**	**Tm**
PromMetF_BamHI_F	CACggatccGCAAAAGTGCGGTCATAAAAAATG	63
PromMetF_ovl_sfGFP_R	CTTTGCTAACCATAAAGTTGCCTCGTGTTTTC	61
PromLsrR_BamHI_F	CACggatcCTTTTACCTGCCGCATAGAAT	61
PromLsrR_ovl_sfGFP_R	CTTTGCTAACCATATGATTTATTTGTTTTTATTGATTTTGGG	61
sfGFP_ovl_PromMetF_F	AGGCAACTTTATGGTTAGCAAAGGTGAAGAAC	62
sfGFP_ovl_PromLsrR_F	AATAAATCATATGGTTAGCAAAGGTGAAGAAC	62
rrnBT2 terminator_XbaI_R	CACtctagaAAAAGGCCATCCGTCAGG	63

2.9. Heat shock transformation

E. coli DH5α competent cells were transformed according to the *Standard Operating Procedure*, to propagate pJT4 plasmid. After leaving to thaw competent cells on ice for 20 minutes, 100 ng of purified plasmid DNA was added into 50 µL of competent cells. The mixture was incubated on ice for 20 minutes and subsequently placed into a 42°C water bath for 60 sec; then returned the tube mixture on ice for 2 minutes. Transformed cells were recovered on a shaking incubator for 1 hour at 37°C, adding 1 ml of 2xTy broth (Sigma-Aldrich cat. Y2377). Consequently, transformed bacteria were plated on 2xTy agar plates containing Kanamycin [50mg/ml] and incubated overnight at 37°C.

2.10. Genomic DNA extraction

Genomic DNA was extracted using the phenol/chloroform method without using commercial kits.

1) Lysis phase - An overnight culture of *A. actinomycetemcomitans* grown in Brain Heart Infusion broth (BHI). After centrifugation with max speed to pellet 1,5 ml of overnight culture, the mix has been resuspended with 600 µl of Lysis Buffer, composed of TE buffer (10mM Tris-Cl, 1Mm EDTA, pH 8.0) and 0,6% of SDS. The suspension was incubated on a rotatory platform for 10 minutes mixed with Lysozyme solution [20 mg/ml] (e.g. Sigma-Aldrich cat. 62971) and RNAse A solution [10 mg/ml] (e.g. Sigma-Aldrich cat. R5503 or Thermo Fisher Scientific Cat. EN0531). consequently, Proteinase K solution [20 mg/ml] was added. The mixture was incubated overnight at 50°C.

An equal amount of phenol/chloroform/isoamyl alcohol solution (25:24:1) (e.g. Sigma-Aldrich cat. P2069) was added. Serial spin (at max speed, RT) is essential for forming the phenol/chloroform interface. The upper aqueous phase is transferred in a new 1,5 ml Eppendorf tube.

2) Precipitation of gDNA - 2.5 volume of cold absolute ethanol has been added to precipitate the gDNA, and consequently incubated at -20°C for 30 minutes. Following spinning with max speed for 15 minutes at 4°C; the DNA pellet was rinsed with 70% ethanol. Dried DNA was resuspended in 100 µl of TE buffer previously used. The isolated gDNA was checked on 0,8% agarose gel.

2.11. Preparation of electrocompetent *A. actinomycetemcomitans*

ATCC 29523 has been streaked out of frozen glycerol stock 80% into a BHI plate without antibiotics and lived overnight at 37°C. The next day, 400 ml of BHI broth have been inoculated with a single colony. Subsequently an overnight growth at 37°C with 200 rpm shaking, the subculture was inoculated with 400 ml of pre-warmed BHI medium. After reaching an OD_{600} which corresponds to 0.3, the culture was split into four sterile centrifuge tubes. Cells have been collected by centrifugation at 5000 x g for 10 min at room temperature (RT). Pellets were resuspended with 25 ml of RT Deionized water (Milli-Q). The step of centrifugation is repeated another time. Now, each pellet was resuspended in 10 ml of 10% glycerol and centrifugated at 5000 x g for 10 minutes. Consequently, the pellet is resuspended with a proportional less amount of 10% glycerol (5 ml, 2,5 ml, 1 ml, 400 ul). 50 ul aliquots were dispensed in 1.5 ml Eppendorf tubes and stored at -80°C.

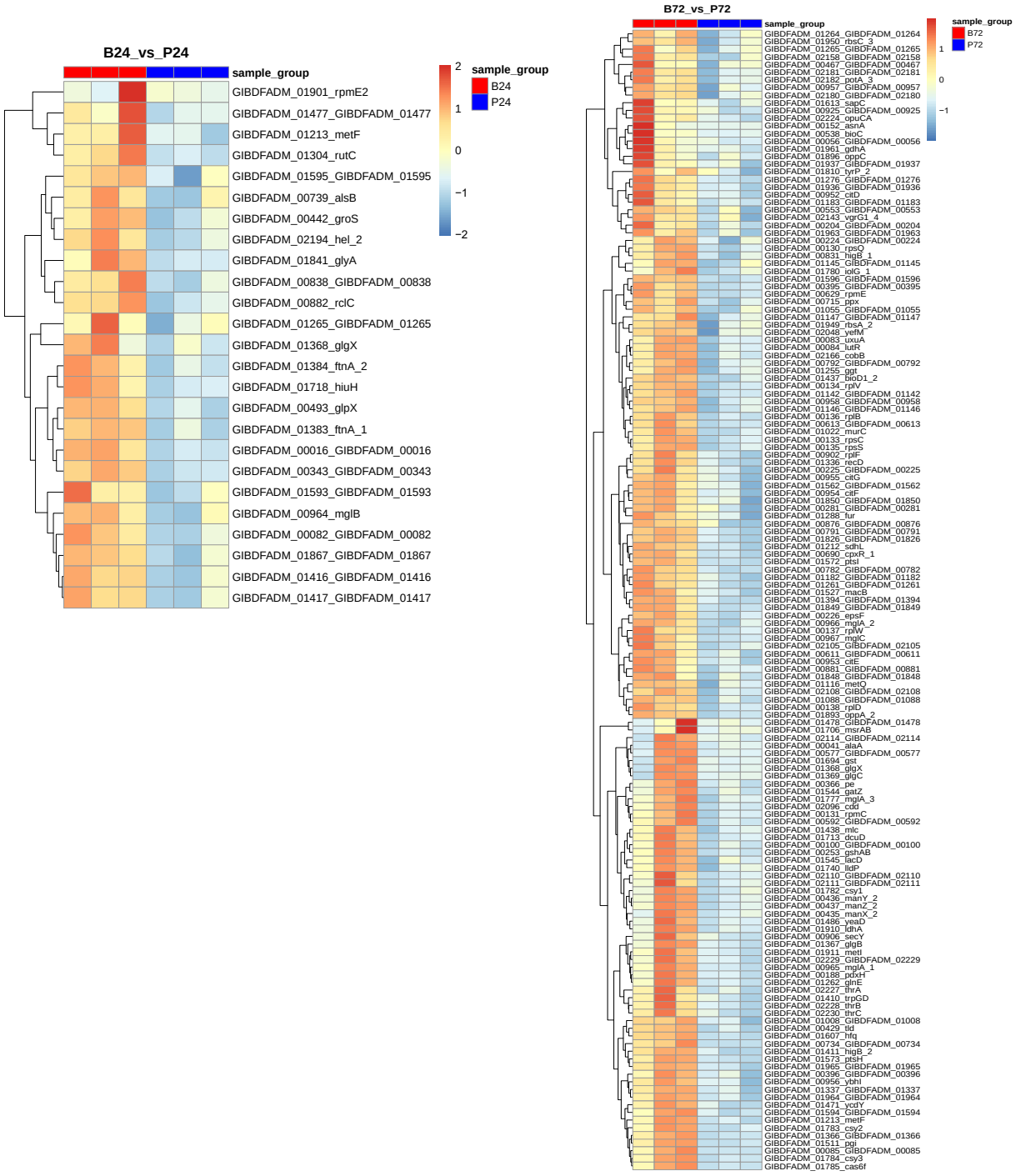
2.12. Electroporation of *A. actinomycemcomitans*

Competent cells were collected from -80°C and placed on ice for 5 min. Competent cells were enriched with 1-5 μg of purified pJT5 plasmid and transferred in a pre-cooled 0,1 mm electroporation cuvette (Gene Pulser/MicroPulser Electroporation Cuvettes, 0.1 cm gap #1652093). It used a Biorad Electroporator to pulse cells at 25 kV/cm 100 Ω and 25 mF (the time constant is usually about 4.8 – 5.8 ms). After the pulsation, it has been added fresh BHI media (1 ml). Cells were transferred into 15 ml round-shaped tubes and incubated for 4 hours at 37°C. After 4 hours, electroporated cells were spread on BHI plates supplemented with selective antibiotic Kanamycin [50 μg/ml]. 24h/48h of incubation was necessary for the appearance of distinct colonies.

3. RESULTS

3.1. NGS and DEG analysis

RNA-Seq Transcriptomic analysis was performed using RNA samples of *A. actinomycetemcomitans* ATCC 29523 strain, collected respectively in the planktonic and biofilm phases. For each gene, the transcript level was the average of 3 biological replicates in each growth condition (biofilm and planktonic 24-72 hours). Differential gene expression (DEG) analysis was outsourced to a transcriptomics laboratory in UPO-CAAD (Center for Autoimmune and Allergic Diseases, AOU). Heatmaps graphs below depict the differential expression of a wide range of genes by comparing biofilm 24h vs planktonic 24h (table 1) and biofilm 72h vs planktonic 72h (table 2). Notably, the number of genes expressed in Biofilm 72h and Planktonic 72h is higher compared to genes expressed in Biofilm 24h vs Planktonic 24h.


B24_vs_P24
sample_group
GIBDFADM_01901_rpmE2
GIBDFADM_01477_GIBDFADM_01477
GIBDFADM_01213_metF
GIBDFADM_01304_rutC
GIBDFADM_01595_GIBDFADM_01595
GIBDFADM_00739_alsB
GIBDFADM_00442_groS
GIBDFADM_02194_hel_2
GIBDFADM_01841_glyA
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GIBDFADM_01417_GIBDFADM_01417
2
1
0
−1
−2
sample_group
B24
P24
B72_vs_P72
sample_group
GIBDFADM_01264_GIBDFADM_01264
GIBDFADM_01950_rbsC_3
GIBDFADM_01265_GIBDFADM_01265
GIBDFADM_02158_GIBDFADM_02158
GIBDFADM_00467_GIBDFADM_00467
GIBDFADM_02181_GIBDFADM_02181
GIBDFADM_02182_potA_3
GIBDFADM_00957_GIBDFADM_00957
GIBDFADM_02180_GIBDFADM_02180
GIBDFADM_01613_sapC
GIBDFADM_00925_GIBDFADM_00925
GIBDFADM_02224_opuCA
GIBDFADM_00152_asnA
GIBDFADM_00538_bioC
GIBDFADM_00056_GIBDFADM_00056
GIBDFADM_01961_gdhA
GIBDFADM_01896_oppC
GIBDFADM_01937_GIBDFADM_01937
GIBDFADM_01810_tyrP_2
GIBDFADM_01276_GIBDFADM_01276
GIBDFADM_01936_GIBDFADM_01936
GIBDFADM_00952_citD
GIBDFADM_01183_GIBDFADM_01183
GIBDFADM_00553_GIBDFADM_00553
GIBDFADM_02143_vgrG1_4
GIBDFADM_00204_GIBDFADM_00204
GIBDFADM_01963_GIBDFADM_01963
GIBDFADM_00224_GIBDFADM_00224
GIBDFADM_00130_rpsQ
GIBDFADM_00831_higB_1
GIBDFADM_01145_GIBDFADM_01145
GIBDFADM_01780_ioIG_1
GIBDFADM_01596_GIBDFADM_01596
GIBDFADM_00395_GIBDFADM_00395
GIBDFADM_00629_rpmE
GIBDFADM_00715_ppx
GIBDFADM_01055_GIBDFADM_01055
GIBDFADM_01147_GIBDFADM_01147
GIBDFADM_01949_rbsA_2
GIBDFADM_02048_yefM
GIBDFADM_00083_uxuA
GIBDFADM_00084_lutR
GIBDFADM_02166_cobB
GIBDFADM_00792_GIBDFADM_00792
GIBDFADM_01437_bioD1_2
GIBDFADM_00134_rplV
GIBDFADM_01142_GIBDFADM_01142
GIBDFADM_00958_GIBDFADM_00958
GIBDFADM_01146_GIBDFADM_01146
GIBDFADM_00136_rplB
GIBDFADM_00613_GIBDFADM_00613
GIBDFADM_01022_murC
GIBDFADM_00133_rpsC
GIBDFADM_00135_rpsS
GIBDFADM_00902_rplF
GIBDFADM_01336_recD
GIBDFADM_00225_GIBDFADM_00225
GIBDFADM_00955_citG
GIBDFADM_01562_GIBDFADM_01562
GIBDFADM_00954_citF
GIBDFADM_01850_GIBDFADM_01850
GIBDFADM_00281_GIBDFADM_00281
GIBDFADM_01288_fur
GIBDFADM_00876_GIBDFADM_00876
GIBDFADM_00791_GIBDFADM_00791
GIBDFADM_01826_GIBDFADM_01826
GIBDFADM_01212_sdhL
GIBDFADM_00690_cpxR_1
GIBDFADM_01572_ptsI
GIBDFADM_00782_GIBDFADM_00782
GIBDFADM_01182_GIBDFADM_01182
GIBDFADM_01261_GIBDFADM_01261
GIBDFADM_01527_macB
GIBDFADM_01394_GIBDFADM_01394
GIBDFADM_01849_GIBDFADM_01849
GIBDFADM_00226_epsF
GIBDFADM_00966_mglA_2
GIBDFADM_00137_rplW
GIBDFADM_00967_mglC
GIBDFADM_02105_GIBDFADM_02105
GIBDFADM_00611_GIBDFADM_00611
GIBDFADM_00953_citE
GIBDFADM_00881_GIBDFADM_00881
GIBDFADM_01848_GIBDFADM_01848
GIBDFADM_01116_metQ
GIBDFADM_02108_GIBDFADM_02108
GIBDFADM_01088_GIBDFADM_01088
GIBDFADM_00138_rplD
GIBDFADM_01893_oppA_2
GIBDFADM_01478_GIBDFADM_01478
GIBDFADM_01706_msrAB
GIBDFADM_02114_GIBDFADM_02114
GIBDFADM_00041_alaA
GIBDFADM_00577_GIBDFADM_00577
GIBDFADM_01694_gst
GIBDFADM_01368_glgX
GIBDFADM_01369_glgC
GIBDFADM_00366_pe
GIBDFADM_01544_galZ
GIBDFADM_01777_mglA_3
GIBDFADM_02096_cdd
GIBDFADM_00131_rpmC
GIBDFADM_00592_GIBDFADM_00592
GIBDFADM_01438_mlc
GIBDFADM_01713_dcuD
GIBDFADM_00100_GIBDFADM_00100
GIBDFADM_00253_gshAB
GIBDFADM_01545_lacD
GIBDFADM_01740_lldP
GIBDFADM_02110_GIBDFADM_02110
GIBDFADM_02111_GIBDFADM_02111
GIBDFADM_01782_csy1
GIBDFADM_00436_manY_2
GIBDFADM_00437_manZ_2
GIBDFADM_00435_manX_2
GIBDFADM_01486_yeaD
GIBDFADM_01910_ldhA
GIBDFADM_00906_secY
GIBDFADM_01367_glgB
GIBDFADM_01911_metI
GIBDFADM_02229_GIBDFADM_02229
GIBDFADM_00965_mglA_1
GIBDFADM_00188_pdxH
GIBDFADM_01262_glnE
GIBDFADM_02227_thrA
GIBDFADM_01410_trpGD
GIBDFADM_02228_thrB
GIBDFADM_02230_thrC
GIBDFADM_01008_GIBDFADM_01008
GIBDFADM_00429_lld
GIBDFADM_01607_hfq
GIBDFADM_00734_GIBDFADM_00734
GIBDFADM_01411_higB_2
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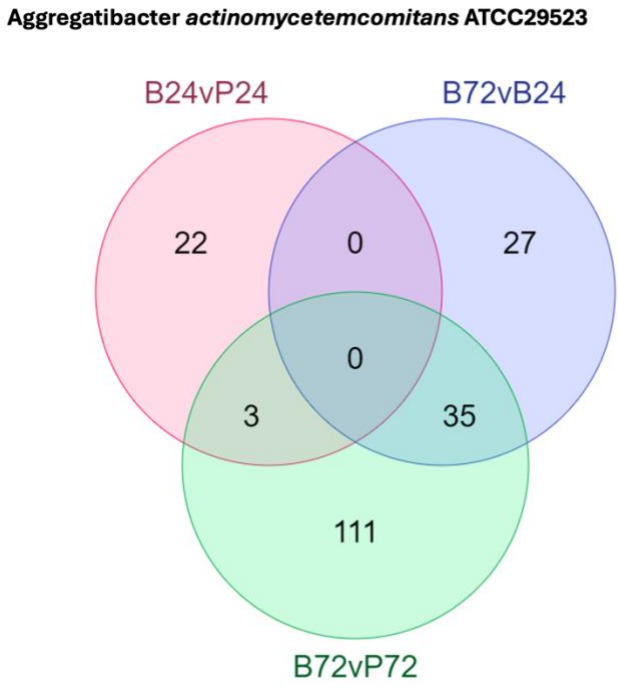
GIBDFADM_01471_ycdY
GIBDFADM_01594_GIBDFADM_01594
GIBDFADM_01213_metF
GIBDFADM_01783_csy2
GIBDFADM_01366_GIBDFADM_01366
GIBDFADM_01511_pgi
GIBDFADM_00085_GIBDFADM_00085
GIBDFADM_01784_csy3
GIBDFADM_01785_cas6f
1
0
−1
sample_group
B72
P72

3.2. Identification of genes upregulated at 24-72 h

A Venn diagram was generated to select upregulated genes that are in common within the two experimental group conditions (Biofilm *vs* Planktonic at 24h and 72h), and Biofilm 72h vs Biofilm 24h). None of the DEG analysis upregulated genes selected belongs to the intersection of the three groups. According to this, we decided to select six upregulated genes that were present in the intersection between B24vsP24 and B72vsP72 minus B72vsB24 and the union between B24vsP24 and B72vsB24 minus B72vsP72. Selected genes represent the MetF gene, GlgX gene, and four hypothetical proteins GIBDFADM_00611, GIBDFADM_00734, GIBDFADM_00204, and GIBDFADM_01265.



(B24vP24 ∩ B72vP72) − B72vB24

"GIBDFADM_01213_metF"
"GIBDFADM_01265_GIBDFADM_01265"
"GIBDFADM_01368_glgX"

B72vP72 − (B24vP24 ∪ B72vB24)

"GIBDFADM_00734_GIBDFADM_00734"
"GIBDFADM_00204_GIBDFADM_00204"
"GIBDFADM_00611_GIBDFADM_00611"

3.3. Hypothetical proteins physicochemical properties analysis

Further in-depth, we retrieved a few different upregulated genes during biofilm formation, compared to the planktonic phase, through DEG expression analysis. These genes are hypothetical proteins named GIBDFADM_00611, GIBDFADM_00734, GIBDFADM_00204, and GIBDFADM_01265, respectively. Starting from an annotated genome A. actinomycetemcomitans, followed by searching the NCBI database (uniprot.org/blast/uniprotkb/ncbiblast/), a sequence-based peptide search was performed to determine whether or not these proteins are redundant. Chemical and physical attributes of the hypothetical proteins were assessed using the ProtParam tool on the ExPASSy website (https://web.expasy.org/protam/). The website provides theoretical metrics of molecular mass, amino acids composition, totally positive and negative residue count, extinction coefficient, theoretical pH, aliphatic index (AI), instability index (II), and grand average of hydropathicity. HP GIBDFADM_00734 results having redundant homology with Transposase enzyme, involved in the cutting and splicing of large quantities of DNA. HP GIBDFADM_00611, instead, has similarities with different types of proteins such as Competence C protein and Fimbrial assembly protein. HP GIBDFADM_00204 and GIBDFADM_01265 show high sequence redundancy with DUF4123 and CRISPR-associated proteins, respectively (Masum MHU et al, 2023).

3.3.1. GIBDFADM_00734

Protoparam parameters	Values
Number of amino acids	375
Molecular weight (MW)	32120.70
Theoretical pI	5,09
Formula	$C_{1180}H_{1987}N_{375}O_{510}S_{79}$
Total number or atoms	4131
Extinction coefficient	4875 Abs 0.1% (=1 g/l) 0.152, assuming all pairs of Cys residues form cystines
Estimated half-life	7.2 hours (mammalian reticulocytes, in vitro).

	>20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).
Instability index	44.80 This classifies the protein as unstable.
Aliphatic index	22.13
Grand average of hydropathicity (GRAVY)	0.591

3.3.2. GIBDFADM_00611

Protoparam parameters	Values
Number of amino acids	525
Molecular weight (MW)	44182.10
Theoretical pI	5.06
Formula	$C_{1627}H_{2731}N_{525}O_{694}S_{107}$
Total number or atoms	5684
Extinction coefficient	6625 Abs 0.1% (=1 g/l) 0.150, assuming all pairs of Cys residues form cystines
Estimated half-life	7.2 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).
Instability index	17.94 This classifies the protein as stable.
Aliphatic index	25.52
Grand average of hydropathicity (GRAVY)	0.657

3.3.3. GIBDFADM_01265

Protoparam parameters	Values
Number of amino acids	2034
Molecular weight (MW)	166542.91
Theoretical pI	4.93
Formula	$C_{6116}H_{10200}N_{2034}O_{2509}S_{442}$
Total number or atoms	21301
Extinction coefficient	27625 Abs 0.1% (=1 g/l) 0.166, assuming all pairs of Cys residues form cystines
Estimated half-life	4.4 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).
Instability index	39.24 This classifies the protein as stable.
Aliphatic index	32.35
Grand average of hydropathicity (GRAVY)	0.872

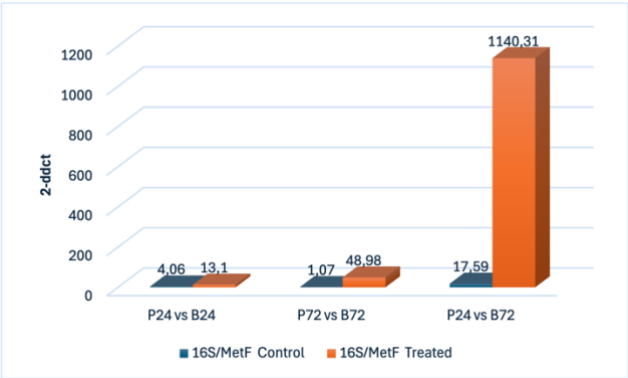
3.3.4. GIBDFADM_00204

Protoparam parameters	Values
Number of amino acids	1179
Molecular weight (MW)	99082.50
Theoretical pI	5.03
Formula	$C_{3635}H_{6093}N_{1179}O_{1509}S_{269}$
Total number or atoms	12685
Extinction coefficient	16750 Abs 0.1% (=1 g/l) 0.169, assuming all pairs of Cys residues form cystines
Estimated half-life	30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo
Instability index	46.75 This classifies the protein as unstable.
Aliphatic index	29.69
Grand average of hydropathicity (GRAVY)	0.831

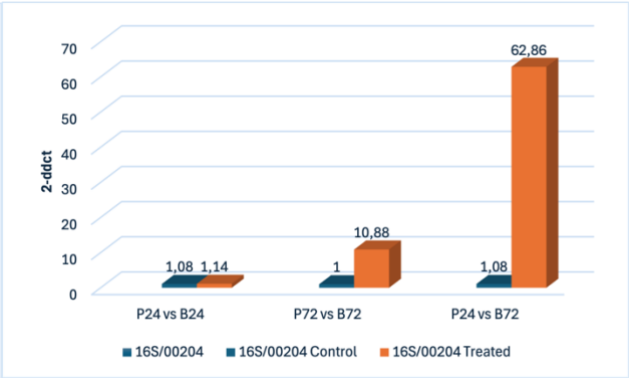
3.4. qRT-PCR

To confirm the expression levels obtained by RNA-Seq, quantitative real-time PCR (qRT-PCR) was also performed on selected genes MetF, glgX, GIBDFADM_00611, GIBDFADM_00734, GIBDFADM_00204, and GIBDFADM_01265, housekeeping gene 16S v3 isoform of *A. actinomycetemcomitans*. The primers used are shown in the materials and methods section. *A. actinomycetemcomitans* RNA (Biofilm24 vs Planktonic24, Biofilm72 vs Planktonic72, Biofilm72 vs Planktonic24 used) were samples previously collected and used for RNA-seq Transcriptomics analysis.

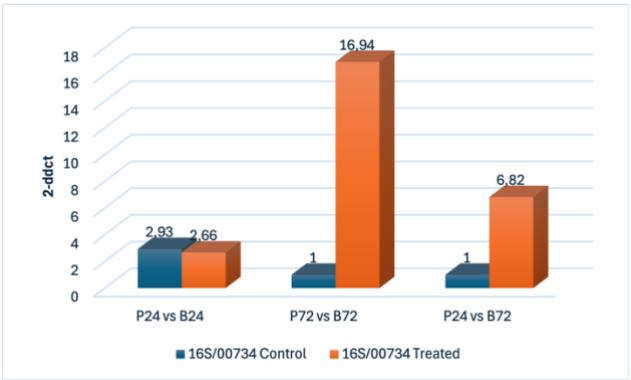
A)



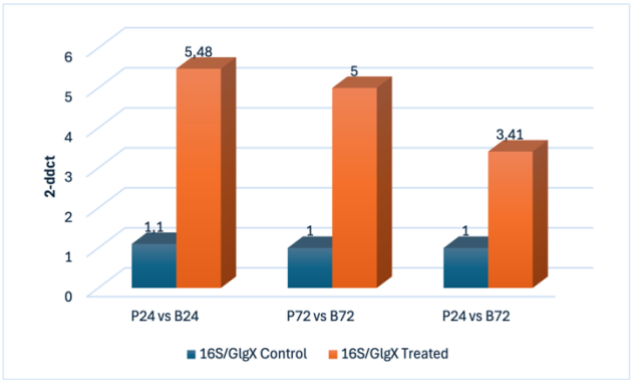
B)



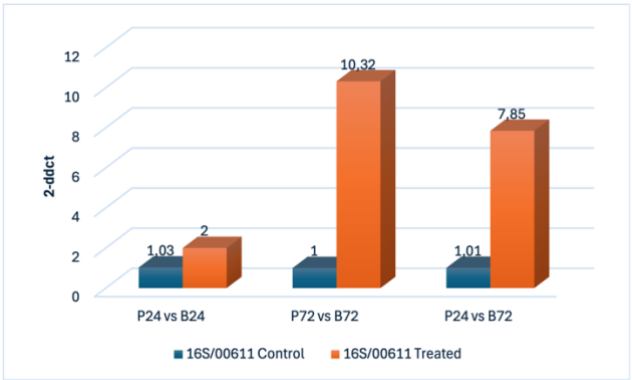
C)



D)

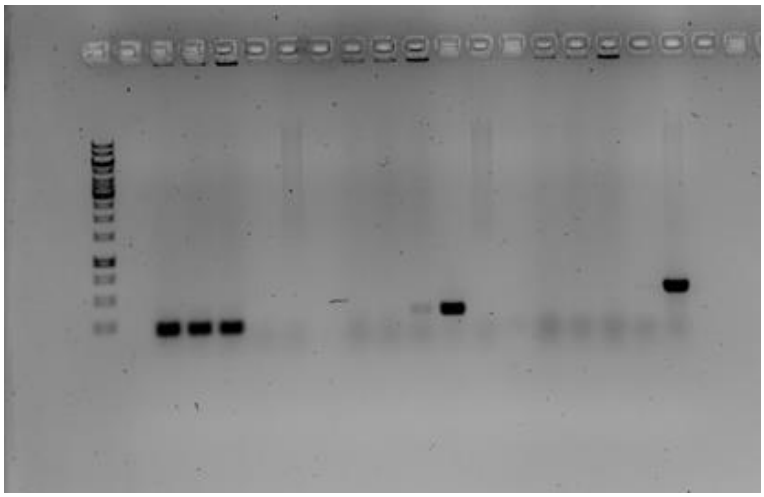


E)



3.5. Strain confirmation

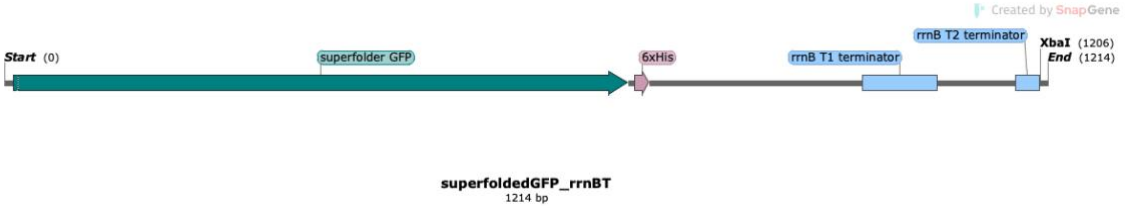
To validate the *Aggregatibacter actinomycetemcomitans* strain ATCC 29523, we performed a quantitative PCR using 16S v3 housekeeping genes of *A. actinomycetemcomitans*, Staphylococcus Aureus ATCC 43300, Staphylococcus epidermidis ATCC 51625, *Escherichia coli* DH5α as positive control.



3.6. Cloning of MetF and lsrR promoter into pJT4 plasmid

Selected MetF, a Methylenetetrahydrofolate reductase, as an upregulated gene, we extracted the promoter into the *A. actinomycetemcomitans* annotated genome. The length of the MetF promoter amplicon is 177 bp. As a positive control, chosen a constitutive promoter of the lsrR gene, which encodes for a transcriptional regulator involved in the expression control of gene encoding toxins and bacterial virulence factors, whose evidenced importance in biofilm formation. The lsrR promoter used is 246 bp in length. The DNA fragment containing the reporter gene sfGFP was amplified from the commercially available plasmid *pBAD-sfGFP* (Addgene) (A), and inserted downstream of methylenetetrahydrofolate reductase (MetF) gene promoter (B), upregulated in biofilm phases, and gene lsrR promoter (C) as a positive control. Obtained inserts using Overlap PCR, both fragments were cloned into pJT4 plasmid, previously described (D and E).

A)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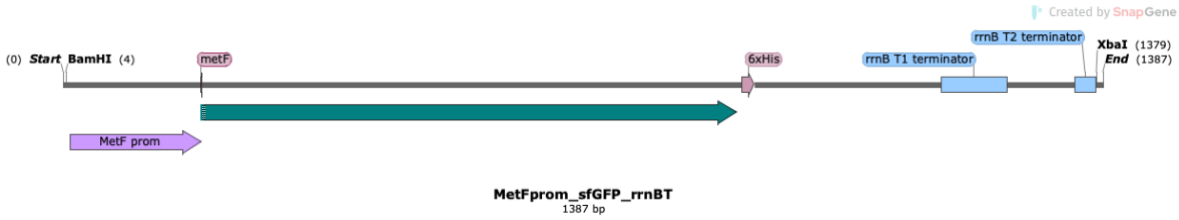
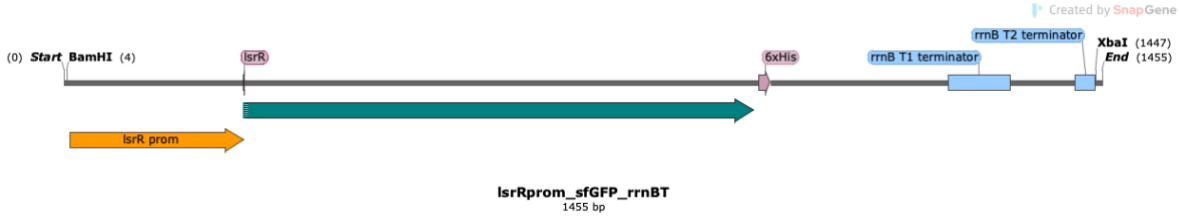
Start (0)
superfolder GFP
6xHis
rrnB T1 terminator
rrnB T2 terminator
XbaI (1206)
End (1214)
superfoldedGFP_rrnBT
1214 bp

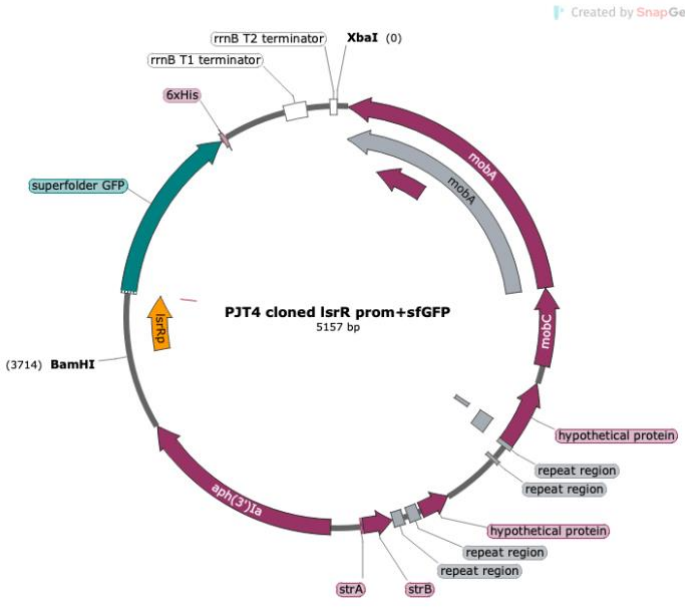
B)


Created by SnapGene
(0) Start BamHI (4)
metF
6xHis
rrnB T1 terminator
rrnB T2 terminator
XbaI (1379)
End (1387)
MetF prom
MetFprom_sfGFP_rrnBT
1387 bp

C)


Created by SnapGene
(0) Start BamHI (4)
lsrR
6xHis
rrnB T1 terminator
rrnB T2 terminator
XbaI (1447)
End (1455)
lsrR prom
lsrRprom_sfGFP_rrnBT
1455 bp

D)


Created by SnapGene
rrnB T2 terminator
XbaI (0)
rrnB T1 terminator
6xHis
superfolder GFP
mobA
mobA
lsrRp
PJT4 cloned lsrR prom+sfGFP
5157 bp
(3714) BamHI
mobC
hypothetical protein
repeat region
repeat region
aph(3')Ia
hypothetical protein
repeat region
repeat region
strA
strB

E)

4. Discussion

Periodontitis is a chronic multifactorial inflammatory disease resulting in the progressive destruction of the supporting periodontal tissues, understood to be driven by the colonization of dysbiotic plaque biofilm in a susceptible host (Lamont RJ et al, 2015). “Inflammation-mediated polymicrobial-emergence and Dysbiotic Exacerbation” (IMPEDE) is a designed model that proposes a central role of host inflammation in modulating dysbiosis, shifted in hypothesis away from specific pathogens as initial causal agents. The model focused on the importance of genetic susceptibility and environment, which seems to be leading inflammation processes (Van Dyke TE et al, 2020). However, *Aggregatibacter actinomycetemcomitans* is often pointed out as the prior cause of chronic periodontitis in juveniles and adults. Especially, a particular clone of *A. actinomycetemcomitans*, JP2, possesses a deletion in the promoter region of the leukotoxin gene operon which increases its leukotoxic activity and may enhance its ability to the host immune system (Cortelli JR et al, 2020). The interaction between *A. actinomycetemcomitans* and its role in the inflammation environment it may be driven by different mechanisms, that comprise the interaction with commensal microbiota and nutrient availability.

A putative model of infection starts from a poor hygiene condition, that leads to the formation of dental plaque and periodontal pathogens invasion. Tissue invasion and periodontal diseases are causative of bleeding episodes, connecting oral events with systemic events, such as exposure to bacteria and lipopolysaccharides, increasing the production of HSP60-like molecules and cytokine production (Xu Q. et al,1994). Cardiovascular events in connection with *A. actinomycetemcomitans* are poorly linked and far from a complete understanding; the exposure to lipopolysaccharides potentially follows a damaged cascade of heart valves, enhanced by vasculature and coagulation complications. By damaging valve components, a direct reflection seems to be the development of Infectious endocarditis (Nakano K et al, 2007) and Atherosclerosis (A. actinomycetemcomitans and P. gingivalis in plaque), followed by Coronary heart diseases.

It has been largely explored the role of Biofilm-formant bacteria and their role in antibiotic resistance. The oxygen gradient present within a biofilm, that is higher near the surface and declines toward the center, controls the metabolic activity stratification. Growth and protein synthesis with a high rate in the surface and no or very low rate in the center resulting in less penetration and consumption of antibiotics in the biofilm (Werner E. et al,2004). The modulation of phenotypic diversity in a biofilm context leads to differential gene expression, and drug tolerance through the regulation of important genes, such as DNA repair, lipid biosynthesis, toxin efflux, and ion sequestration (Fux CA et al, 2005). Given this, acute and chronic infections caused by biofilm-forming bacteria are hard to eradicate utilizing gold-standard treatments such as tetracyclines and penicillin antibiotics. A better view of the role of this bacterium in the mechanisms of infection and disease should also provide a stone for developing new hypothetical strategies that move beyond classical antibiotics. As previously stated, the production of virulence factors such as LxtA and CDT, among others, is highly connected with the tissue environment and the presence of nutrients, making it extremely difficult to develop a comprehensive experimental model that takes all of these parameters into account. The co-habitation within different bacteria species can also endorse the thesis of having a complex and unstable environment, difficult to translate into in vitro study models. This led to a loss of complete understanding of virulence factors' role and disease development.

An example of a preliminary animal model was described, where *A. actinomycetemcomitans* biofilm was established in vitro on titanium implants before placement in rat oral cavity (Freire MO et al, 2011), leading to an inflammatory response, osteolysis, and tissue destruction, which may have a potential use for investigation of host response to biofilm pathogens and antibiofilm therapy. Schreiner H. and colleagues performed, instead, a comparison of *A. actinomycetemcomitans* virulence traits in a rat model for periodontal disease, unexpectedly discovering that both leukotoxin A (LtxA) and Cytolethal Distending toxin (Cdt) exerts a significant effect on host immune system and evaluating the bone loss in a rat model of A.a.-induced periodontitis (Schreiner H et al, 2013).

Notably, all experimental models agree in asserting that virulence factors play a crucial role in this game, and there is a strong need to predict and prevent the cascade of events leading to this intriguing picture's establishment. Nowadays we know different virulence factors involved, such as factors that promote colonization and persistence in the oral cavity (Adhesins, invasins, bacteriocins, antibiotic resistance), that interfere with the host's defenses (leukotoxin,

Chemotactic inhibitors, immunosuppressive proteins, Fc-binding proteins), host tissue destruction (cytotoxins, collagenase, bone resorption agents, stimulator of inflammatory mediators), and factors that inhibit host repair of tissues (inhibitors of fibroblast proliferation, inhibitors of bone formation).

Upregulated genes that were found according to differential expression analysis (DEG) are comprehensive hypothetical enzymes and proteins involved in biofilm formation and maturation. Hypothetical protein HP GIBDFADM_00734 results having redundant homology with Transposase enzyme, involved in the cutting and splicing of large quantities of DNA. HP GIBDFADM_00611, instead, has similarities with different types of proteins such as Competence C protein and Fimbrial assembly protein. HP GIBDFADM_00204 and GIBDFADM_01265 show high sequence redundancy with DUF4123 and CRISPR-associated proteins, respectively (Masum MHU et al, 2023). In Escherichia coli, the GlgX gene is involved in bacterial glycogen synthesis and/or degradation; specifically, it encodes an isoamylase-type debranching enzyme (Dauvillée D et al, 2005). LsrACDBFG is an operon largely expressed in *E. coli* and *A. actinomycetemcomitans* species. Upstream and divergently transcribed from the LsrACDBFG operon resides lsrRK, a sequence that encodes for a repressor of lsrACDBFG, lsrR. An intergenic region (IGR) site between lsrACDBFG and lsrRK genes contains one promoter that drives expression of both operons, negatively regulated by lsrR and positively regulated by cyclin AMP (cAMP)-cyclin AMP receptor protein (CRP) complex. lsrR, due to the binding with other elements, such as lsrK, can regulate lsrACDBFG expression, using an AI-2-dependent manner (Autoinducer-2 pathway). AI-2 signal molecules can regulate genes encoding toxins and bacterial virulence factors, evidenced by their importance in biofilm formation. Proper regulation of the *lsr* locus is essential for biofilm growth in *A. actinomycetemcomitans* (Torres-Escobar A. et al, 2013). lsrR promoter is sconstitutively expressed in A. actinomycetemcomitans, underlining its potential usage in virulence process tracking.

Met gene, therefore, encodes for a Methylenetetrahydrofolate reductase (MetF or MTHFR), that is fundamental for folate reduction and homocysteine metabolism driving a catalysis reaction of 5,10- methylenetetrahydrofolate to 5- methyltetrahydrofolate. MetF encodes for a protein of 307 amino acids expressed as leaderless mRNA. Destruction of the metF gene led to methionine auxotrophy. In Escherichia coli and Salmonella typhimurium, metF is part of a met regulon, that encodes methionine biosynthesis (Blanco J et al., 1998).

In this study, we decided to focus our attention on the construction of a novel tool that should be able to track *Aggregatibacter actinomycetemcomitans* infection. Following this purpose, the constructed plasmid containing the MetF gene promoter cloned upstream of the super-folded Green fluorescent protein (GFP) can be used for a better understanding of the infection development. The novel tools designed should also provide a better picture of the biofilm environment, elucidating the complex process of progression and maturation. Also, being *A. actinomycetemcomitans* involved in different diseases can be useful even in understanding the association between this bacterium and other clinical contexts.

To conclude, identifying naïve target genes, involved in biofilm maturation and maintenance, and better elucidating the mechanisms of actions of the known virulence factors could help, in the future, to prevent and manage properly Biofilm-formation infections, as well as avoid sanitary problems bound with antibiotic resistance and mortality worldwide.

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