Original article

Potential Drug Target Identification in *Porphyromonas gingivalis* using *In-silico* Subtractive Metabolic Pathway Analysis

Prachi Sao[1], Yamini Chand[2], Atul Kumar[3], Sachidanand Singh[4]

Abstract:

Introduction: *Porphyromonas Gingivalis (P. gingivalis)* a primary periodontal disease pathogen. This bacterium affects sub-gingival tissue and leads to loss of teeth and alveolar bone destruction in the acute stage. In recent years, *P. gingivalis* is often connected with other diseases such as rheumatoid arthritis, diabetes, Alzheimer's, and heart disease, though the aetiology is still unclear. ***Objective:*** The use of commonly available drugs to treat periodontitis results in various side effects, in particular multi-drug resistant strains. As the development of multidrug-resistant strains frequently urges the identification of novel drug targets, the aim of this study is to identify specific targets in the narrow spectrum to combat oral pathogens. ***Methodology:*** This study used a comparative and subtractive pathway analysis approach to identify potential drug targets specific to *P. gingivalis*. ***Results:*** The *in-silico* comparison of the *P. gingivalis* and *Homo sapiens (H. sapiens)* metabolic pathways resulted in 13 unique pathogen pathways. A homology search of the 67 enzymes in the unique bacterial pathway using the BLASTp program against the Homo sapiens proteome resulted in fifteen possible targets that are non-homologous to the human proteome. Thirteen genes among 15 potent target encoders are key DEG genes indispensable for *P. gingivalis's* survival. A comprehensive analysis of the literature identified three potential therapeutic drug targets. ***Conclusions:*** The three most relevant drug targets are Arabinose-5-phosphate isomerase, UDP-2,3-diacylglucosamine hydrolase, and *Undecaprenyl diphosphatase*. Upon corroboration, these targets may give rise to narrow-spectrum antibiotics that can specificallytreat thedental infection.

Keywords: *Porphyromonas Gingivalis*; Periodontitis; Comparative Pathway Analysis; Homology Search; Drug Resistance

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Introduction

Oral hygiene plays a consequential role in maintaining the overall health of a human being. 95% of the people in India suffer from the periodontal disease at some point in their age and only 2% visit a dental professional[1]. Periodontitis, an inflammation of the tissues around teeth, which in the acute stage leads to tooth loss and alveolar bone ravagement affecting 10 to 15% of the world population[2].

Even though periodontitis is a polymicrobial disease of over 500 bacterial species in a subgingival region[3], a host of work strongly shows that *P. gingivalis* is an etiological primary agent at 85% of the site of the disease[4,5]. The bacterium belongs to the "red

1. Prachi Sao
2. Yamini Chand

   Faculty of Biotechnology, Institute of Biosciences and Technology, Shri Ramswaroop Memorial University, Barabanki, 225003, India.
3. Atul Kumar, Faculty of Medicine, Biomedical Center, Lund University, Lund, Sweden.
4. Sachidanand Singh, Faculty of Biotechnology, Institute of Biosciences and Technology, Shri Ramswaroop Memorial University, Barabanki, 225003, India.

Correspondence to: Sachidanand Singh, Faculty of Biotechnology, Institute of Biosciences and Technology, Shri Ramswaroop Memorial University, Barabanki, 225003, India. Email: prachi1627@gmail.com

complex" and crucial for biofilm formation[6]. There is a strong link between the increasing size of the periodontal pocket and colonizing *P. gingivalis*[5, 7,8,9]. *P. gingivalis*is a gram-negative, rod shape, obligately anaerobic, ebony-pigmented, non-motile bacteria. This notorious pathogen has great potential for the incursion, it can invade cells, tissues, and macrophages to elude the host's innate immune system[10]. Once it is established in the host cell it interferes with the host immune system and deregulates it[11,12]. The recent study suggests that *P. gingivalis* contributes to various diseases such as rheumatoid arthritis, a diabetic, heart condition, oral cancer, and Alzheimer's[13,14,15.16].

In its virulence factors, *P. gingivalis* varies between its different strains, with a wide range of virulence factors like capsules, fimbria, lipopolysaccharide (LPS), proteases, and external membrane. It can use host immune cells for its proliferation, develop resistance to oxidative killing by neutrophils, can have a vast amount of gene rearrangement and intracellular recombination. As a result, *P. gingivalis* can regulate the gene expression of virulence factors on environmental grounds[17, 18], and therefore it can easily develop resistance to antibiotics.

With the increasing number of antibiotics, the risk of the resistant strain also increases. Existing antibiotics such as cefuroxime, minocycline, doxycycline, metronidazole, amoxicillin, chlorhexidine, ampicillin, and ofloxacin[19,20,21] do not effectively control periodontitis because it is related to biofilm and involves multiple organisms[22]. At present, conventional broad-spectrum antibiotics are given as additional treatments for periodontitis[24,25] and the prolonged use of antibiotics not specific to a pathogen is always known to cause a variety of side effects on the host[25]. It also destroys the essential microbiome and increases the risk of antibiotic resistance. Since *P. gingivalis* is a key player in biofilm development [26] therefore, eradication of *P. gingivalis* may disturb the whole biofilm. As a result, pathogen-specific targets are constantly required against which an effective drug molecule can be developed and which do not interfere with the metabolism of the host.

Comparative and subtractive path analysis can be used to obtain pathogen-specific targets[27]. The whole-genome sequencing has created a huge number of data that can be used to understand the pathogenicity and survival of the pathogen in a specific environment. This *in-silico* comparative and Subtractive Pathway Analysis is an important and time-saving tool for identifying pathways specific to the pathogen[28,29,30]. This can further be used to understand the connections between cells, the cell components, communication within cells, and the role of the essential proteins present in the metabolic pathway which is very important for identifying potential targets for drugs[31]. As specific bacterial pathways exist and their proteins are essential for the survival of the pathogens, these can be potential medications targets[33,34].

In this work, the strain TDC60 of *P. gingivalis*is selected because of its greater pathogenicity over the other two strains W83 and ATCC3327[33]. It comprises 2319 genes, 87 pathways, and 650 RefSeq. We perform a comparative analysis of the metabolic pathways between the host (*H. Sapiens*) and pathogen (*P. gingivalis)*. The pathways unique to pathogen *P. gingivalis* were selected and all the enzymes involved in it were fed to BLASTp against human proteins for a homology search. The aforementioned enzymes which are non-homologous to the host proteins were subjected to homology search against the DEG database to find enzymes essential for the survival of pathogens. Pathogen-specific enzymes that have no significant similarity for any protein in the host organism and inactivation of such proteins can be lethal to the pathogen can be a potential target because there is little risk of a drug interfering negatively with the host metabolism. Further filtration of the potential drug targets was performed using the CELLO v2.5 which represented the distribution of targets in the cell and DrugBank which filters out targets which are already in an experiment or approved by the FDA. The study revealed 4 enzymes present in *P. gingivalis TDC6*0 as potential drug targets for the treatment of periodontitis.

Material and Method

Subtractive pathway analysis approach has been used to identify the essential proteins in *P. gingivalis TDC60* for potential drug targets and further analysis has been undertaken. Figure 1 shows the flow chart describing the detailed methodology for the identification of pathogen-specific proteins by the method of subtractive genomics.

The metabolic pathways of pathogen *P.gingivalis TDC60* and the metabolic pathways of host *Homo sapiens* were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [34] a curated database of genomes and metabolic pathways. A comparative analysis of both pathways was performed using an

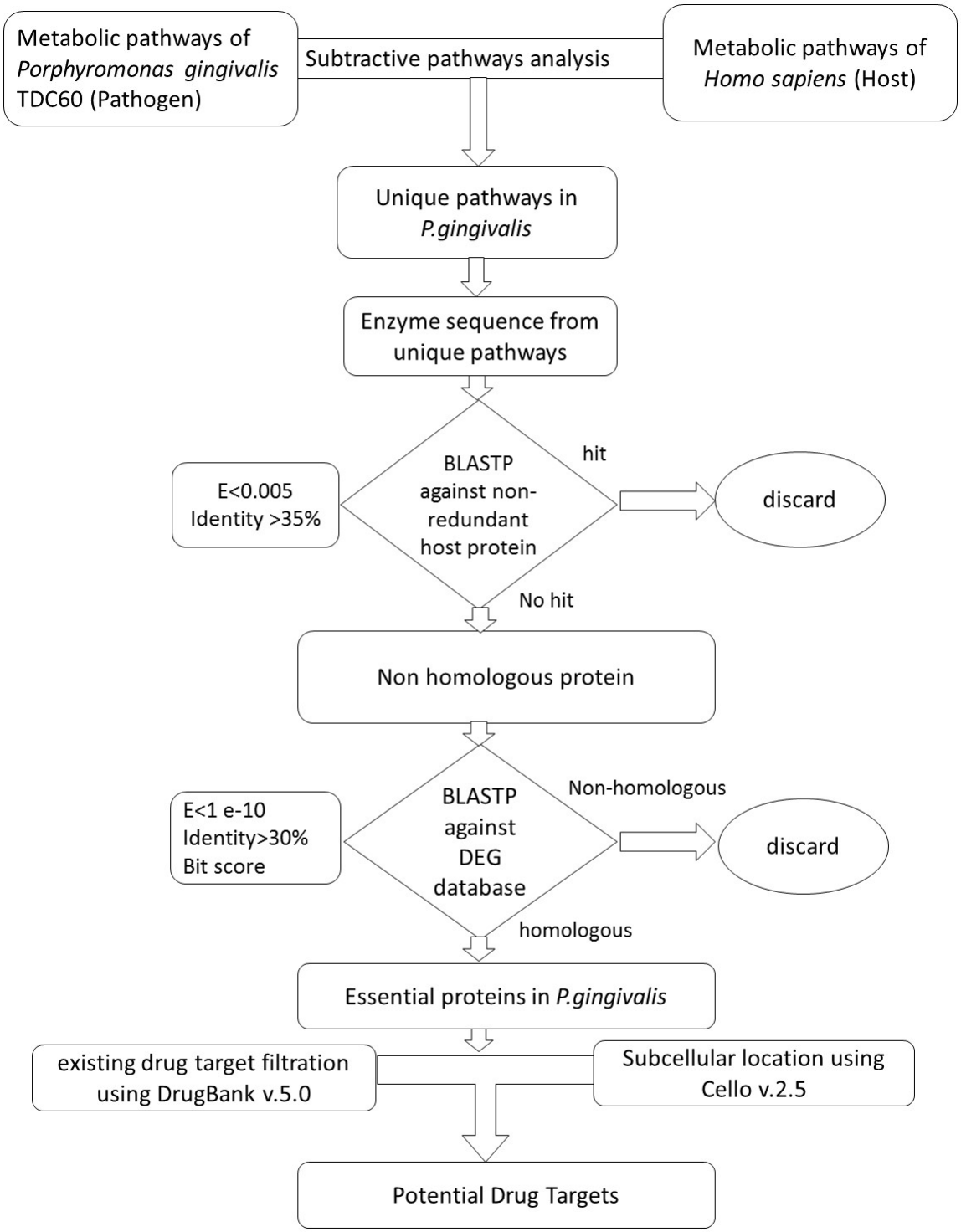

Figure 1: A Schematic Representation of the Workflow of Potential Drug Targets Identification by Subtractive Pathways Analysis Approach

in-house python program. Pathways found on both the host and *P.gingivalis TDC60* were identified as common pathways and pathways found only in pathogen *P.gingivalis TDC60* as unique bacterial pathways. Unique bacterial pathways were considered for further studies. Refseq Protein (enzymes) sequences were retrieved from the National Center for Biotechnology Information (NCBI) Ref-seq database[35] for all genes present in unique bacterial pathways with appropriate Enzyme Commission numbers (EC no.) known from the KEGG database.

Identification of Non-Homologous Proteins

In order to be a potential drug target, a protein must

be unique to the pathogen *P.gingivalis TDC60* and must be essential for the survival of the pathogen. Therefore, a homology search using BLASTp (Protein-protein Basic Local Alignment Search Tool) of afore obtained pathogen protein sequences against the host database, which is *H.sapiens* with Expectation Value (E Value) cut off by 0.005 and identity cut off by 35%, was performed to classify these proteins. Expectation value(E value) and identity cut off were selected based on a similar type of the previous workflow[27, 29]. Proteins that did not display any hit or hit below the above threshold were considered to be non-homologous to the host and further evaluated as a possible drug target.

Identification of Essential Proteins

Further protein filtration was performed based on essential and non-essential proteins for pathogen survival. BLASTp of aforementioned non-homologous protein sequences was thus carried out against other prokaryotic organisms from the Database Essential Genes (DEG 5.0)[37], having an E-value of less than 1e-10, a bit score of more than 100 and an identification of greater than 30%. The hit protein has been identified as homologous to proteins of the other bacteria and accentuates its paramountcy for the survival of the pathogen[38].

Characterization and Prioritization of the Drug Targets

Druggability of the Targets

CELLO versions 2.5 subCELlularLOcalization predictor[38], was used to describe the possible subcellular localization of drug targets, a good understanding of the distribution of pharmaceutical targets in various cell sections is important for identification of potential drug targets. The DrugBank version 5.0[40] was used to further remove already proposed drug targets. It stores data regarding FDA-approved drug targets. DrugBank was submitted with protein sequences of all 15 non-homologous proteins, and no sequence of matches was identified as potential targets.

Ethical Clearance: Not Applicable

Result and Discussion

A comparative study of *P. gingivalis TDC60* and *H. sapiens* metabolic pathways gave 61 common pathways listed in supplementary table 1 and 13 unique bacterial pathways listed in table 1. Unique bacterial pathways were further studied in this work. Several proteins present in each unique pathway were investigated and considered only if proteins were not associated with the common pathway. The unique bacterial pathway has 67 unique pathogen-specific enzymes; details are listed in supplementary table 2. Pathways unique to *P.gingivalis* are Monobactam Biosynthesis, Lysine Biosynthesis, Benzoate Degradation, Cyanoamino Acid Metabolism, D-Alanine Metabolism, Streptomycin Biosynthesis, Polyketide Sugar Unit Biosynthesis, Acarbose, and Validamycin Biosynthesis, Lipopolysaccharide Biosynthesis, Peptidoglycan Biosynthesis, Methane Metabolism, Two-Component System, Bacterial Secretion System. The following pathways are associated with bacterial functions and virulence: secondary-metabolite biosystems, bacterial invaders of epithelial cells, diverse environments of microbial metabolism, aromatic degradation compounds, PBP affinity changes, two-component systems, Beta-Lactam resistance, quorum sensing, bacterial chemotaxis, and special lipopolysaccharide and periplasmic space which doesn't allow antibiotics to reach the cytoplasm[41]. The distribution of enzymes in each unique bacterial pathway is displayed in figure 2.

Table 1: Metabolic Pathways Which Are Unique to the Pathogen *Porphyromonas Gingivalis TDC60*

S. No.	*Metabolic pathway*	*KEGG pathway ID*	*No. of proteins*
1	Monobactam biosynthesis	pgt00261	4
2	Lysine biosynthesis	pgt00300	9
3	Benzoate degradation	pgt00362	3
4	Cyanoamino acid metabolism	pgt00460	2
5	D-Alanine metabolism	pgt00473	2
6	Streptomycin biosynthesis	pgt00521	6
7	Polyketide sugar unit biosynthesis	pgt00523	4
8	Acarbose and validamycin biosynthesis	pgt00525	2
9	Lipopolysaccharide biosynthesis	pgt00540	13
10	Peptidoglycan biosynthesis	pgt00550	15
11	Methane metabolism	pgt00680	13
12	Two-component system	pgt02020	14
13	Bacterial secretion system	pgt03070	11

Identification of Non-Homologous Proteins

We subjected 66 enzymes to protein blast (BLASTp) against the host (H. sapiens) proteome. Screening of 67 enzymes resulted in 41 enzymes that had identity ≤ 35% with the human proteome. Among these 41 enzymes, only 15 enzymes were no-hit proteins. Which means these 15 enzymes are unique to the

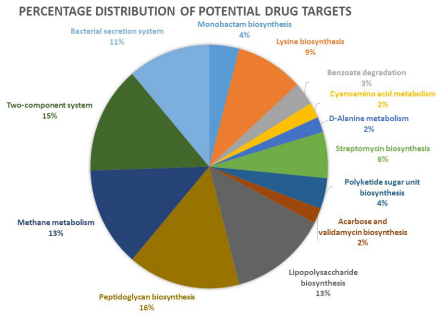

Figure 2 : Percentage Distribution of Novel Drug Targets Involved in Different Unique Bacterial Metabolic Pathways.

pathogen and absent in humans.

During the process of evolution, bacteria may adapt some protein that is common with a host known as homologous to save energy and certain proteins will be unique to a pathogen that plays a significant role in survival, specifically in cell multiplication and protection against host immune system such proteins are known as non-homologous to host.

Identification of Essential Proteins

Even though 41 proteins are non-homologous to host and may be involved in multiple pathways, still all cannot be drug targets, because they may not be necessarily essential for survival or therapeutically beneficial. The essential genes are the minimum number of genes required for any organism to survive[37]. Therefore, protein needs to be unique to pathogens and must be essential for survival. Further filtration of essential protein was done using protein Blast (BLASTp) against other pathogens using the DEG database and analysis shows that 31 enzymes were similar to experimentally proved essential protein. To further narrow down the targets, only those proteins which are completely non-homologous (no-hit) to host and essential for the survival of *P.gingivalis*were selected as potential drug targets. All 15 proteins that were completely non-homologous to host are represented in table 2.

Characterization and Prioritization of the Drug Targets

The identification of subcellular localization of the protein plays a significant role in understating the distribution and nature of the target proteins. Especially for pathogenic bacteria secretion of enzymes can be selective to the environment and cellular condition, therefore identification of location can explain the accessibility of the target protein by drug molecules. Subcellular localization of proteins can also describe the properties of protein as a good target, in general, cytoplasmic proteins are significant over membrane-bound proteins because later one is difficult to isolate and study *in-vitro*[38]. In present work 10 enzymes were present in the cytoplasmic region, 3 in the inner membrane and 1 each in the periplasmic and outer-membrane region of *P.gingivalis. Percentage* distribution is represented in figure 3. One of the non-homologous proteins was a hypothetical protein, for present work we have eliminated the hypothetical protein, a further analysis of which may relevel drug target properties. Therefore, finally, 14 proteins were selected for druggability using DrugBank.

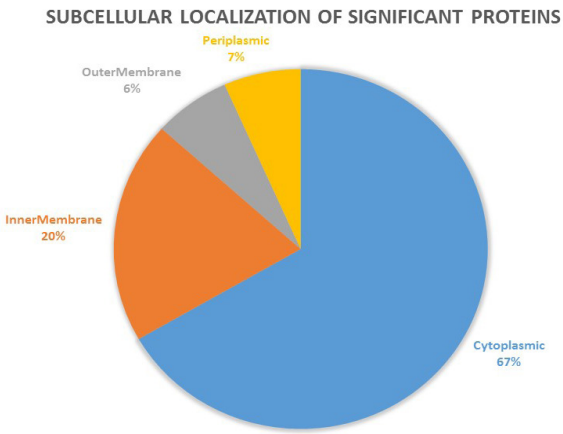

Figure 3: Percentage Distribution of the Subcellular Localization of Significant Proteins Specific to *Porphyromonas Gingivalis*

Druggability of the Targets

DrugBank runs a similarity search of the putative drug target sequence against already known US-FDA approved drug targets sequences if the target sequence is matched with a known sequence, then for that target similar drug can be used, therefore it cannot be a novel drug target. Using the DrugBank database, filtration of 14 non-homologous proteins was done. Four already approved drug targets are involved in Peptidoglycan biosynthesis. Six drugs were under experiment, two of which belong to Lysine biosynthesis, one each belongs to Monobactam biosynthesis Lipopolysaccharide biosynthesis, Peptidoglycan biosynthesis, Methane metabolism, and Two-component system, displayed in figure 4. Four proteins show no search against the available approved or experimental target sequences in the DrugBank, out of which 3 were essential for *P.gingivalis,* and it was selected as a potential drug target.

Table 2: List of Completely Non-Homologous Proteins of *Porphyromonas Gingivalis*

S.NO	KEGG GENE ID	KEG ORTHOLOGY	EC NO.	PATHWAYS AND DESCRIPTION	ESSENTIAL	SUBCELLULAR LOCALIZATION
1	PGTDC60_1695	K00133	[EC:1.2.1.11]	aspartate-semialdehyde dehydrogenase ; asd; aspartate-semialdehyde dehydrogenase (A)	YES	Cytoplasmic
2	PGTDC60_1700	K01928	[EC:6.3.2.13]	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase ; murE; UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (A)	YES	Cytoplasmic
3	PGTDC60_1925	K03340	[EC:1.4.1.16]	diaminopimelate dehydrogenase ; Gfo/Idh/MocA family oxidoreductase (A)	YES	Cytoplasmic
4	PGTDC60_0076	K00979	[EC:2.7.7.38]	3-deoxy-manno-octulosonate cytidylyltransferase (CMP-KDO synthetase) ; kdsB; 3-deoxy-manno-octulosonate cytidylyltransferase (A)	YES	Cytoplasmic
5	PGTDC60_0366	K06041	[EC:5.3.1.13]	arabinose-5-phosphate isomerase ; SIS domain-containing protein (A)	YES	Cytoplasmic
6	PGTDC60_1358	K03269	[EC:3.6.1.54]	UDP-2,3-diacylglucosamine hydrolase ; probable UDP-2,3-diacylglucosamine hydrolase (A)	YES	Cytoplasmic
7	PGTDC60_0712	K19302	[EC:3.6.1.27]	undecaprenyl-diphosphatase ; PAP2 superfamily protein (A)	NO	Inner-Membrane
8	PGTDC60_0760	K06153	[EC:3.6.1.27]	undecaprenyl-diphosphatase ; undecaprenol kinase, putative (A)	YES	Inner-Membrane
9	PGTDC60_1699	K03587	[EC:3.4.16.4]	cell division protein FtsI (penicillin-binding protein 3) ; penicillin-binding protein 2, putative (A)	YES	Periplasmic
10	PGTDC60_2040	K07259	[EC:3.4.16.4 3.4.21.-]	serine-type D-Ala-D-Ala carboxypeptidase/endopeptidase (penicillin-binding protein 4) ; dacB; D-alanyl-D-alanine carboxypeptidase (A)	NO	OuterMembrane
11	PGTDC60_2071	K05515	[EC:3.4.16.4]	penicillin-binding protein 2 ; penicillin-binding protein 2, putative (A)	YES	Cytoplasmic
12	PGTDC60_2130	K00075	[EC:1.3.1.98]	UDP-N-acetylmuramate dehydrogenase ; murB; UDP-N-acetylenolpyruvoylglucosamine reductase (A)	YES	Cytoplasmic
13	PGTDC60_0013	K05979	[EC:3.1.3.71]	2-phosphosulfolactate phosphatase ; hypothetical protein (A)	NO	Cytoplasmic
14	PGTDC60_1126	K00625	[EC:2.3.1.8]	phosphate acetyltransferase ; pta; phosphotransacetylase (A)	YES	Cytoplasmic
15	PGTDC60_0827	K00426	[EC:7.1.1.7]	cytochrome bd ubiquinol oxidase subunit II; cydB; cytochrome d ubiquinol oxidase, subunit II (A)	NO	Inner-Membrane

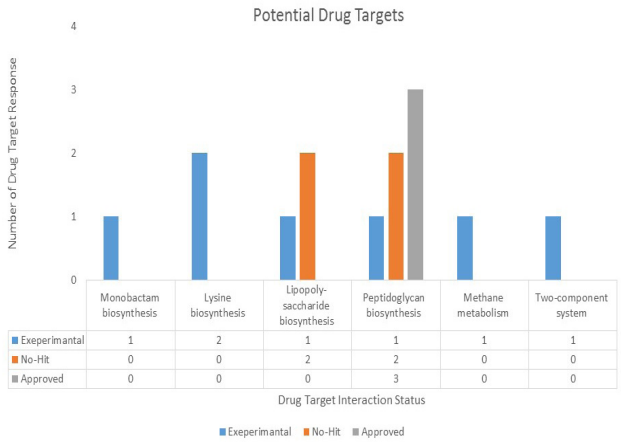

Figure 4: Distribution of Non-Homologous Essential Proteins of *Porphyromonas Gingivalis* Similar to Binding Partners of FDA Approved Drugs as Inferred from Drugbank

Arabinose-5-phosphate isomerase [EC:5.3.1.13] and *UDP-2,3-deacylglucosamine hydrogenase* [EC:3.6.1.54]were from lipopolysaccharide biosynthesis pathway and plays a significant role in cell-envelope biosynthesis which is unique to gram-negative bacteria. Whereas *Uundecaprenyl-diphosphatase* [EC:3.6.1.27] was from the peptidoglycan biosynthesis pathway which is the main component of cell wall formation. Lipopolysaccharides biosynthesis and peptidoglycan biosynthesis pathways are promising targets because of their exquisite gram-negative bacteria. If these pathways are disturbed by targeting a single protein or assembly of proteins, it can make gram-negative bacteria susceptible to antimicrobial agents.

Arabinose-5-phosphate isomerase [EC:5.3.1.13]

Arabinose-5-phosphate isomerase (API), encoded by gene KdsD, and belongs to isomerases class of enzymes. API takes part in the first step of 3-deoxy-d-manno-octulosonic acid (Kdo) biosynthesis. It catalyzes the interconversion of d-ribulose-5-phosphate and d-arabinose-5-phosphate, a key component of Gram-negative bacterial lipopolysaccharide[41]. *UDP-2,3-deacylglucosamine hydrogenase* plays a crucial role in lipid A biosynthesis which plays a significant role in stimulating the innate immune system[43]. *Uundecaprenyl-diphosphatase*is involved in peptidoglycan biosynthesis, it anchors lipopolysaccharides and other proteins to the outer membrane helps in maintaining cell shape, adverse effect of internal osmotic pressure, and transport of hydrophilic molecules[44]. The higher number of approved and experimental drug targets belong to the cell-wall biosynthesis which emphasizes to study it in detail and also justify the aforementioned three proteins as potential drug targets

Overall as per the different analytic approaches performed, three significant target proteins are involved in the structural composition of *P.gingivalis* which marks its crucially towards attachment to the surface, protection against anti-bacterial agents, absorption of nutrients and production of proteases. The *in vitro* and *in vivo* validation and evaluation of the identified target proteins will combat the infection and diseases caused by *Porphyromonas Gingivalis* and will enable support of the development of drug and vaccine candidates against it.

Conclusion

P.gingivalis is a key pathogen of oral biofilm development, with several studies suggesting that eradication of *P.gingivalis* can disturb the entire biofilm and prevent periodontitis. Consequently, a narrow spectrum *P.gingivalis*-specific antibiotics can be very important. The constant resistance of pathogens to pre-existing antibiotics also promotes the discovery of new drug targets which are pathogens-specific and interact less with host metabolism.

In-silico comparative and subtractive pathway analysis approach has helped us to reduce the number of targets considered for the drug discovery workflows. The analysis of the metabolic pathway between the pathogen *P.gingivalis* and the host *H. sapiens* using the BLASTp program has found bacterial enzymes that do not have any similarities to human proteins. This study has prosperously identified potential drug targets in *P.gingivalis* that can be further validated with *in vitro* studies. Therefore three proteins namely *Arabinose-5-phosphate isomerase [EC:5.3.1.13], UDP-2,3-deacylglucosamine hydrogenase [EC:3.6.1.54] and UDP-2,3-deacylglucosamine hydrogenase [EC:3.6.1.54]* can be considered as potential drug targets against which a drug to cure

periodontitis can be developed. Since the targets are pathogen-specific, not found in the host, chances of development of drug resistance and side effects are greatly reduced. Further experimental studies on the above-mentioned drug targets may reveal the broad-spectrum antibiotic effect to cure an infection caused by oral pathogens.

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Authors Contribution:

Data gathering and idea owner :Prachi Sao, Yamini Chand, Dr. Sachidanand Singh

Writing, editing final draft and Submission of the manuscript: Prachi Sao

Approval of final draft: Dr. Sachidanandsingh and Dr. Atul Kumar

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