GSC Advanced Research and Reviews

eISSN: 2582-4597 CODEN (USA): GARRC2

Cross Ref DOI: 10.30574/gscarr

Journal homepage: https://gsconlinepress.com/journals/gscarr/

(RESEARCH ARTICLE)

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Amylase from *Streptococcus pyogenes* inhibits biofilm formation in *Streptococcus salivarius*

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GSC Advanced Research and Reviews, 2023, 14(02), 047–053

Publication history: Received on 26 December 2022; revised on 01 Februay 2023; accepted on 04 February 2023

Article DOI: https://doi.org/10.30574/gscarr.2023.14.2.0050

Abstract

Biofilms are extracellular constituents composed of polysaccharides and other substances necessary for bacteria to defend themselves against foreign enemies. Amylase of *Streptococcus pyogenes* is an enzyme that degrades polysaccharides and other substances and produces nutrients for invasion of epithelial cells. *S. salivarius* is an oral commensal bacterium that shows antibacterial activity against *S. pyogenes*. We investigated the relationship between *S. pyogenes* and *S. salivarius* to determine whether the amylase of *S. pyogenes* affects the biofilm of *S. salivarius*. The *amyA* gene-deficient strains were generated from *S. pyogenes* 1529 and MDYK strains, and the amylase production ability of the wild-type and gene-deficient strains were compared. Amylase production in mutant strain was significantly reduced compared to the wild-type strain. Next, the biofilm-forming ability of *S. pyogenes* was compared between wild-type and mutant strains, and significantly increased biofilm-forming ability was observed in the gene-deficient strains. Next, *S. salivarius* was cultured to create biofilms, and then wild-type and mutant strains of *S. pyogenes* were added to the culture. Significantly, the biofilms of *S. salivarius* with the gene-deficient strains were higher than those with the wild strains. As the biofilm-forming ability of *S. salivarius* co-cultured with *S. pyogenes* was compared, the biofilm-forming ability of *S. salivarius* co-cultured with the mutant strain of *S. pyogenes* was also significantly increased. These results were common findings for the 1529 and MDYK strains of *S. pyogenes*. Our results suggest that amylase from *S. pyogenes* inhibits biofilm formation in *S. salivarius*.

Keywords: *Streptococcus pyogenes*; *Streptococcus salivarius*; Amylase; Biofilm

1. Introduction

Streptococcus pyogenes is a gram-positive coccus that infects the upper respiratory tract, including the tonsils and pharynx, and causes post-infectious diseases such as rheumatic fever and glomerulonephritis [1]. As it is a typical upper respiratory tract infection in children, but it has been reported that the presence of *S. salivarius* strains of oral streptococci that produce antimicrobial active substances decreases the frequency of *S. pyogenes* acquisition in the oral cavity of children [2]. *S. salivarius* is an oral streptococcus that inhabits many human oral epithelial surfaces and one of the first bacteria to become established in the oral cavity of newborns [3, 4]. It is a commensal bacterium that establishes itself in the oral cavity and saliva and produces salivaricin A, salivaricin A2, salivaricin B or enosin, which have bactericidal activity [5-7]. Three salivaricin genes, *salA,* salA2, and *sbo*, encode salivaricin A, salivaricin A2, and salivaricin B, respectively [6, 7]. It has been reported that salivaricin A is responsible for the decreased frequency of *S. pyogenes* acquisition in the oral cavity of children [2]. *S. pyogenes* forms colonies in the pharynx before invading host tissues. If *S. salivarius* then colonizes the site of *S. pyogenes* invasion, further colony formation of *S. pyogenes* may be inhibited by antimicrobial active substances derived from *S. salivarius*. We have reported that *S. pyogenes* is resistant to

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S. salivarius through the interaction of the Streptococcal Inhibitor of Complement (SIC) protein of *S. pyogenes* with antimicrobial active substances from *S. salivarius* [8]. However, this phenomenon alone does not fully explain the interaction between *S. pyogenes* and *S. salivarius* under pharyngeal colonization conditions. Biofilms are biological and non-biological surfaces associated with highly structured invertebrate complexes produced by bacteria, which anchor themselves to a self-productive extracellular matrix of carbohydrate polysaccharides and exopolysaccharides (EPS) along with proteins, DNA and other small molecules [9]. Bacteria adhering to medical devices and damaged tissues can cause persistent infections through biofilm formation [10]. Antibiotics traditionally used to treat biofilm-forming pathogens such as Streptococcus do not target biofilms, but rather target floating bacteria, exacerbating the problem of drug-resistant bacteria by overloading the bacteria and causing them to acquire resistance to antibiotics [11]. Although biofilm is essential for bacterial survival, it is possible that biofilm formation may be involved in the competition for survival between bacteria, such as between *S. pyogenes* and *S. salivarius*. Polysaccharides and other materials are used to form biofilms, and *S. pyogenes* produces an amylase enzyme that degrades these polysaccharides [12]. In this study, we investigated the effect of *S. pyogenes* amylase on *S. salivarius* biofilm formation.

2. Material and methods

2.1. Bacteria and culture conditions

M1 serotype *Streptococcus pyogenes* strains 1529, and MDYK used in this study were clinical isolates from hospital patients in Japan with invasive *S. pyogenes* infections [13]. *S. salivarius* JCM5707 strain was obtained (Riken Bio Resource Center, Tsukuba, Japan) [8]. Streptococci were usually cultured in 10 mL of Todd Hewitt broth (Difco Laboratories Inc., Detroit, MI, USA) containing 0.2% yeast extract (Difco Laboratories) (THY) for 18 hours at 37 ℃ without agitation. *Escherichia coli* JM109 were maintained on Luria–Bertani (LB) agar (Difco Laboratories) or were grown in LB broth with aeration at 37℃.

2.2. Gene manipulation techniques

Escherichia coli JM109 was used to propagate plasmid constructions. Nonpolar inactivated mutants of the *amyA* gene were constructed via double-crossover allelic replacement in *S. pyogenes* 1529 and MDYK chromosomes with some modifications of the methodology of a previously report [8]. The primers used in this study are listed in Table 1.

Table 1 Oligonucleotide primers used in this study

Primer	Sequence
amyA F1	GGAAGACAGGTCATGAAGCACA
amyA F2	ATTCTGAAACGCAGCTTAGG
amyA R1	GTTCTAGGAATTCCTGCTTTAC
amaA R2	TCCACTAACTGTTTCTGCTG

2.3. Measurement of amylase activity

Bacterial amylase activity was measured using a kit (α-amylase assay kit, Kikkoman BioChemifa Co., Tokyo, Japan). Briefly describing the method, bacterial culture supernatant was added to N3-G5-β-CNP, and glucoamylase and β-glucosidase were further added. After mixing at 37 °C for 10 minutes, sodium carbonate was added to stop the enzymatic reaction. The optical absorbance of the mixture was measured at 400 nm. Milli-Q water was used as a control. The absorbance value obtained by subtracting the control from the sample and multiplying by 89.5 was defined as the activity of α-amylase (U/mL).

2.4. Bacterial biofilm measurement

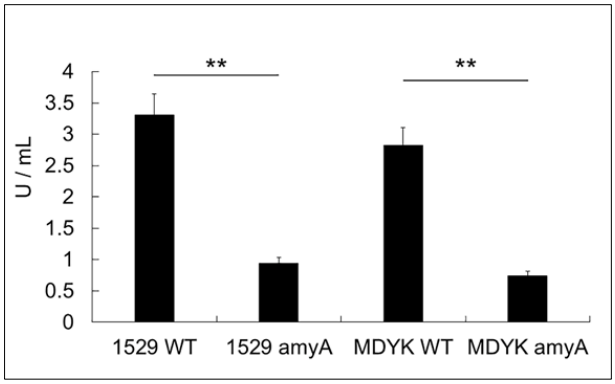
Biofilm assay was performed with some modifications [14, 15]. Each bacterium (1×10^6 CFU) was seeded onto 96-well polystyrene plates (Thermo Fisher Scientific, MA, USA), which were then incubated in THY broth at 37 °C for 48 hours. After removal of the media, the plates were washed three times with PBS, and adherent bacteria were stained with 0.2% crystal violet (Fujifilm Wako Pure Chemical) at room temperature for 10 min, after which, they were gently washed three times with PBS. Each biofilm was quantitated by measuring the absorbance at 570 nm (A570). Wells incubated without bacteria were used as blanks. The absorbance of blank wells was subtracted from the test values.

2.5. Statistical Analysis

We expressed the study data as the means ± standard deviation (SD). We also conducted the statistical analysis using the Tukey's multiple comparison t-tests for the differences among multiple groups. The *p*-values < 0.05 indicated statistical significance.

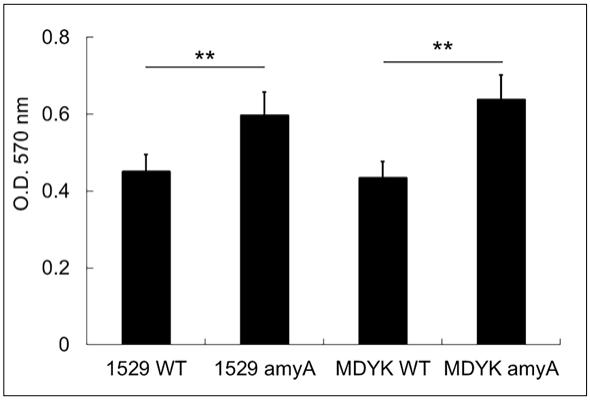
3. Results

First, to confirm that the mutant strain lacking the amylase gene does not possess amylase activity, the amylase activity of the culture supernatant of the wild and mutant strains was measured. As a result, we were able to confirm that the amylase activity observed in the wild strain was significantly reduced in the mutant strain, as shown in Figure 1. The same trend was observed for two independent bacterial strains (1529 and MDYK).



** : $p < 0.01$

Figure 1 Comparison of amylase activity of *S. pyogenes*. The amylase activity of bacterial culture supernatants was determined. 1529 WT: 1529 wild-type strain, 1529 amyA: 1529 amyA-deficient strain, MDYK WT: wild-type strain, MDYK amyA: MDYK amyA-deficient strain

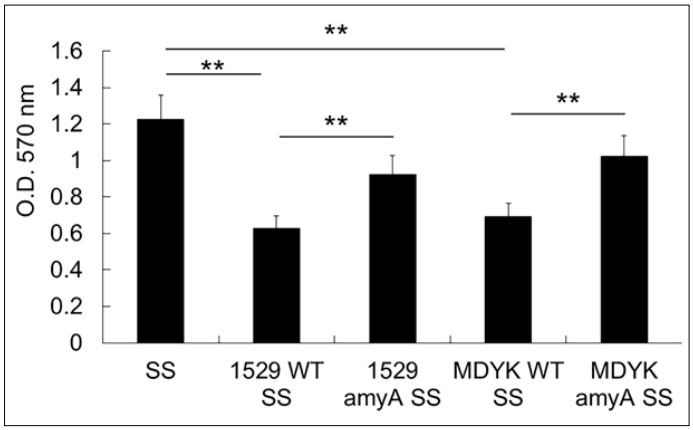


** : $p < 0.01$

Figure 2 Comparison of biofilm forming ability of *S. pyogenes* only. Bacterial biofilms were stained with crystal violet and their absorbance was measured. 1529 WT: 1529 wild-type strain, 1529 amyA: 1529 *amyA*-deficient strain, MDYK WT: wild-type strain, MDYK amyA: MDYK *amyA*-deficient strain

We next examined the ability of *S. pyogenes* alone to form biofilms. After 48 hours of incubation of the wild-type and the amylase gene-deficient mutant strain, biofilms stained with crystal violet were measured with a spectrophotometer. As shown in Figure 2, biofilm formation was enhanced in the mutant strain compared to the wild-type strain. This same trend was also observed in two independent bacterial strains (1529 and MDYK).

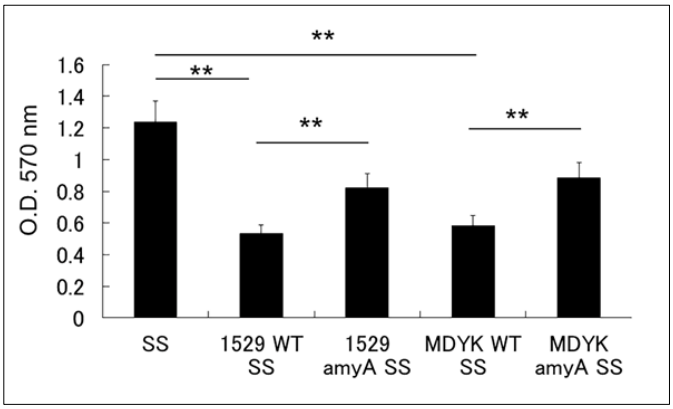
After another 48 hours of incubation, wild-type and mutant strains of *S. pyogenes* were added to *S. salivarius* and incubated for another 48 hours, and the biofilm stained with crystal violet was measured with a spectrophotometer. As shown in Figure 3, biofilm formation was inhibited in *S. pyogenes* -added *S. salivarius* compared to *S. salivarius* alone. And compared to the addition of wild-type *S. pyogenes* strain, the biofilm formation of mutant *S. pyogenes* strain-added *S. salivarius* was increased. We also observed the same trend in two independent bacterial strains.



** : $p < 0.01$

Figure 3 Comparison of biofilm forming ability of *S. salivarius* (SS) after addition of *S. pyogenes*. Bacterial biofilms were stained with crystal violet and their absorbance was measured. 1529 WT: 1529 wild-type strain, 1529 amyA: 1529 *amyA*-deficient strain, MDYK WT: wild-type strain, MDYK amyA: MDYK *amyA*-deficient strain

S. salivarius were then added to the wild-type and mutant strains of *S. pyogenes* were added and incubated simultaneously for 48 hours, after which crystal violet-stained biofilms were measured with an absorbance spectrophotometer. As shown in Figure 4, biofilm formation was inhibited in *S. pyogenes* -added *S. salivarius* compared to *S. salivarius* alone. And compared to the addition of the wild-type strain, the biofilm formation of the mutant *S. pyogenes* strain-added *S. salivarius* was increased. The same trend was also observed in two independent bacterial strains.



** : $p < 0.01$

Figure 4 Comparison of biofilm-forming ability of *S. salivarius* (SS) and *S. pyogenes* co-cultures. Bacterial biofilms were stained with crystal violet and their absorbance was measured. 1529 WT: 1529 wild-type strain, 1529 amyA: 1529 *amyA*-deficient strain, MDYK WT: wild-type strain, MDYK amyA: MDYK *amyA*-deficient strain

4. Discussion

Our results showed that the amylase gene-deficient streptococci had a reduced capacity to produce amylase compared to wild-type strains. And wild-type *S. pyogenes* were found to inhibit biofilm formation in *S. salivarius* compared to amylase gene-deficient *S. pyogenes* strain. The adherent biofilm reached maximum thickness between 24 and 48 hours and demonstrated a dependence on both temperature and growth medium [16]. Taking this result into account, we determined the evaluation time of the biofilm.

Previous concepts of polysaccharide degradation by amylase of *S. pyogenes* have been described as follows. The major site of *S. pyrogens* infection and colonization in human is the oropharynx [17]. Alpha-glucans, such as starch and glycogen, are polysaccharides composed of repeating D-glucose monomers linked by a-links and are found in high concentrations in the human oropharynx [18]. Since the molecular weight of glucans is typically over 100,000, in order to be used as an energy source, they must be digested by extracellular enzymes and become smaller molecules that can be transported into the bacterial cell and enter energy production pathways. Some strains of *S. pyogenes* are capable of degrading starch [19]. Alpha-glucans can be degraded to linearly bound glucose (i.e., maltodextrins) by enzymes called amylases or pullulanases [20]. Alpha-glucan degradation in *S. pyogenes* may involve both of these enzymes may be important. AmyA has been suggested to be a virulence factor for *S. pyogenes* based on its function and extracellular location [21]. Furthermore, the ability of *S. pyogenes* to degrade a-glucans has been speculated to contribute to its virulence [22]. The cyclomaltodextrin and alpha-glucan digestion products produced by AmyA increase the permeability of pharyngeal epithelial cells and enhance the translocation ability of *S. pyogenes* [23]. On the other hand, *S. pyogenes* strains containing *amyA* gene can degrade a-glucan via AmyA. It has been previously reported that the alpha-glucan degrading enzyme of *S. pyogenes* is AmyA and that the cyclomaltodextrin produced by the alpha-glucan degradation of AmyA inhibits normality of human pharyngeal epithelial cells and promotes bacterial migration [24]. Our findings explain the novel pathogenic role of AmyA in *S. pyogenes*. Several studies of enzymes that inhibit bacterial biofilms have been reported for clinical treatment purposes. As a potential treatment for *Pseudomonas aeruginosa* infections, a combined enzyme approach to inhibit biofilms, *P. aeruginosa* self-produced glycosyl hydrolysis enzyme PslG binds to *P. aeruginosa* N-acylhomoserine lactonase AidH to destroy the biofilm. As a result, no bacterial biofilm was observed at the bottom of the NEST glass bottom cell culture dish during the combined enzyme intervention for *P. aeruginosa* [25]. Glycoside hydrolases are a type of enzyme that degrades biofilm substrates and enhances the effectiveness of antibiotics. An infection-responsive delivery system combining the glycoside hydrolyzing enzymes alginate lyase and gentamicin was investigated for the treatment of Pseudomonas biofilms and found that treatment with a liquid crystal containing alginate lyase and gentamicin resulted in a 2 log or greater reduction [26]. Some researchers investigated whether NucB, a novel non-toxic deoxyribonuclease effective in dispersing a variety of single and mixed species bacterial biofilms from marine isolates of *Bacillus licheniformis*, could also destroy and remove mixed species biofilms from tracheoesophageal valves and found that the non-toxic deoxyribonuclease, NucB, was effective in releasing more microorganisms from tracheoesophageal valve biofilms, reflecting NucB's ability to break down and disperse biofilms [27]. Two glycosidic hydrolytic enzymes, alpha-amylase and cellulase, which further degrade complex polysaccharides, effectively destroy monoculture and coculture biofilms of *Staphylococcus aureus* and *P. aeruginosa*, and glycoside hydrolase Treatment significantly reduced EPS biomass and converted the bacteria to a planktonic state, potentially making them more susceptible to conventional antimicrobial agents, and *in vitro* and *in vivo* cultured *S. aureus* and *P. aeruginosa* biofilms were treated with alpha-amylase and cellulase solutions resulted in a significant reduction in biomass, biofilm lysis, and increased efficacy of subsequent antibiotic treatment. These results support our present results and suggest the possibility of developing *S. pyogenes* amylase as an anti-biofilm agent in the future [28]. Finally, we describe the limitations of this study. We have not confirmed biofilm inhibition by adding recombinant protein of *S. pyogenes* amylase directly to biofilms of *S. salivarius*. Nor did the study of the inhibitory effect of amylase on *S. salivarius* biofilms with 3D images comparing live and dead bacteria. The absence of the above results does not preclude the result that amylase of *S. pyogenes* inhibits biofilm formation of *S. salivarius*. However, these questions will be addressed in future studies.

5. Conclusion

In this study, the relationship between *S. pyogenes* and *S. salivarius* was explained in terms of amylase produced by the *S. pyogenes* inhibiting *S. salivarius* biofilms. Further mechanistic elucidation of the survival relationship between *S. pyogenes* and *S. salivarius* is a topic for future research.

Compliance with ethical standards

Acknowledgments

We would like to thank Mr. Masashi Ishihara, Ms. Miwako Fujimura, and Prof. Tadao Hasegawa for excellent support through this investigation.

Disclosure of conflict of interest

All authors declare no conflict of interest of regarding the publication of this paper.

References

[1] Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev. 2000 Jul, 13(3):470-511. doi: 10.1128/CMR.13.3.470.

[2] Dierksen KP, Inglis M, Tagg JR. High pharyngeal carriage rates of *Streptococcus pyogenes* in Dunedin school children with a low incidence of rheumatic fever. N Z Med J. 2000 Nov 24, 113(1122):496-9.

[3] Walls T, Power D, Tagg J. Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media? J Med Microbiol. 2003 Sep, 52(Pt 9):829-833. doi: 10.1099/jmm.0.05259-0.

[4] Carlsson J, Grahnén H, Jonsson G, Wikner S. Early establishment of *Streptococcus salivarius* in the mouth of infants. J Dent Res. 1970 Mar-Apr, 49(2):415-8. doi: 10.1177/00220345700490023601.

[5] Sanders CC, Sanders Jr WE. Enocin: an antibiotic produced by *Streptococcus salivarius* that may contribute to protection against infections due to group A streptococci. J Infect Dis. 1982 Nov, 146(5):683-90. doi: 10.1093/infdis/146.5.683.

[6] Upton M, Tagg JR, P Wescombe P, Jenkinson HF. Intra- and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. J Bacteriol. 2001 Jul, 183(13):3931-8. doi: 10.1128/JB.183.13.3931-3938.2001.

[7] Hyink O, Wescombe PA, Upton M, Ragland N, Burton JP, Tagg JR. Salivaricin A2 and the novel lantibiotic salivaricin B are encoded at adjacent loci on a 190-kilobase transmissible megaplasmid in the oral probiotic strain *Streptococcus salivarius* K12. Appl Environ Microbiol. 2007 Feb, 73(4):1107-13. doi: 10.1128/AEM.02265-06. Epub 2006 Dec 28.

[8] Minami M, Ohmori D, Tatsuno I, Isaka M, Kawamura Y, Ohta M, Hasegawa T.The streptococcal inhibitor of complement (SIC) protects *Streptococcus pyogenes* from bacteriocin-like inhibitory substance (BLIS) from *Streptococcus salivarius*. FEMS Microbiol Lett. 2009 Sep, 298(1):67-73. doi: 10.1111/j.1574-6968.2009.01696.x.

[9] Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol. 2005 Jan, 13(1):20-6. doi: 10.1016/j.tim.2004.11.006.

[10] Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001 Jul 14, 358(9276):135-8. doi: 10.1016/s0140-6736(01)05321-1.

[11] Kania RE, Lamers GEM, Vonk MJ, Dorpmans E, Struik J, Huy PTB, Hiemstra P, Bloemberg GV, Grote JJ. Characterization of mucosal biofilms on human adenoid tissues. Laryngoscope. 2008 Jan, 118(1):128-34. doi: 10.1097/MLG.0b013e318155a464.

[12] Crowley N. The action of streptococcal amylase in relation to the synthesis of an amylosaccharide by amylolytic strains of *Streptococcus pyogenes*. J Gen Microbiol. 1955 Oct, 13(2):218-25. doi: 10.1099/00221287-13-2-218.

[13] Minami M, Ichikawa M, Ohta M, Hasegawa T. The cell envelope-associated protein, LytR, regulates the cysteine protease SpeB in *Streptococcus pyogenes*. APMIS. 2012 May, 120(5):417-26. doi: 10.1111/j.1600-0463.2011.02847.x.

[14] Minami M, Konishi T, Takase H, Makino T. Shin'iseihaito (Xinyiqingfeitang) suppresses the biofilm formation of *Streptococcus pneumoniae in vitro*. Biomed Res Int. 2017, 2017:4575709. doi: 10.1155/2017/4575709.

[15] Minami M, Takase H, Nakamura M, Makino T. Methanol extract of Lonicera caerulea var. emphyllocalyx fruit has antibacterial and anti-biofilm activity against *Streptococcus pyogenes in vitro.* Biosci Trends. 2019 May 12, 13(2):145-151. doi: 10.5582/bst.2019.01005.

[16] Akiyama H, Morizane S, Yamasaki O, Oono T, Iwatsuki K. Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy. J Dermatol Sci. 2003 Sep, 32(3):193-9. doi: 10.1016/s0923-1811(03)00096-3.

[17] Peter G, Smith AL. Group A streptococcal infections of the skin and pharynx (second of two parts). N Engl J Med. 1977 Aug 18, 297(7):365-70. doi: 10.1056/NEJM197708182970706.

[18] Mörmann JE, Mühlemann HR. Oral starch degradation and its influence on acid production in human dental plaque. Caries Res. 1981, 15(2):166-75. doi: 10.1159/000260514.

[19] Crowley N.The degradation of starch by strains of group A streptococci having related antigens. J Gen Microbiol. 1950 May, 4(2):156-70. doi: 10.1099/00221287-4-2-156.

[20] Bertoldo C, Antranikian G. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Curr Opin Chem Biol. 2002 Apr, 6(2):151-60. doi: 10.1016/s1367-5931(02)00311-3.

[21] Hytönen J, Haataja S, Finne J. *Streptococcus pyogenes* glycoprotein-binding strepadhesin activity is mediated by a surface-associated carbohydrate-degrading enzyme, pullulanase. Infect Immun. 2003 Feb, 71(2):784-93. doi: 10.1128/IAI.71.2.784-793.2003.

[22] van Bueren AL, Higgins M, Wang D, Burke RD, Boraston AB. Identification and structural basis of binding to host lung glycogen by streptococcal virulence factors. Nat Struct Mol Biol. 2007 Jan, 14(1):76-84. doi: 10.1038/nsmb1187. Epub 2006 Dec 24.

[23] Shelburne Iii SA, Keith DB, Davenport MT, Beres SB, Carroll RK, Musser JM. Contribution of AmyA, an extracellular alpha-glucan degrading enzyme, to group A streptococcal host-pathogen interaction. Mol Microbiol. 2009 Oct, 74(1):159-174. doi: 10.1111/j.1365-2958.2009.06858.x. Epub 2009 Sep 3.

[24] Shelburne 3rd SA, Keith DB, Davenport MT, Horstmann N, Brennan RG, Musser JM. Molecular characterization of group A Streptococcus maltodextrin catabolism and its role in pharyngitis. Mol Microbiol. 2008 Jul, 69(2):436-52. doi: 10.1111/j.1365-2958.2008.06290.x.

[25] Zhang Y, Wei W, Wen H, Cheng Z, Mi Z, Zhang J, Liu X, Xinjiong Fan X. Targeting multidrug-recalcitrant *Pseudomonas aeruginosa* biofilms: Combined-enzyme treatment enhances antibiotic efficacy. Antimicrob Agents Chemother. 2023 Jan 24, 67(1):e0135822. doi: 10.1128/aac.01358-22.

[26] Thorn CR, Clive A Prestidge CA, Boyd BJ, Nicky Thomas N. Pseudomonas Infection Responsive Liquid Crystals for Glycoside Hydrolase and Antibiotic Combination. ACS Appl Bio Mater. 2018 Aug 20, 1(2):281-288. doi: 10.1021/acsabm.8b00062.

[27] Shakir A, Elbadawey MR, Shields RC, Jakubovics NS, Burgess JG. Removal of biofilms from tracheoesophageal speech valves using a novel marine microbial deoxyribonuclease. Otolaryngol Head Neck Surg. 2012 Sep, 147(3):509-14. doi: 10.1177/0194599812442867. Epub 2012 Apr 2.

[28] Fleming D, Chahin L, Rumbaugh K. Glycoside Hydrolases Degrade Polymicrobial Bacterial Biofilms in Wounds. Antimicrob Agents Chemother. 2017 Jan 24, 61(2):e01998-16. doi: 10.1128/AAC.01998-16.