Biofilm Formation By Staphylococcus Aureus And Detection Of Biofilm Producing Staphylococci

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Abstract- I Look out and give an increasing attention has to be focused on understanding bacterial biofilms and this growth relation to human diseases. In this review we explore the biofilm formation, maturation and its detection methods in the context of gram- positive cocci, Staphylococcus aureus. In addition, we discuss diseases and host immune response and current therapies associated with Staph aureus biofilm infections and prevention strategies. Staphylococcus aureus is an important food borne pathogen able to form biofilms. The ability of the strains to form biofilms was investigated microtiter polystyrene plates. Biofilms are the aggregation of microbial cells, which are associated with the surface in almost an irreversible manner. It exists in variety of forms like dental plaque, pond scum, or the slimy build up in sink. Biofilm formation involves sequence of steps like conditioning, attachment, metabolism, and detachment. Biofilm consists of water channels, EPS (Exopolysaccharide), and eDNA (Environmental DNA), which plays an important role in nutrient circulation, its development, and structure stabilization. Resistance of planktonic bacteria against antimicrobial agents gets increased on the formation of biofilm, which may be the presence of diffusive barrier EPS or neutralizing enzyme, cells undergoing starvation, or due to spore formation. There are numerous factors, which affects biofilm formation such as substratum effects, conditioning film on substratum, hydrodynamics, characteristics of the aqueous medium, cell characteristics, and environmental factors. Biofilm can cause industrial, medical, and household damage and is a reason for loss of billions of dollars every year. Development of biofilm on catheters, medical implants, and devices is a major cause of infections and diseases in humans. Examples include Plaque, Native Valve Endocarditis, Otitis media, Prostatitis, Cystic fibrosis, Periodontitis, Osteomyelitis, and many more.

Index Terms- Biofilm, Staphylococci, Virulence, Pathogenesis, Detection methods like Tissue culture plate, Tube method, Congo red agar, therapy, vaccine.

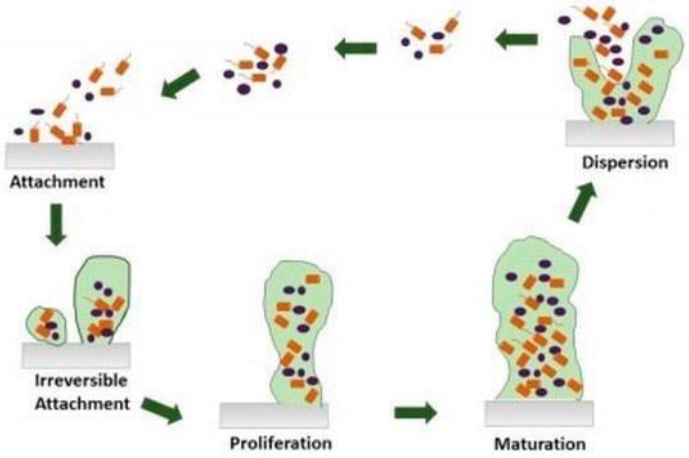
I. INTRODUCTION

A biofilm is an assemblage of surface associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms. Biofilm associated organisms also differ from their planktonic counter parts with respect to the genes that are transcribed. Biofilm may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or portable water system piping, or natural aquatic systems. The biofilm channels allow the supply of nutrients, water, air to each cell giving it new properties-multicellular properties.

Biofilm are a common mode of bacterial growth in nature and their presence has an enormous impact on many aspects of our lives, such as sewage treatment, corrosion of materials, food contamination during processing, formation of dental plaques or problems related to medical implants.

A biofilm may be composed of one microbial species or many microbial species found on a variety of living or non-living surfaces. However, mixed species biofilms form the majority in most of the environments and single species biofilms host the surface of medical implants and hence being the reason of infections.

The initiation of biofilm formation has some requirements as the bacteria must be capable of attaching itself to and moving on the surface, detecting their cell density and ultimately to form a 3-D mesh of cells enclosed by exo-polysaccharide. There is also an important role of cell membrane proteins, extracellular polysaccharides and signalling molecule



The primary organisms responsible for this condition are S. epidermidis, S. aureus, Streptococcus species, GNB, Diphtheroid, Enterococci and Candida species.

Detection methods are tissue culture plate (TCP), Tube method (TM) Congo Red Agar (CRA) and these three are invitro phenotypic methods. Other methods are bioluminescent assay, piezoelectric sensors and fluorescent microscopic examinations. Tissue culture plate assay as described by Christensen's et al;1995 is the most widely used methods and is considered as the standard method for detection of biofilm formation.

Congo red agar method as described by Freeman et al;1989 it is a simple qualitative method to detect the biofilm formation. This method involves use of special media that is BHIA with sucrose and Congo red.

II. OBJECTIVES OF THE STUDY

The aim of this study was to evaluate the formation of biofilm by staphylococcus and its detection methods.

III. REVIEW OF LITERATURE

Staphylococcus was first observed in pus by von Recklinghausen (1871) and was first cultured in liquid medium by Louis Pasteur (1880). It was named staphylococcus means bunch of grapes "by Sir Alexander Ogston. Rosenbach named two species of staphylococci based on pigmentation of colonies as S. aureus (golden yellow colonies) and S. albus (white colonies). Later Passet (1885) named a third species as S. citrus (lemon yellow colonies).

Among Staphylococcus species ***Staphylococcus aureus*** is the most pathogenic and it is catalase positive, coagulase positive, facultative anaerobe, non-motile, non-sporing and occasionally capsulated. They are spherical cocci, about 1µm in diameter, arranged in grape-like clusters. This arrangement is due with daughter cells remaining close together. S. aureus is the most virulent species among staphylococci; produces infections which range from localized pyogenic to life-threatening systemic infections in man. Golden yellow pigmentation and it increased in the presence of CO2 and also at room temperature. Pigmentation can be induced by culturing bacteria into 30% milk agar, potato, and 1% glycerol monoacetate or phosphate agar.

Selective media for ***Staphylococcus*** are

1. 7-10% salt agar
2. Mannitol salt agar
3. Tellurite glycine agar
4. Phenolphthalein phosphate agar
5. Polymyxin B agar (75 µg/ml)

Staph aureus is the leading cause of skin and soft tissue infections such as **abscesses (boils), furuncles, and cellulitis**. Although most staph infections are not serious, S. aureus can cause serious infections such as bloodstream infections, pneumonia, or bone and joint infections.

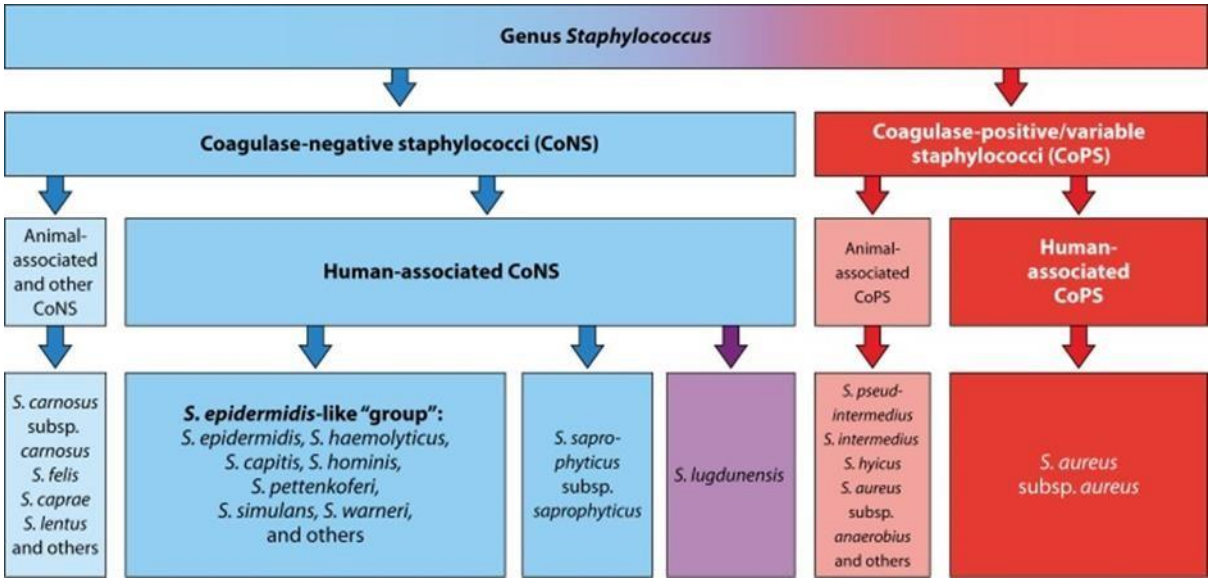
Scientific classification of *Staphylococcus aureus* is given as:

- Domain: Bacteria
- Phylum: Firmicutes
- Class: Bacilli
- Order: Bacillales
- Family: Staphylococceae
- Genus: *Staphylococcus*
- Species: S. *aureus*

List of coagulases-positive staphylococci (Cops)
Staphylococcus aureus, Staphylococcus felis, Staphylococcus intermedius, Staphylococcus lutrae

List of coagulases- negative staphylococci (Cons)

Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus lugdunensis, Staphylococcus schleiferi, Staphylococcus haemolyticus and Staphylococcus warneri.



Staphylococcus aureus can cause the following diseases like-

1. Abscess
2. Conjunctivitis
3. Corneal ulcer
4. Septicaemia
5. Endocarditis
6. Pneumonia
7. Mastitis: It is an inflammation of the breast.
8. Empyema: It is an accumulation of pus in the body cavity.
9. Food poisoning
10. Staphylococcal Scalded Syndrome
11. Toxic Shock Syndrome (TSS)-enterotoxin F
12. Septic arthritis
13. Meningitis
14. Osteomyelitis

Staphylococcus Aureus Virulence Factors

To cause infection, a bacterium needs to first gain access to the host. This is preceded by attaching to the host cells or tissues. S. aureus has numerous surface proteins that promote attachment to host proteins such as laminin and fibronectin that form part of the extracellular matrix.

Fibronectin is also present on epithelial and endothelial surfaces and is also a part of blood clots. The bacteria have a fibrinogen/fibrin binding protein that helps them to attach to blood clots and traumatized tissue. This is the reason why S. aureus is capable of producing wound infections and post-surgery infections.

The virulence factors of Staphylococcus aureus include antigens, enzymes and toxins like:

1) Antigens: Capsule, Adhesins
2) Enzymes: Coagulase, Lipase, Hyaluronidase, Staphylokinase, Nuclease.
3) Toxins: α –Toxin, β –Toxin, δ-Toxin, P-V Leucocidin, Enterotoxin, Exfoliative toxin, Toxic Shock Syndrome Toxin

One major virulence factor of S. aureus is alpha-toxin (aka alpha-hemolysin, hla), which was first described for its lytic activity toward rabbit erythrocytes (17). Alpha- toxin is secreted as a monomer and forms heptameric pores upon binding to the host cell membrane, resulting in death of the target cell. Alpha toxin is a 33,000 D polypeptide produced by most S. aureus strains that cause disease in humans. The toxin disrupts the smooth muscle of the blood vessels and is toxic to many cells. It is a mediator of tissue damage in staphylococcal disease.

GENETICS

S. aureus, biofilm formation is mainly encoded by 12 different genes, i.e., fibrinogen-binding proteins (fib) gene, fibronectin-binding proteins (fnbA and fnbB) genes, intercellular adhesion (icaA, B, C and D) genes, clumping factor (clfA and B), elastin binding protein (ebps), laminin binding protein (eno) and collagen, these are detected by PCR method.

The identified genes involved in the biosynthesis of surface molecules required for the formation of biofilms, such as capsule, poly-beta-1,6-N-acetyl-D-glucosamine (PGA), and pilin. The three most common ways that bacteria diversify their DNA are transformation, conjugation, and transduction.

BIOFILM

Biofilms are the aggregation of microbial cells, which are associated with the surface in almost an irreversible manner, i.e., cannot be removed by gently rising. They are attached with a biotic or abiotic surface integrated into the matrix that they have produced. Biofilm formation is a process whereby microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription. Bacteria form biofilms in response to environmental stresses such as UV radiation, desiccation, limited nutrients, extreme pH, extreme temperature, high salt concentrations, high pressure, and antimicrobial agents. Both gram-positive and gram- negative bacteria can form biofilms on medical devices, but the most common forms are Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridians, E. coli, Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas aeruginosa. Sites for biofilm formation include all kinds of surfaces: natural materials above and below ground, metals, plastics, medical implant materials—even plant and body tissue. Wherever you find a combination of moisture, nutrients and a surface, you are likely to find biofilm. Biofilm formation is commonly considered to occur in four main stages: (1) bacterial attachment to a surface, (2) microcolony formation, (3) biofilm maturation and (4) detachment (also termed dispersal) of bacteria. Bacterial biofilms are clusters of bacteria that are attached to a surface and/or to each other and embedded in a self-produced matrix. The biofilm matrix consists of substances like proteins (e.g., fibrin), polysaccharide (e.g., alginate), as well as eDNA. In addition to the protection offered by the matrix, bacteria in biofilms can employ several survival strategies to evade the host defence systems. Biofilm formation increases the bacteria's resistance against the defence mechanisms of the body, as well as antimicrobial treatments, thereby promoting chronic infections. Biofilms may also function as an environment that accumulate different bacterial species as well as bacterial numbers in certain locations. This can result in deleterious effects on host cells due to concentrated, sequential, and/or synergistic activities by the present bacteria. Furthermore, the mere presence of persistent biofilms may modulate the local immune response in several ways, e.g., by stimulating a local inflammatory response that can cause or aggravate tissue damage.

Staphylococcus aureus is a leading cause of such infections (2). Bacterial biofilm formation proceeds in three steps: initial adhesion, proliferation, and detachment. Adhesion may occur onto virtually any biotic or abiotic surface. Biofilm development has been proposed to occur in three stages: (1) attachment, (2) proliferation/structuring, and (3) detachment/dispersal. During the attachment phase planktonic bacteria adhere to a biotic surface, such as human tissue or a human matrix-covered indwelling device, by non- covalent interactions between human matrix protein and dedicated bacterial surface binding proteins. After attachment is accomplished, biofilm cells multiply producing an extracellular biofilm matrix that is composed of variety of

macromolecules. Several proteins and eDNA have been implicated in invitro staphylococcal biofilm formation. And thus, proteases and nucleases were found to contribute to biofilm structuring and dispersal invitro.

S. aureus, biofilm formation is mainly encoded by 12 different genes, i.e., fibrinogen- binding proteins (fib)gene, fibronectin-binding proteins genes, intracellular adhesion genes, clumping factor, elastin binding proteins, laminin binding protein and collagen. In many bacterial species, the intracellular signalling molecule, c-di-GMP, stimulates the synthesis of biofilm matrix components, particularly polysaccharides.

Bacteria responsible for biofilm production are Staphylococcus aureus, Enterococcus faecalis, Staphylococcus epidermidis, Streptococcus viridians, E. coli, Klebsiella pneumonia, Proteus mirabilis. Formation of Staphylococcus aureus biofilm causes significant infections in human body. Biofilm forms through the aggregation of bacterial species and brings about many complications. It mediates drug resistance and persistence and facilitates the recurrence of infection at the end of antimicrobial therapy. Microbes decide to form biofilms mainly when they are capable of specific or non-specific attachment sites on a surface and also due to exposure of planktonic cells to sub-inhibitory concentrations of antibiotics.

Quorum sensing is a type of decision-making process used by a decentralized group to coordinate behaviour. It can occur within a single bacterial species as well as between diverse species. Many species of bacteria use it to coordinate their gene expression according to the local density of their population. Molecules used as signals in gram positive bacteria are oligopeptides, in gram negative bacteria AHL, also, in both types of bacteria AL-2. These biochemicals diffuse through water channels seen in matrix.

Biochemicals regulate the transcription of various genes as per the requirement of community to produce various proteins, enzymes, etc. It serves as a major means of communication that allows the sessile organisms to survive and grow in the presence of unsuitable environmental conditions and antibiotics or detergents.

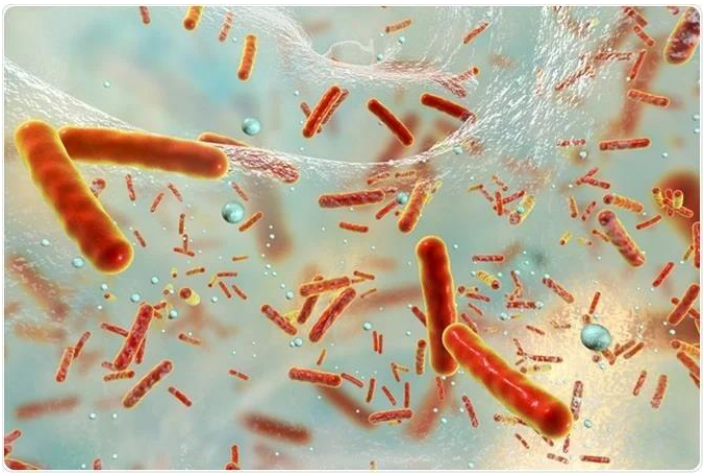
FUNCTIONING OF BIOFILM

Outermost layer –

Highest concentration of oxygen and nutrients, resemble their planktonic counterparts. They slough off and initiate biofilm formation downstream.

Second layer-

Organism here downregulates in their metabolic activity. Although they can clearly utilize the nutrients, exchange genes and have the potential for multiple drug resistance. The benefit is obtained from their alignment in this layer which depends upon spatial arrangement, physiologic heterogeneity and non-uniformity Innermost layer- Attached to the substratum and is the earliest part of biofilm. Also efficiently downregulate and are least metabolically active. Most persisters are found there and provides inheritance for future populations that transfer laterally.



Antibiotic resistant bacteria inside a biofilm, 3D illustration. Biofilm is a community of bacteria where they acquire antibiotic resistance and communicate with each other by quorum sensing molecules.

Biofilm association also provides a mechanism for selecting and promoting the spread of bacterial resistance to antimicrobial age. Biofilms are the aggregation of microbial cells, which are associated with the surface in almost an irreversible manner. It exists in variety of forms like dental plaque, pond scum, or the slimy build up in sink. Biofilm formation involves sequence of steps like conditioning, attachment, metabolism, and detachment. Biofilm consists of water channels, EPS (Exopolysaccharide), and eDNA (Environmental DNA), which plays an important role in nutrient circulation, its development, and structure stabilization. Resistance of planktonic bacteria against antimicrobial agents gets increased on the formation of biofilm, which may be the presence of diffusive barrier EPS or neutralizing enzyme, cells undergoing starvation, or due to spore formation. There are numerous factors, which affects biofilm formation such as substratum effects, conditioning film on substratum, hydrodynamics, characteristics of the aqueous medium, cell characteristics, and environmental factors. Biofilm can cause industrial, medical, and household damage and is a reason for loss of billions of dollars every year. Development of biofilm on catheters, medical implants, and devices is a major cause of infections and diseases in humans.

Examples include Plaque, Native Valve Endocarditis, Otitis media, Prostatitis, Cystic fibrosis, Periodontitis, Osteomyelitis.

Some common Biofilm infections

2) Dental biofilms

Dental biofilms, commonly known as plaque are the most studied biofilm in human. It involves hundreds of species of bacteria. Some significant microbes include Porphyromonas gingivalis, Bacteroides forsythias, Actinobacillus actinomycetemcomitans, Treponema denticula, and a number of Streptococci including Streptococcus mutans.

After a good oral wash or dental cleaning, the tooth enamel acquires a coating called as pellicle which is composed of various proteins and glycoproteins of host origin. Then with the help of adhesion molecules and pili, first Streptococci then Actinomycetes colonizes the teeth surface. Bacterial cells start interacting with

each other on the pellicle and a number of Streptococci and related organisms starts synthesizing insoluble glucan via glucan binding protein. After few successive colonization with few more organisms, demineralization of tooth enamel starts (which leads to caries) by the acids which are produced by fermentation of the dietary sucrose and other carbohydrates.

2) Otitis media

It is a condition of chronic ear infection caused due to inflammation of mucoperiosteal lining. In the middle ear cavity, fluid gets accumulated which ultimately affects speech development and learning capability of the patient. However, its complete etiology is still under research. Various organisms responsible for otitis media include S. pneumoniae, H. influenzae, Moraxella catarrhalis, S. epidermidis, P. aeruginosa, etc. As due to limited penetration of antibiotic, its low concentration is present in middle ear fluid, hence strong antibiotics like amoxicillin, cefaclor, erythromycin, and clarithromycin are needed for combating otitis media.

Inhibition of staphylococcal biofilm formation by nitrite

Several environmental stresses have been demonstrated to increase polysaccharide intercellular adhesin (PIA) synthesis and biofilm formation by the human pathogens Staphylococcus aureus and Staphylococcus epidermidis. Nitrite-mediated inhibition of S. aureus biofilm formation was abrogated by the addition of nitric oxide (NO) scavengers, suggesting that NO is directly or indirectly involved. Nitrite also repressed biofilm formation of S. epidermidis RP62A.

Antibiotics such as fluoroquinolones, rifampin, and ampicillin penetrate well through the matrix, even though they fail to eradicate 100% of biofilm bacteria. Moreover, even in the case of compounds slowly diffusing within biofilms, most antibiotics ultimately reach all biofilm bacteria. The primary and most effective treatment of biofilm infections is physical removal followed by inhibition of reconstitution with antibiofilm agents (ABF), antibiotics (ABX), and selective biocides.

The enzymes **amylase, cellulase, protease, DNase, alginate, and lyase** are reported to support removal of biofilms from medical devices (Stiefel et al., 2016). Therefore, enzymes can be considered natural agents for degradation of biofilm **Foods and food-based supplements such as turmeric (containing Curcumin), garlic (containing ajoene and allicin), apple cider vinegar, vanilla beans, oregano oil (containing carvacrol) pomegranate (containing ellagic acid), and cinnamon (to name but a few) have been scientifically proven to disrupt or prevent biofilm formation.**

Table 1. Partial list of human infections involving biofilms.

Infection or disease	Common biofilm bacterial species
Dental caries	Acidogenic Gram-positive cocci (e.g. *Streptococcus*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g., *Staphylococci*)
Necrotizing fascitis	Group A *Streptococci*
Biliary tract infection	Enteric bacteria (eg., *Escherichia coli*)
Osteomyelitis	Various bacterial and fungal species – often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	*Viridans* Group *Streptococci*
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
Meliodiosis	*Pseudomonas pseudomallei*
Nosocomial infections	
ICU pneumonia	Gram-negative rods
Sutures	*Staphylococcus epidermidis* and *S. aureus*
Exit sites	*S. epidermidis* and *S. aureus*
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Schleral buckles	Gram-positive cocci
Contact lens	*P. aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*S. epidermidis, K. pneumoniae, E. faecalis, Proteus mirabili.*
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
IUDs	*S. epidermidis, S. aureus, Corynebacterium* sp., *Micrococcu* *Enterococcus* sp., *Candida albicans*, Group B *Streptococci.*
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	*S. epidermidis* and *C. albicans*
Central venous catheters	*S. epidermidis, S. aureus, E. faecalis, K. pneumoniae, P. aerugin*
Mechanical heart valves	*Viridans streptococci, Enterococci*
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	*Hemolytic streptococci, Enterococci, P. mirabilis, Bacteroid* *aeruginosa, E. coli*
Pentile prostheses	*S. aureus* and *S. epidermidis*

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