B21: "What key regulatory mechanisms control the release of extracellular DNA (eDNA) from *Staphylococcus aureus* during autolysis, which significantly contributes to the structural integrity of biofilm?"

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RESPONSE/RECOMMENDATION: Extracellular DNA (eDNA) contributes to the structure, growth and immune-evasive properties of *S. aureus* biofilms in orthopaedic infections, and is derived from both the bacteria that undergo autolysis and neutrophils that undergo NETosis at the infection site. eDNA release via autolysis is mediated by murein hydrolase, which is encoded by the *atl* gene. The cidABC and lrgAB operons modulate murein hydrolase activity and autolysis, while the CidR and lytSR transcription regulators control the expression of these operons, respectively. Other murein hydrolase-independent mechanisms of eDNA release have also been reported, such as the *gdpP*-encoded phosphodiesterase and *nuc*-encoded thermonuclease pathways.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: [% vote], Disagree: [%], Abstain: [%]

RATIONALE: A comprehensive literature search was conducted using the terms "biofilm", "Staphylococcus aureus" and "extracellular DNA" within PubMed and Scopus, which initially yielded 374 potentially relevant unique studies. These were screened by two independent reviewers, of which 146 were selected for full-text review and 37 were finally included for evaluation. *Staphylococcus aureus* is a highly prevalent cause of musculoskeletal infections [1]. Importantly, *S. aureus* is known to form biofilms, which consists of cells embedded in an extracellular matrix comprising proteins, polysaccharides, lipids, and extracellular DNA (eDNA). The exact composition of this matrix is highly strain-, time- and condition-dependent [2–4]. Although research has focused on the protein and polysaccharide constituents of the matrix [5,6], it has been increasingly recognized that eDNA contributes to the structure, stability, growth and immune-evasive properties of *S. aureus* biofilms [7]. Given the critical role of eDNA, understanding and counteracting the mechanisms that control the release of eDNA could provide an alternative therapeutic target for bacterial eradication.

eDNA in *S. aureus* biofilms is composed of DNA released by autolysis of a subfraction of the population [8,9], and autolysis-independent pathways have not yet been demonstrated in *S. aureus* [10]. As a result, eDNA is believed to encompass all chromosomally encoded genes [10] as well as some amount of extrachromosomal plasmid DNA [9], which contrasts with findings in other bacterial species. In particular, murein hydrolase plays a key role in autolysis as by degrading the peptidoglycan cell wall [11], lysing the cell and releasing genomic DNA [8,9]. Mutations in the gene coding for murein hydrolase, *atl*, result in defective biofilm formation [12] with decreased eDNA content [13,14]. Similarly, when the biofilm of an *atl* mutant was treated with DNase I, no significant difference in biomass was found compared to that of a wild-type [4].

The cidABC and lrgAB operons regulate cell death and lysis [8]. The cidA gene encodes a holin membrane-associated protein that oligomerizes and forms pores in the membrane [11,15]. This allows murein hydrolase, the endolysin, to access the cell wall and facilitate lysis [16]. In contrast, the lrgA gene encodes an antiholin protein that prevents holins from oligomerizing, thus suppressing lysis [8,15]. These operons work in tandem to modulate murein hydrolase activity and autolysis. Expression of cidA and lrgA have been shown to be affected by local oxygen concentrations [17].

CidR, a LysR-type transcriptional regulator, controls the expression of the cidABC operon, and its expression may in turn be affected by glucose levels [18]. In the presence of acetic acid, a byproduct of glucose metabolism, CidR enhances the transcription of cidA [18,19], which is likely a pH-independent interaction. While CidA plays a significant role in cell lysis, one study has shown that CidB and CidC may also contribute to this process [16].

In the same vein, the lrgAB operon is controlled by the lytSR regulatory system [20], which encompasses two signal transduction pathways: first, it detects reductions in membrane potential and enhances the transcription of lrgA [19,20]; additionally, it activates lrgAB in response to excess glucose metabolism.

Other murein hydrolase-independent mechanisms of eDNA release have also been reported. For instance, the *gdpP* gene encodes a phosphodiesterase responsible for cleaving cyclic-di-AMP. Previous studies have demonstrated that deletions of *gdpP* increase peptidoglycan cross-linking and enhance resistance to antibiotics targeting the cell envelope, which support the theory that reduced cyclic-di-AMP levels compromise cell wall integrity and promote cell lysis [21]. Higher glucose levels have also been found to decrease cyclic-di-AMP and enhance autolysis [21]. A separate study identified a mutation in the purine biosynthesis pathway (ΔpurF) that significantly decreased cyclic-di-AMP levels, biofilm formation, and eDNA levels [22]. When these mutants were supplemented with exogenous cyclic-di-AMP, eDNA production levels were comparable to those of the wild-type.

Another gene implicated is the *nuc* gene, which encodes staphylococcal thermonuclease that degrades eDNA, aiding *S. aureus* in its defense against neutrophil extracellular traps (NETs) [23] and potentially facilitating the release of a subpopulation cells from the biofilm [24]. Strains with reduced thermonuclease activity exhibit greater biofilm formation, thus there is an inverse relationship between *nuc* expression and eDNA levels in the biofilm [25,26].

The intercellular adhesion locus, *ica*, present in *S. aureus* is required for biofilm formation. Previous studies have attempted to categorize methicillin-resistant *S. aureus* (MRSA) biofilms as primarily composed of protein and eDNA (*ica*-independent), while classifying methicillin-sensitive *S. aureus* (MSSA) biofilms as polysaccharide-based (*ica*-dependent). However, this characterization does not apply universally to all strains, since most *S. aureus* isolates harbor the *ica* operon. By contrast, its expression is highly regulated and influenced by various environmental factors [27]. *Ica*-dependent biofilms have observed to contain lower levels of eDNA compared to *ica*-independent biofilms [2], although this may not apply to all *ica*-dependent strains [4]. However, whether this lower level of eDNA adversely affects structural support of biofilm remains to be seen, since both *ica*-dependent and *ica*-independent biofilms were equally susceptible to

DNase I treatment [28]. Another study analyzing 47 clinical isolates of *S. aureus* also found eDNA present in biofilms of all strains, regardless of their methicillin resistance status [2].

Subinhibitory concentrations of beta-lactam antibiotics have been shown to enhance both eDNA release and biofilm formation in certain strains of *S. aureus* [29,30]. This is intuitive as cell wall damage would lead to increased eDNA release, thus promoting biofilm formation. Furthermore, subinhibitory levels of other classes of antibiotics such as clindamycin, a protein synthesis inhibitor, may also influence eDNA release in *S. aureus* biofilms, although this effect could be strain-specific [31]. Subinhibitory ceftriaxone treatment was also found to upregulate *atl* expression, contributing to increased eDNA levels within the biofilm [32]. Conversely, some antibiotics have demonstrated the opposite effect. For instance, subinhibitory levels of nisin, which disrupts cell wall depolarization and inhibits peptidoglycan synthesis, were found to reduce eDNA content in *S. aureus* biofilms [33,34]. Similarly, subinhibitory concentrations of tunicamycin, a teichoic acid synthesis inhibitor, led to decreased eDNA release [35].

The critical role of eDNA in *S. aureus* biofilms as well as its ubiquitous nature across various strains have rendered eDNA an important target for modern therapeutics. It is evident that the mechanisms of eDNA release may be influenced by various strain and environmental factors, including but not limited to local oxygen concentration, glucose levels and culture media. More importantly, further research is needed to understand the influence of subinhibitory antibiotic levels on *S. aureus* eDNA release, given its immense clinical implications for physicians treating musculoskeletal infections.

Active research in the field of in vivo biofilm formation is also focused on host biomolecule contributions including eDNA from NETs [36]. During NETosis, DNA is released from neutrophils recruited to the infection site through a process involving bacterial activation of NADPH oxidase, which generates reactive oxygen species that lead to chromatin decondensation by enzymes that modify histones (e.g. PAD4) [37,38]. Enzymes including myeloperoxidase (MPO) and neutrophil elastase (NE) then rupture the nuclear membrane to release the chromosomal DNA from the neutrophil in NETs during "lytic NETosis", where the cell dies, or “vital NETosis”, which produces NETs and anuclear cytoplasts that phagocytose bacteria. Pathogens including *S. aureus* can usurp NETs by secreting endonucleases that remodels host DNA into biofilm eDNA [39]. Thus, it is unclear how effective targeting bacterial eDNA formation can be as a treatment for orthopaedic infections if the majority of eDNA in biofilm is actually derived from NETs, as some investigators have hypothesized [40].

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