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Original Article

Attenuation of alginate lyase by chlorhexidine in the alginate pathway of *Pseudomonas aeruginosa* biofilm formation

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ABSTRACT

Ventilator-associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* poses a significant clinical challenge due to the pathogen's robust biofilm formation and resistance mechanisms. Chlorhexidine (CHX), a commonly used antiseptic, has shown potential in biofilm inhibition, though its exact mechanism remains unclear. Our previous work focusing on quorum sensing associated proteins identified strong CHX binding to PslG, a key protein in the Psl pathway. However, the potential for CHX interactions within other biofilm pathways, such as alginate, has not been explored. This study explored CHX's potential interaction with proteins involved in the alginate pathway of *P. aeruginosa* biofilm formation using reverse docking. Structures of eight alginate-associated proteins were retrieved from the Protein Data Bank. The CHX structure was obtained from PubChem and docking simulations were conducted using Fpocket and SMINA to predict binding affinities. A total of 735 docking poses were generated for the analyzed alginate-associated proteins. Among these, CHX showed the strongest binding affinity for alginate lyase, a key enzyme that degrades alginate and has been implicated as a therapeutic target for biofilm disruption. This finding suggests that CHX may exert its anti-biofilm effects by targeting the alginate matrix through alginate lyase, potentially enhancing biofilm degradation. Given the promising application of alginate lyase in biofilm control strategies, the interaction between CHX and alginate lyase merits further experimental validation to explore its therapeutic impact in clinical settings.

Keywords: Ventilator-associated pneumonia, Pseudomonas aeruginosa, Chlorhexidine, Alginate lyase, Alginate pathway

Introduction

Pseudomonas aeruginosa is a ubiquitous and opportunistic Gram-negative bacterium that can cause severe infections in immunocompromised individuals, particularly those with cystic fibrosis or chronic wounds [1]. A key virulence factor of *P. aeruginosa* is its ability to form biofilms, which protect the bacteria from host immune responses and antimicrobial agents [2]. Biofilm formation is a complex process that involves the production of extracellular polysaccharides, proteins, and DNA, which provide stability and adhesion to the bacterial community. Biofilms are a key factor in the development of ventilator-associated pneumonia (VAP) caused by *P. aeruginosa* [3]. Understanding the molecular mechanisms underlying biofilm formation is crucial for developing effective therapies to combat *P. aeruginosa* infections.

Biofilm formation by *P. aeruginosa* is driven by a variety of regulatory pathways and genetic factors. The Pel, Psl, and Alg operons are responsible for the biosynthesis of the extracellular polysaccharides that form the biofilm matrix, while the GGDEF-EAL domain-containing enzymes play a role in regulating the levels of the secondary messenger cyclic di-GMP, which is a key signal for biofilm formation [4]. The extracellular polysaccharide alginate, along with Psl and Pel polysaccharides, play critical roles in the structural integrity and stability of *P. aeruginosa* biofilms. The biosynthesis of these three key biofilm matrix components is regulated by complex networks of enzymes and regulatory proteins involved in their respective biosynthesis

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pathways [4, 5]. Within the biofilm, *P. aeruginosa* cells experience stringent growth conditions and must adapt to survive, often through the acquisition of genetic changes and horizontal gene transfer [6]. This heightened resistance to antimicrobial agents makes *P. aeruginosa* biofilm infections particularly challenging to treat.

Numerous strategies have been developed to combat *P. aeruginosa* biofilms, including the use of antimicrobial agents, quorum-sensing inhibitors, and enzymes that degrade the biofilm matrix [2]. Among these approaches, chlorhexidine (CHX)—a widely used antiseptic and disinfectant—has garnered attention for its broad-spectrum antimicrobial properties [7]. Known for its extensive application in medical and dental fields, CHX has been particularly effective in preventing plaque formation and managing gingivitis. More recently, its potential role in preventing VAP caused by *P. aeruginosa* has been investigated [8]. Emerging evidence suggests that CHX-based oral care regimens may reduce the incidence of VAP by disrupting the formation of Pseudomonas biofilms within the respiratory tract [9]. However, the precise molecular mechanisms by which CHX disrupts biofilms in this context remain poorly understood.

To unravel the underlying mechanisms of CHX's biofilm inhibition, we previously performed reverse docking analyses targeting six quorum-sensing proteins in *P. aeruginosa*, including LasR, LasL, PslG, PelA, PelB, and RhlR (in press). Our results revealed that CHX exhibits the highest binding affinity for PslG, a critical regulator in the construction of the *P. aeruginosa* biofilm matrix. While the potential of CHX for Psl and Pel-associated biofilms has been explored, relatively little attention has been given to the alginate and its associated proteins in biofilm development. This study seeks to explore the potential of CHX to target the alginate biosynthesis pathway through molecular docking analysis, focusing on interactions with key alginate-associated proteins. By identifying specific alginate biosynthesis proteins that CHX may inhibit or activate, our study could provide novel insights into additional biofilm disruption strategies. Understanding these molecular interactions may further enhance the therapeutic value of CHX as a versatile antiseptic in clinical settings aimed at controlling biofilm-associated infections, such as VAP.

Materials and Methods

Data preparation

We obtained the three-dimensional structures of eight alginate-associated proteins from the bacterium *P. aeruginosa*: AlgD (PDB ID:1MV8), Alg44 (PDB ID:4RT0), AlgK (PDB ID:3E4B), AlgE 4(PDB ID: XNK), AlgG (PDB ID:4NK6), AlgX (PDB ID:4KNC), AlgL (PDB ID:4OZW), and AlgJ (PDB ID:4O8V). To prepare the proteins for analysis, we employed a sanitization process using the LePro software. This process involved adding hydrogen atoms while accurately accounting for the protonation states of histidine residues. During this sanitization, all crystal waters, ions, small ligands, and cofactors were removed to preserve the structural integrity of the proteins. We then selected the antimicrobial compound CHX as the ligand of interest. We retrieved the isomeric SMILES representations of CHX from the PubChem database and used Pybel to generate 3D structures of the ligand. Finally, the ligand was optimized through 500 steps of the MMFF94s force field.

Pocket detection and molecular docking

We first cleaned the protein structures and then used pockets to identify potential binding pockets, focusing on those with a volume of at least 250 Å^3 [10, 11]. We processed the identified pockets using PyMOL and identified the grid box to determine their centers and dimensions, extending the selection by 4.0 Å. We performed molecular docking analysis on these pockets using Smina software, with parameters set for the pocket center coordinates, dimensions, exhaustiveness of 8, and up to 5 binding modes [12]. The docking results were saved in SDF format for further analysis. We utilized Py3Dmol to examine the 3D interactions between the proteins and ligands. Finally, the SDF files were parsed to extract essential information such as binding modes and affinities, which were compiled into a data frame for detailed examination.

Fingerprint analysis

To further analyze the protein-ligand interactions, we performed in-depth interaction fingerprinting using the ProLIF Python library [13, 14]. We focused our analysis on the three binding poses with the highest docking scores for each selected pocket and protein complex. By generating detailed interaction fingerprints, we were able to gain valuable insights into the key interactions between the protein and ligand, allowing us to thoroughly visualize and characterize the binding modes at an atomic level.

Results and Discussion

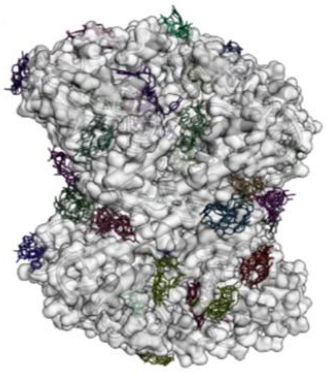
Our comprehensive analysis of the alginate biosynthesis proteins uncovered a wide array of candidate binding sites across eight key targets **(Table 1)**. We applied a stringent criterion, focusing only on pockets with a minimum volume of 250 Å^3 to enhance the relevance of our findings. Notably, AlgD had 36 identified pockets, Alg44 revealed 4, AlgK showed 25, AlgE uncovered 26, AlgG had 11, AlgX displayed 17, AlgL presented 13, and AlgJ exhibited 15 binding pockets **(Table 2)**. For each of these sizable binding pockets, five distinct docking poses were generated, leading to a comprehensive set of 735 docking simulations across all proteins **(Table 2)**. This dataset provides a rich landscape for studying potential molecular interactions, offering crucial insights into how CHX might interact with components of the biofilm matrix. A detailed visualization of the detected binding sites and their respective ligand interactions is presented in **Figure 1**, laying the groundwork for further in-depth analysis and experimental validation.

Table 1. Top 10 Binding Affinity Scores from Molecular Docking

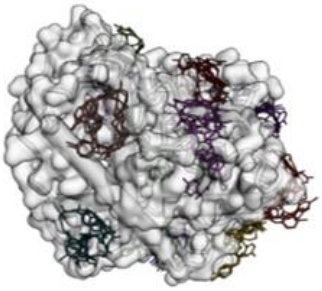
Protein	Pocket ID	Affinity (kcal/mol)	Ligand
AlgL	4OZW_pock_6.sdf	1	-9.06051
AlgD	1MV8_pock_17.sdf	1	-8.89037
AlgE	4XNK_pock_2.sdf	1	-8.71466
AlgD	1MV8_pock_13.sdf	1	-8.66897

The docking analysis identified notable binding affinities for CHX across several key proteins in the alginate biosynthesis pathway. The highest affinity was observed for AlgL (PDB ID: 4OZW) in pocket id 6 in this study, with a binding energy of -9.06 kcal/mol, suggesting a strong interaction potential with this enzyme **(Table 1, Figure 2)**. Following closely, AlgD (PDB ID: 1MV8) demonstrated strong affinities in two distinct pockets, with pocket id 17 exhibiting a binding energy of -8.89 kcal/mol and pocket id 13 showing -8.67 kcal/mol. Additionally, AlgE (PDB ID: 4XNK) showed a noteworthy binding affinity in pocket id 2, with a binding energy of -8.71 kcal/mol **(Table 1)**. Among these, AlgL—an alginate lyase—appears to be the most promising binding partner of CHX if the compound disrupts *P. aeruginosa* biofilms via the alginate pathway. AlgL plays a pivotal role in breaking down the alginate polymer within the biofilm matrix, implying that CHX's interaction with this enzyme could potentially modulate the integrity of the biofilm structure. Further experimental studies are needed to clarify the mechanistic role of CHX in this context. Our results indicate that the protein-ligand interactions between CHX and AlgL are characterized by a combination of hydrophobic and PiCation interaction as well as hydrogen and Van der Walls bonds. The two chlorguanides of CHX are involved in hydrophobic and Van der Walls's interaction with the aromatic and polar residues. Additionally, the one of chlorguanide groups forms hydrogen bonds with Lys66. These interactions likely contribute to the overall binding affinity and stability of the CHX-AlgL complex **(Figure 2b)**.

Reverse docking has emerged as a powerful strategy for identifying previously unknown protein targets that can interact with a specific ligand. This method allows for the discovery of novel drug targets for existing compounds, thereby offering opportunities for drug repositioning and expanding therapeutic applications. One of the key advantages of reverse docking is its ability to reveal unexpected off-target proteins that may bind to the compound of interest. While this can lead to challenges, such as potential side effects, it also opens up new avenues for drug development. In this study, we utilized a reverse docking approach to explore potential protein targets of CHX—a widely used antiseptic—in the inhibition of *P. aeruginosa* biofilm formation, particularly through the alginate biosynthesis pathway. Among the eight selected proteins studied, we found that AlgL exhibited the strongest binding affinity to CHX, suggesting that it could serve as a critical target in disrupting biofilm formation.



a)



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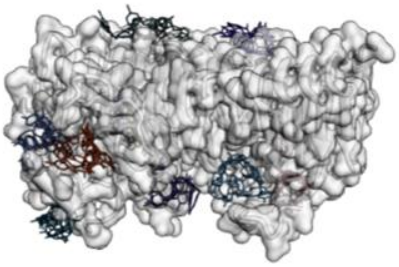

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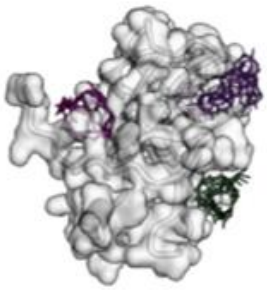

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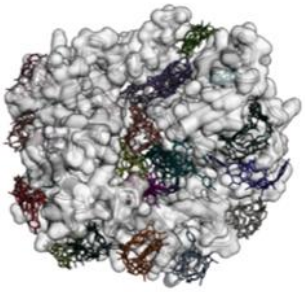

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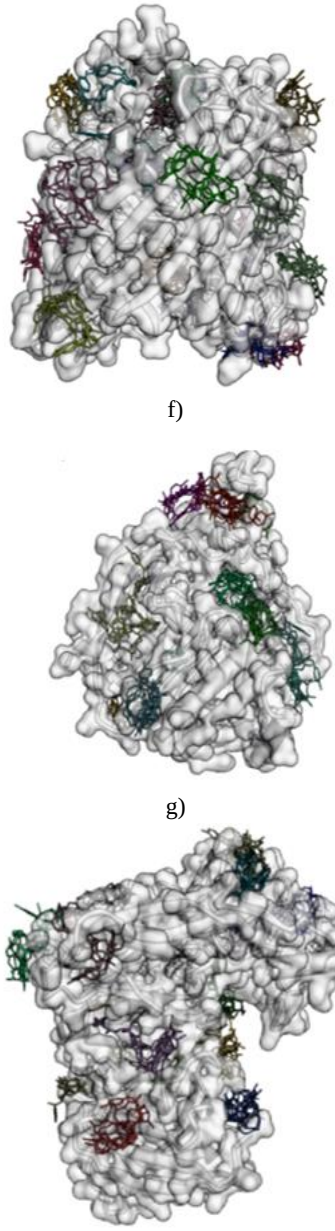

Figure 1. Molecular docking of a) LasI, b) PslG, c) LasR, d) PelA, e) PelB, and f) RhlR with chlorhexidine at identified pockets. Each pocket shows five poses of the ligand bound to the site.

In *P. aeruginosa*, the alginate biosynthetic pathway is a tightly regulated process essential for its survival in challenging environments, particularly in the lungs of individuals with cystic fibrosis [15]. This intricate pathway involves a series of enzymatic reactions that convert simple sugars into the final alginate polymer. The process begins in the cytoplasm with AlgD playing a critical role in the formation of the precursor molecule, GDP-mannuronic acid [4]. This precursor is then transported across the inner membrane into the periplasm, where the real action begins. AlgA takes center stage, polymerizing GDP-mannuronic acid units into a growing alginate chain. AlgG, an epimerase, then steps in to fine-tune the polymer's properties by converting some D-mannuronic acid residues to L-guluronic acid. Meanwhile, AlgE, AlgK, and AlgX work together to transport the growing alginate chain across the periplasm, ensuring its proper localization. AlgJ and AlgI collaborate to acetylate the alginate, further modifying its characteristics. Finally, AlgL acts as a quality controller, removing any errant D-mannuronic acid residues that could disrupt the polymer's structure [4, 16]. This complex interplay of proteins in *P. aeruginosa* highlights the importance of alginate biosynthesis for this opportunistic pathogen.

Considering the regulatory role of AlgL in maintaining the structural integrity of alginate and its strong binding affinity to CHX in our study, it may represent a key target through which CHX disrupts *P. aeruginosa* biofilms. It is uncertain whether CHX acts as an agonist, potentially enhancing alginate degradation, or as an inhibitor, impeding the function of AlgL and thus stabilizing the biofilm matrix. Understanding the precise nature of this interaction is crucial for determining how CHX may interfere with biofilm formation or persistence in the respiratory tract of patients at risk for infections such as VAP. Further studies are needed to validate the identified binding interactions and assess the impact of CHX on AlgL activity and *P. aeruginosa* biofilm formation. Experimental techniques such as surface plasmon resonance, isothermal titration calorimetry, and enzyme activity assays could provide valuable insights into the binding kinetics and thermodynamics of the CHX-AlgL interaction [17-20]. Additionally, in vitro and in vivo studies evaluating the effects of CHX on *P. aeruginosa* biofilm development and alginate production would help to elucidate the potential of this compound as an anti-biofilm through alginate biosynthetic pathway.

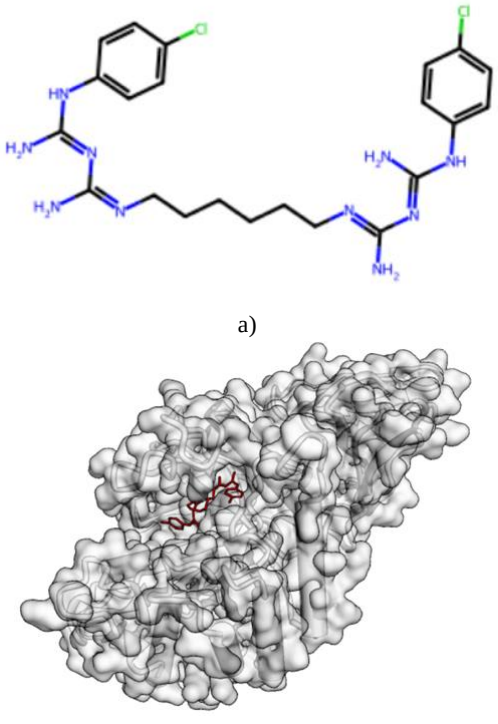

Figure 2. a) Visualization of Chlorhexidine molecular structure and b) Interaction of Chlorhexidine with PslG Pocket 9 which has the highest binding affinity in this study.

Conclusion

Our study identifies AlgL, an enzyme involved in the alginate biosynthetic pathway, as a highly probable binding partner of CHX, particularly if this compound disrupts biofilms through interference with the alginate pathway. The strong binding affinity observed between CHX and AlgL suggests that CHX may impair biofilm integrity by targeting key components of the alginate matrix, thereby enhancing biofilm degradation. These findings present new opportunities for the therapeutic use of CHX in biofilm-associated infections by targeting alginate lyase as a crucial factor in biofilm control. However, further experimental validation is crucial to confirm the efficacy of this interaction and to assess its clinical relevance, particularly in the treatment of persistent infections such as VAP.

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