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RESEARCH ARTICLE

The Effect of Autoinducer Analogs on the Quorum Sensing Systems of *E. coli* K12

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ABSTRACT: Virulent *E. coli* in clinic settings causes UTIs, meningitis, among others. Novel drugs to combat these bacteria are developed, but a resistant strain will inevitably arise. Therefore, a non-antibiotic approach using autoinducer analogs (AIAs) was taken to inhibit the biofilms of *E. coli* K12. The effects of the AIAs N-Acetylglucosamine and S-(5′-Adenosyl)-L-homocysteine on the LuxI/LuxR and LuxS quorum sensing systems of *E. coli* K12 were studied to determine which quorum sensing system of *E. coli* K12 is most susceptible to inhibition. This experiment measured the reduction of biofilm formation of *E. coli* K12 using AIAs on the strain's quorum sensing systems: LuxI/LuxR and LuxS. *E. coli* K12 had a 10-sample control group along with a 10-sample AIA group for each AIA. The biofilm counts were recorded for all three groups in 24-hour intervals. The LuxI/LuxR group resulted in the greatest biofilm inhibition meaning the LuxI/LuxR quorum sensing system of *E. coli* is most susceptible to inhibition. This non-antibiotic approach for inhibition will save millions of dollars for pharmaceutical companies that are spent on developing antibiotics to combat multidrug-resistant bacteria.

KEYWORDS: Biochemistry; General Biochemistry; Autoinducer Analogs; *E. coli*; Quorum Sensing.

Background

A study done by Microbiologists in Japan was on the effects of autoinducer analogs on both the free-floating (planktonic) and concentrated (biofilms) cells of *Pseudomonas aeruginosa*. *This gram-negative bacterium* can cause serious illnesses such as cystic fibrosis.[1] In general, bacteria can be divided into two types: gram-positive and gram-negative. The key difference between both types of bacteria is that gram-positive bacteria have a thicker layer of peptidoglycan than gram-negative bacteria in the extracellular matrix area. Regardless, both types of bacteria use the process of quorum sensing to form biofilms, which cause infections. A biofilm is an aggregation of bacteria with a slimy layer of proteins that covers the bacteria. Using quorum sensing, bacteria release autoinducers: ligands that bind to specific receptors with similar signaling pathways. Thus, these ligands act as indicators that alert the bacteria to move to a common location forming adhesive clumps known as biofilms.[2] Biofilms are multi-drug resistant from garnering many bacterial strains effective against antibiotics, especially in clinical settings. Novel antibiotics are similar to pre-existing ones in terms of chemical composition, thereby allowing biofilms to continue their resistance. Additionally, antibiotics are developed at a slow rate because of the US Food and Drug Administration's limited approvals contributing to the quick adaptability of biofilms.

A possible solution to stop the formation of biofilms is to stop the autoinducer ligands from binding to the receptors. Thus, autoinducer analogs are competitive inhibitors that prevent the binding of autoinducers to the receptors and help stop the aggregation of bacteria. The researchers purchased two autoinducers (AIA-1 and AIA-2) analogs from Meiji Seika Pharmaceuticals and two antibiotics called azithromycin (AZM) and clarithromycin (CLR). The control group *P. aeruginosa* was grown without antibiotics. They used the killing assay to determine the biofilm cells after using AIA-1 plus various concentrations of AZM, CLR, and AIA-2 plus multiple concentrations of AZM and CLR. The results were that there was at least 100 times less formation of biofilms for both antibiotics. For AZM, specifically, on the highest dosage of 256 micrograms/milliliter, there was at least 100,000 times less biofilm formation.

According to a study on QS and biofilms, the three general classes of signaling pathways for the gram-positive and gram-negative bacteria are defined: LuxI/LuxR, Oligopeptide-two-computing-type, and LuxS.[3] Each of these signaling pathways has its own autoinducer for which respective autoinducer analogs can be used to inhibit the signaling pathways, thereby ceasing biofilm formation. Usually, the bacteria have at least two of the three signaling pathways.[4]

From our background research, discovering which signaling pathways of critical multi-drug resistant bacteria such as *E. coli* are most susceptible to autoinducer analogs would help identify which signaling pathways are most relevant for biofilm inhibition and paving future research in a methodical manner. A Time Kill Assay would be used to measure the reduction of biofilm formation over time with and without the addition AIAs respective to *E. coli*'s quorum sensing systems of LuxI/LuxR and LuxS. Furthermore, the Time Kill Assay is adept at only measuring biofilm inhibition and not the killing of bacteria as biofilms exposed to AIAs may exist in a planktonic state where they are separated from biofilms and alive partially but not visible in the field view, making such bacteria untraceable and unethical to count.[5] The experiment is feasible as all mate

DOI: 10.36838/v5i6.19

rials are within reach for a low price. Additionally, the studies formerly stated are thorough and provide a solid foundation for the experiment.

Methods

An inoculation loop was heated using a Bunsen burner until it was hot red for sterilization and was left alone for a 5-minute cooldown. *E. coli* K12 from its growth medium was transferred to 10 Petri dishes and cultured using the 4-quadrant streaking method in a fume hood, also where all Petri dishes were stored and monitored for biofilm formation. The average number of visible biofilms (Table 1) was calculated from the control groups after 24, 48, and 72 hours. Experimental Petri dishes were prepared for *E. coli* K12's (LuxI/LuxR and LuxS) quorum sensing systems with the autoinducer analogs for *E. coli* K12's (N-Acetylglucosamine and S-(5′-Adenosyl)-L-homocysteine) quorum sensing systems. A precision scale was used to weigh 1000x Minimum Inhibitory Concentration (MIC) for each AIA (Figure 1), which was the most precise level of AIA, in grams, obtainable. MIC values were obtained from IC values initially (maximal inhibitory concentration). The IC100 (100% of maximal inhibition) for LuxS and IC50 (50% of maximal inhibition) values for LuxI/LuxR were converted to MIC values. The measured amounts were diluted by 1mL of DI water for the concentrations of 1.8 mM and 0.5 mM for LuxI/LuxR and LuxS groups. Each AIA solution was transferred using sterile pipettes to their respective groups on the 3rd and 4th quadrants of the Petri dishes, as that is the area of bacterial growth. The number of isolated colonies formed for both autoinducer analog groups of *E. coli* K12 were averaged after 24, 48, and 72 hours. The averaged biofilm counts of the autoinducer analog groups for *E. coli* K12 were compared to each other and their control using an ANOVA test and Tukey's HSD to identify the significance of the results. In addition, a Time Kill Assay was generated to determine which quorum sensing system was most susceptible to inhibition.

Results

Table 1: The table shows the recorded number of biofilms for each group (Control, LuxS, and LuxI/LuxR) for all ten samples of each group over 24-hour intervals (0, 24, 48, and 72 hours). LuxI/LuxR showed the lowest number of biofilms or the greatest susceptibility.

Number of Biofilms by Group									
	Control			LuxS			LuxI/LuxR		
Sample #	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
1	0	105	297	0	300	450	0	5	180
2	0	237	435	0	70	220	0	35	250
3	0	138	415	0	150	350	0	120	150
4	0	300	595	0	200	396	0	15	250
5	0	204	600	0	147	358	0	19	50
6	0	196	572	0	133	305	0	57	80
7	0	203	543	0	62	172	0	69	130
8	0	300	589	0	119	208	0	19	260
9	0	59	225	0	118	210	0	14	200
10	0	23	189	0	48	90	0	16	80

Table 2: The table shows an ANOVA test for the three groups: Control, LuxS, and LuxI/LuxR. The null hypothesis is rejected, and the result is a p-value of less than 0.05, indicating the statistical significance of the data.

	Groups			
	Control (T1)	LuxS (T2)	LuxI/LuxR (T3)	Total
N	20	20	20	60
∑X	6225	4106	1999	12330
Mean	311.25	205.3	99.95	205.5
∑X2	2610473	1107384	344759	4062616
Std.Dev.	188.1967	117.9702	87.3465	160.9717
f-ratio	11.75677			
p-value	0.000053			

Table 3: The table shows Tukey's HSD, which identified the significance of every pair in this study. As indicated in blue, the p values are less than 0.05 for the possible combinations of Control (T1), LuxS (T2), and LuxI/LuxR (T3) groups.

Pairwise Comparisons		$HSD_{.05}$ = 104.8609 $HSD_{.01}$ = 132.2162	$Q_{.05}$ = 3.4032 $Q_{.01}$ = 4.2910
T_1:T_2	M_1 = 311.25 M_2 = 205.30	105.95	Q = 3.44 (*p* = .04714)
T_1:T_3	M_1 = 311.25 M_3 = 99.95	211.30	Q = 6.86 (*p* = .00003)
T_2:T_3	M_2 = 205.30 M_3 = 99.95	105.35	Q = 3.42 (*p* = .04870)

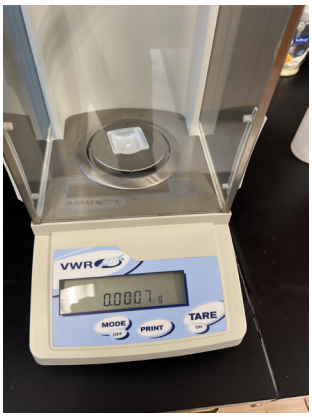

Figure 1: 1000x MIC of LuxS was 0.0007g which was weighed using the shown VWR precision scale.

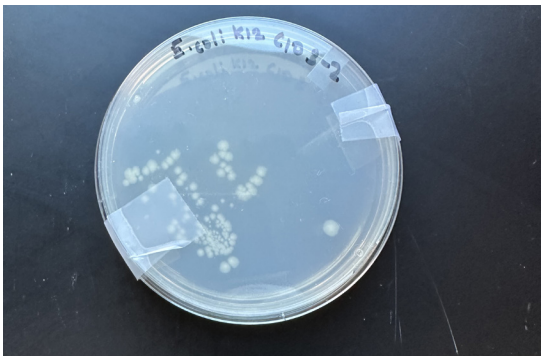

Figure 2: Sample #10 of Control after 72 hrs. of incubation.

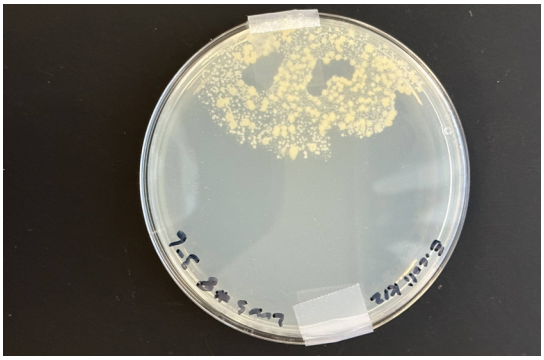

Figure 3: Sample #8 of LuxS after 72 hrs. of incubation.

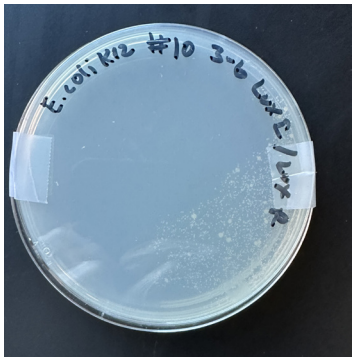

Figure 4: Sample #10 of LuxI/LuxR after 72 hrs. of incubation.

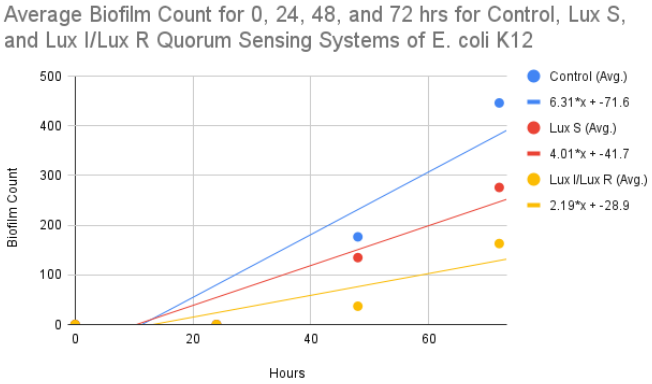

Figure 5: The Time Kill Assay of *E. coli* K12 biofilm count over 24-hour intervals (0, 24, 48, and 72) of the average number of biofilms for the groups of control, LuxS, and LuxI/LuxR shows that the LuxI/LuxR quorum sensing system is more susceptible to inhibition than the LuxS quorum sensing system of *E. coli* K12.

Results and Discussion

Data Significance Validation:

To understand if our results are significant, we conducted an ANOVA test for which we received a p-value of 0.000053, as shown in Table 1, which is significant when p is less than 0.05. To further verify the significance of our results, we conducted Tukey's HSD, shown in Table 2, which compared all possible combinations of pairs of the three groups. The result was once again p values of less than 0.05 which are shown to the immediate right of the Q values in blue. Once again, our data has shown to be statistically significant at p less than 0.05.

Data Interpretation:

As our results were significant, we interpreted our data by generating a Time Kill Assay (Figure 5), which shows the averages of the biofilm counts of all ten samples for each group in 24-hour intervals along with their lines of best-fit or trendlines. The number of biofilms was not referred to as CFUs as we did not count for the number of cells in total but instead for the number of isolated colonies. Once again, it is important to note that the Time Kill Assay measures biofilm inhibition rather than the killing of bacteria. Furthermore, the counted number of biofilms is equivalent to the number of isolated colonies visible in the 3rd and 4th quadrants of the Petri dishes. Distinctive biofilm - multiple isolated colonies - growth in the 1st and 2nd quadrants of the Petri dishes would lead to discarding the Petri dishes. As shown in Figure 5, the overall virulence of *E. coli* has decreased as the biofilm concentration has seen inhibition which can be seen as the trendlines for both the LuxS and LuxI/LuxR groups are significantly lower than the control group's trendline.

Initially, when deciding between spread plating, a common technique used to measure antibiotic effectiveness, and 4-quadrant streaking for proper biofilm yield, we concluded with 4-quadrant streaking because we considered not only the distribution of the bacteria but also the distribution of the compound. spread plating considers antibiotic effectiveness based on the diameter of the zone of inhibition. As AIAs are not like commonly tested antibiotics, there is no existing research on pre-established diameters for zones of inhibition. Additionally, the AIA solutions would not fully dissolve after dilution, so it would have been difficult to disperse the undissolved compounds evenly across a Petri dish as done in spread plating. In contrast, the spreading is limited to only the 3rd and 4th quadrants in 4-quadrant streaking, which was our method of biofilm growth. From this experiment, we identified the quorum sensing system of *E. coli* that is most susceptible to inhibition: LuxI/LuxR. This result can be observed in the Time Kill Assay (Figure 5) as the trendline for the biofilm count for the LuxI/LuxR group has the lowest slope meaning that it was the group with the greatest amount of inhibition.

Potential Errors and Growth:

Our experiment may have been prone to potential errors. First, some biofilms were not separate but rather one big chunk of overlapping biofilms, making it difficult to separate for some. For instance, one chunk may have been three biofilms combined as one but could have been considered 2 or 4 biofilms in the counting process, which is not the most accurate.

Another error would be the cooling time of the inoculation loops. The 5-minute cooling period for the 4-quadrant streaking method was used for the control group. Still, the cooling period was shortened to approximately 3 to 4 minutes for the experimental groups to increase work efficiency. This may have resulted in some bacteria not being killed by the heat, meaning there could have been a greater parent population size for the experimental groups relative to that of the control group.

Additionally, we can observe from Figures 2, 3, and 4 that the biofilms of the LuxI/LuxR sample are smaller than those of the control and LuxS samples, which showed that the partial inhibition of quorum sensing from inhibiting one of the two quorum sensing systems led to the breakdown of more extensive biofilms to smaller biofilms some of which may not have been visible to the naked eye. As the visible larger biofilms were counted, the smaller biofilms were not, which could be made more accurate using microscopy techniques in the future. Although our data were statistically significant, future studies should have more data samples, high-end microscopy techniques, and tests for a larger representation and greater accuracy in counting biofilms to answer the research question better.

Conclusion

Previous research not only for *E. coli* but also for infectious bacteria has tested the efficacy of certain antibiotics of the popular *Pseudomonas aeruginosa* model.[1] The lack of research in clinical bacteria such as *E. coli* prompted us to identify autoin-

DOI: 10.36838/v5i6.19

ducer analogs that would inhibit the growth of *E. coli.* Additionally, there needed to be more research on identifying the sensitivity of the quorum sensing systems to inhibitors. As we were looking forward to researching non-antibiotic methods of inhibiting biofilm growth, we decided to conduct this specific experiment to discover the quorum sensing system of *E. coli* most susceptible to inhibiting biofilm growth.

Our experiment showed that inhibiting the LuxI/LuxR system led to the greatest decrease in biofilm count. As the autoinducer analogs were specific inhibitors of their quorum sensing pathways, we concluded that the LuxI/LuxR pathway is more susceptible to inhibition than the LuxS pathway.

This experiment is crucial for pharmaceutical companies developing antibiotics to inhibit biofilm growth. Our experiment solidifies the concept that autoinducer analogs may be the future of non-antibiotic approaches to inhibition of *E. coli* and such bacteria, thereby saving millions of dollars in the drug development process. Identification of the LuxI/LuxR quorum sensing system as more susceptible to inhibition than the LuxS quorum sensing system forecasts for the development of compounds targeted towards the LuxI/LuxR quorum sensing system for greater efficacy against *E. coli*, thereby lowering nosocomial infections and encouraging similar research for other infectious bacteria.

Acknowledgments

We thank the Rock Ridge High School Science Dept., especially Mr. Dylan Cashman, Dr. Mithra Marcus, and Ms. Madina Mamatova, for their guidance. We also thank the Joanne Nagurny Memorial Fund for recognizing our work at the Loudoun County Public Schools Regional Science and Engineering Fair. Furthermore, we thank Dr. Stephen Burton of George Mason University for providing us this research opportunity.

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