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# Up-Regulating Pyocyanin Production by Amino Acid Addition for Early Electrochemical Identification of *Pseudomonas aeruginosa*

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## ABSTRACT

This work focuses on developing a faster method for electrochemically detecting a *Pseudomonas aeruginosa* infection through the addition of amino acids to cell culture samples. We performed square-wave voltammetry measurements of pyocyanin produced by *P. aeruginosa* using commercially available carbon-based electrodes connected to a Ag/AgCl reference. The electrochemical response resulting from the production of pyocyanin by bacteria was measured in the presence of various amino acids while varying three different culturing parameters: liquid media type (trypticase soy broth vs. M63 minimal media); concentration of amino acids in the solution; and initial concentration of the *P. aeruginosa* in the solution. Our results demonstrate a faster and stronger electrochemical response in media containing tyrosine and valine at elevated concentrations, lending promise to using amino acids as up-regulatory molecules for faster bacterial detection.

## 1. INTRODUCTION

*Pseudomonas aeruginosa* is one of the leading causes of gram-negative bacterial infections in the hospital setting.[1, 2] This opportunistic pathogen is frequently linked to patients with cystic fibrosis,[3-5] severe burn victims, and immunocompromised hosts such as patients with AIDS.[6] Although it is seldom responsible for infections in healthy individuals, several factors contribute to the success of *P. aeruginosa* in the compromised including its ability to thrive in the hospital environment and its increasing resistance to antibiotics.[1] *P. aeruginosa* infections pose serious concerns for this patient population, making it an important bacterium to study in the scientific and medical community.

Unique to *P. aeruginosa* is its production of pyocyanin, a redox-active quorum sensing molecule linked to biofilm formation and therefore a significant contributor in the bacteria's pathogenesis.[7-9] Because it is redox-active, pyocyanin can be detected electrochemically.[10-14] Recently published literature has shown that the addition of amino acids up-regulates the biofilm formation of *P. aeruginosa*.[15, 16] However, these studies do not address the link between pyocyanin production and *P. aeruginosa* growth. Research carried out in the 1950's to determine how amino acids influence pyocyanin production has not been revisited or applied to sensing strategies.[17, 18] The aim of the study is to electrochemically detect the up-regulation of pyocyanin production via amino acid addition, leading to improved detection sensitivity for applications in health care settings.

It is well-known that amino acids are the building blocks for protein synthesis and thus serve as key components for bacterial growth, such as peptidoglycan for cell wall formation.[19] Thorax *et al.* found that amino acid concentrations in sputum were higher for those with more severe cases of cystic fibrosis,[20] leading us to believe that amino acids could be used as

regulatory molecules in bacterial infections. Furthermore, Pierson *et al.* proposed a biosynthetic pathway for pyocyanin whose precursors include a branch-point synthesis of aromatic amino acids, lending credibility to using amino acids as regulatory molecules.[21] As amino acid synthesis is a metabolically expensive process, we expect bacteria, such as *P. aeruginosa*, to adapt their metabolic pathways given an abundance of free amino acids in the environment. The six amino acids (proline, histidine, arginine, leucine, tyrosine, and valine) used in this study were selected based on their ability to up-regulate biofilm formation in *P. aeruginosa* cultures.[15]

The results from this study provide a better understanding of how pyocyanin production is linked to the up-regulation of biofilm formation in *P. aeruginosa,* and give rise to improved electrochemical techniques for early infection identification. By understanding the relationship between pyocyanin production and *P. aeruginosa* behavior, our ability to detect small molecules that are rapidly produced by cells can potentially be extended to other pathogens.

## 2. MATERIALS AND METHODS

### 2.1 Materials

All bacterial tests were completed using wild-type *P. aeruginosa* strain PA14. Cell cultures were routinely grown in trypticase soy broth (TSB) (BD Biosciences 211768) at 37 °C and later stored on TSB agar plates at 4 °C when not in use. All amino acids were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in solution either using TSB or M63 minimal salts medium (Fisher Cat. 50-751-6740) [$(NH_4)_2SO_4$ (15 mM), $KH_2PO_4$ (100 mM)] supplemented with $MgSO_4$ (1 mM) and glycerol (0.027 mM). Electrochemical measurements were performed using commercially available Zensor TE100 (EDAQ ET077) screen-printed electrodes featuring carbon working and counter electrodes. Although the Zensor TE100

electrodes included a silver paste reference, a separate 1 M KCl Ag/AgCl reference electrode (CHI111) was employed to minimize the chances of drift in reference potential during measurements lasting several hours, which can occur when the reference electrode is in direct contact with the sample solution. All electrochemical measurements were recorded using a potentiostat (CHI842C, CH Instruments).

### 2.2 Experimental protocol

Individual amino acids were dissolved at specific concentrations in either 10 mL of TSB or M63 media. Each solution was inoculated with a specific concentration of *P. aeruginosa* and incubated at 37 °C. 100 µL of each solution was removed at designated time points and loaded onto a Zensor electrode for electrochemical testing. Square-wave voltammetric scans were performed at potentials ranging from -0.4 to -0.1 V at an amplitude voltage of 0.050 V and a frequency of 15 Hz. Scans were performed for each sample three times with three replicates for each tested condition. The data was analyzed using OriginPro 9.1 (OriginLab Corporation). Baselines were created for each data set using spline interpolation with 8 base points. The resulting baseline-subtracted data set was used to determine the max currents observed from pyocyanin production.

## 3. RESULTS AND DISCUSSION

### 3.1 Electrochemical detection of *P. aeruginosa* in M63 and TSB media

Liquid cultures of *P. aeruginosa* were grown in either M63 minimal media or TSB to observe the effect of amino acid addition on the bacteria's production of pyocyanin. 10 µL of stock PA14 culture was loaded into 10 mL liquid cultures each containing one of six different

amino acids (final concentration of 4 million cells/mL). To maximize the effect that individual amino acids might have on pyocyanin production, the amino acid concentrations chosen were 16-times higher than the concentrations typically found in patients infected with cystic fibrosis with the exception of tyrosine which was only increased 4-fold due to its lower solubility.[15, 16] Electrochemical scans were taken roughly every two hours over the course of a 24-hour period.

Fig. 1 A and B shows scans taken after 10 hours of growth in M63 minimal media and trypticase soy broth respectively. A control sample with no added amino acids was also tested. Pyocyanin is expected to produce an electrochemical signal around -0.25 V versus a Ag/AgCl reference and this result is observed for all samples grown in the TSB media. However, no peak is observed in any of the samples in M63 minimal media after 10 hours.

Consistent among all tests was that samples grown in TSB produced a pyocyanin signal faster than those grown in M63 media. Cells can grow and divide normally in both media, however, TSB contains a series of additional nutrients not found in the M63 minimal media, such as casein and soybean lysate, which accelerate the bacterial growth rate. However, of greater importance is that select amino acids had an up-regulatory effect on pyocyanin production as demonstrated by the samples containing tyrosine and valine. The results show that the addition of tyrosine to minimal media lowers the amount of time needed to detect the presence of *P. aeruginosa* in a sample via detection of current produced by pyocyanin.

Scans were taken over the course of one day and the maximum currents are reported in Fig. 2. The amino acids had varying effects on *P. aeruginosa's* production of pyocyanin, with tyrosine having the greatest up-regulatory effect in both media. These results link up-regulation of pyocyanin production with increased biofilm formation induced by the addition of individual amino acids.[15] Without the addition of amino acids, it took nearly 24 hours for a pyocyanin peak

to be observed in M63 media, while a peak was seen within 10 hours for the control experiment using TSB. In M63 minimal media, addition of tyrosine and histidine resulted in the appearance of a pyocyanin peak in significantly less time. Addition of amino acid to TSB, however, did not appreciably change the amount of time needed to observe a pyocyanin peak. In all cases, the signal appeared 6-8 hours after the start of the experiment. However, the amount of pyocyanin produced after that point varied significantly. Results for each individual experiment are shown in the Supplementary Information. A small shift towards the positive potential (< 0.10 V) was observed for samples grown in the M63 media, which can be attributed to minor differences in the salt and pH concentration of the surrounding media. The error bars, shown in the figure, increase with time because of heterogeneity in the bacterial population. As the number of cells increases over time, they do not all divide or produce pyocyanin at exactly the same rate. This results in greater variability in the pyocyanin concentration over time, for each experiment. However, as a sensing mechanism, the primary concern is inducing the production of pyocyanin and the actual variance is secondary.

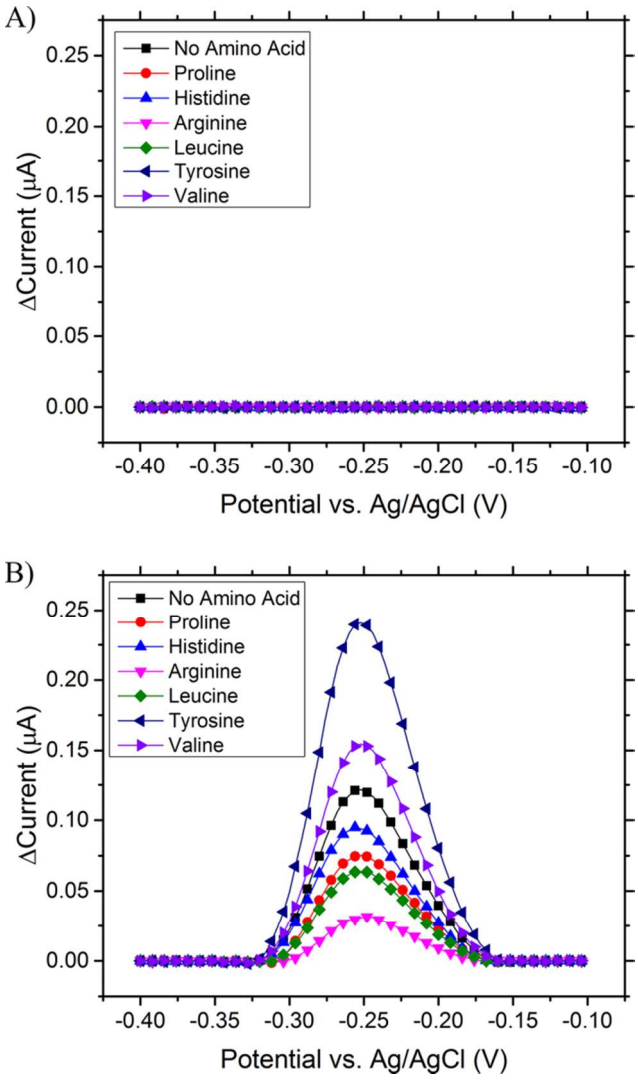


**Fig. 1.** Square-wave voltammograms of *P. aeruginosa* grown for 10 hours in the presence of individual amino acids in A) M63 minimal media and B) trypticase soy broth. Amino acids were used at the following concentrations (mM): proline (27.2), histidine (8), arginine (4.8), leucine (25.6), tyrosine (3.2), and valine (17.6).

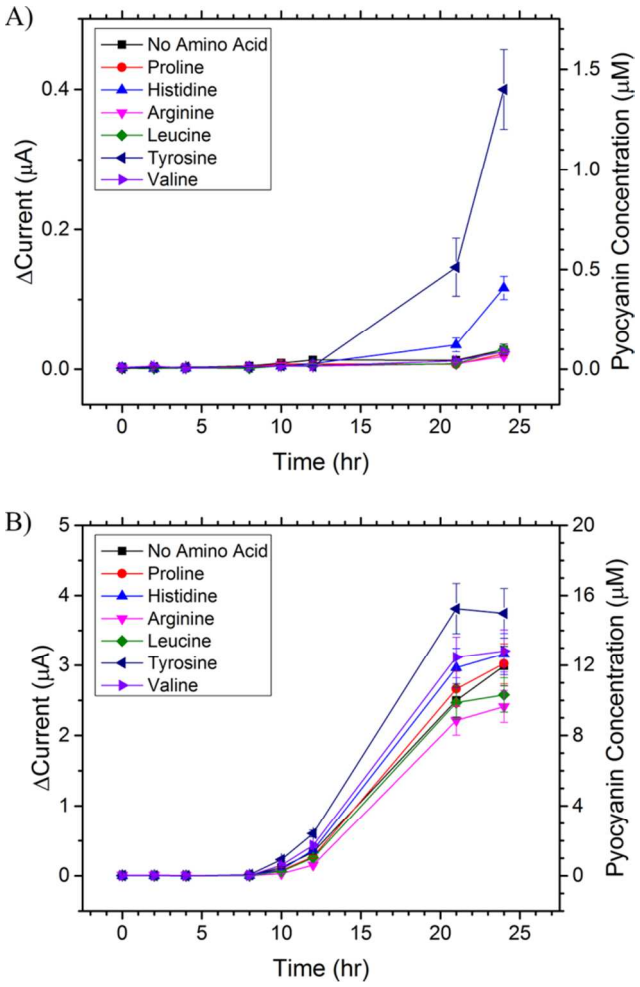


**Fig. 2.** Monitoring *P. aeruginosa's* production of pyocyanin, by plotting the maximum current at -0.25 V vs. Ag/AgCl, over the course of one day in the presence of individual amino acids in A) M63 minimal media and B) trypticase soy broth. Amino acids were used at the following concentrations (mM): proline (27.2), histidine (8), arginine (4.8), leucine (25.6), tyrosine (3.2), and valine (17.6).

### 3.2 Electrochemical detection of *P. aeruginosa* by varying tyrosine and valine concentration

Next, we investigated the optimal concentration at which tyrosine and valine should be present in the growth media to maximize pyocyanin production. Tyrosine and valine were selected as the target amino acids to apply in the TSB media as they demonstrated the largest up-

regulatory effect of the six amino acids tested. As cells grown in TSB growth media gave the fastest pyocyanin response, TSB was chosen as the growth media for the next phase of the study.

Tyrosine and valine were prepared at concentrations ranging from those quantified in typical CF infection levels (tyrosine: 0.2 mM, valine: 1.1 mM) to an 80-fold increase, which reached the solubility limit of the amino acids in TSB. The initial concentration of *P. aeruginosa* loaded in each sample was kept constant at roughly 4 million cells/mL and electrochemical scans were taken every two hours over the course of ten hours. Fig. 3 shows scans taken after eight hours of growth in addition to a control with no amino acid additives. Again, consistent among all scans was the observance of a pyocyanin peak around -0.25 V versus a Ag/AgCl reference. Interesting to note is that the largest pyocyanin concentration for the experiments with valine was recorded for the 17.6 mM concentration and that pyocyanin production decreased when the valine concentration was raised further to 88 mM. It is possible that valine has an inhibitory effect on pyocyanin production at such high concentrations. While such inhibition has been observed for other molecules,[22] *P. aeruginosa* has not been previously studied at valine concentrations that are over 100 times greater than what is typically found in a pseudomonal infection.

Fig. 4 shows the increase in pyocyanin over time for each of the concentrations of tyrosine and valine tested. From the data presented in Fig. 4A, we observe a statistically significant increase in current output between six and eight hours, marking the minimum amount of time necessary to detect electrochemically a *P. aeruginosa* infection in a processed sample. Importantly, varying the concentration of tyrosine and valine added to the solution has a minimal effect on the amount of time needed for cells to up-regulate pyocyanin production, but has a

significant effect on the amounts produced after this critical time point. The rate of pyocyanin production by the cells in 16 mM tyrosine and 17.6 mM valine is nearly identical.

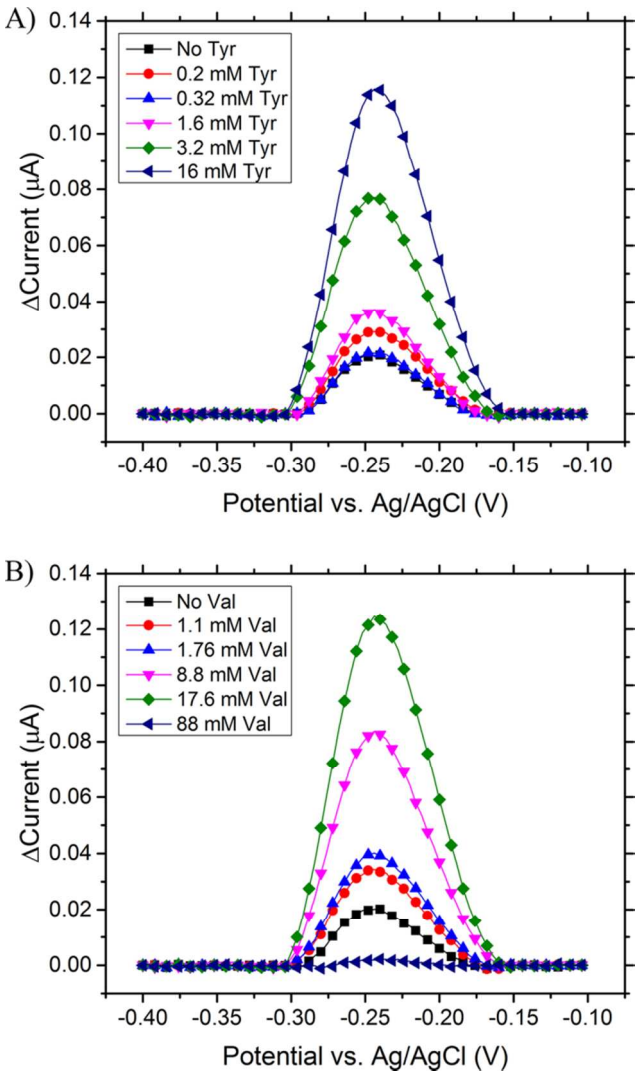


**Fig. 3.** Square-wave voltammograms of *P. aeruginosa* grown in trypticase soy broth for eight hours in the presence of A) tyrosine at various concentrations and B) valine at various concentrations.

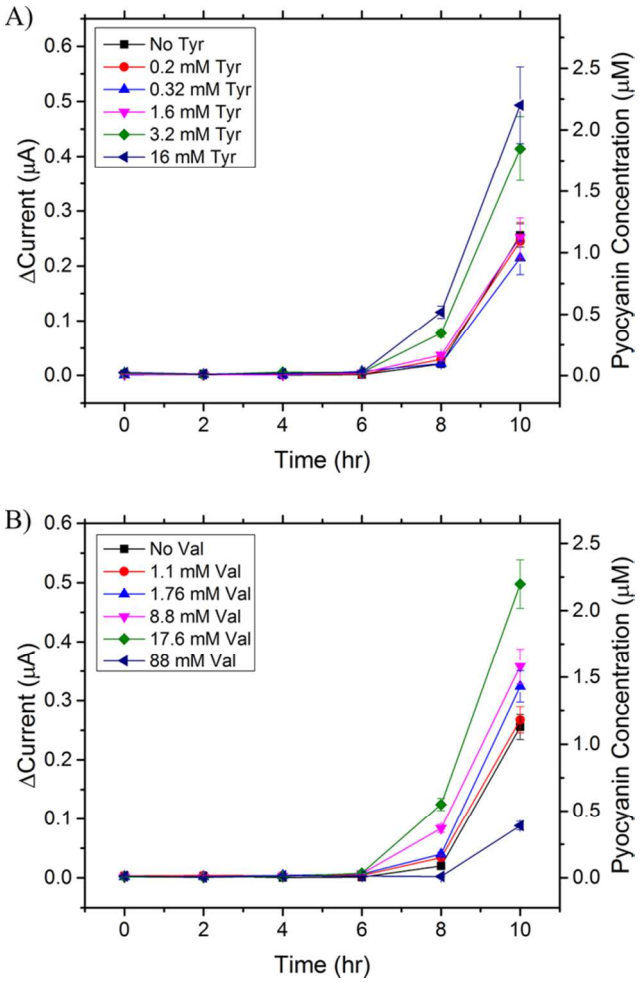


**Fig. 4.** Monitoring *P. aeruginosa's* production of pyocyanin over the course of ten hours in TSB cultures containing A) tyrosine at various concentrations and B) valine at various concentrations.

### 3.3 Electrochemical detection of *P. aeruginosa* by varying initial bacterial concentration

The next set of tests studied how varying the initial *P. aeruginosa* concentration would affect the production of pyocyanin in the presence of amino acids. TSB media was used as the growth media to which tyrosine and valine were added. Tyrosine (16 mM) and valine (17.6 mM) concentrations were held constant while varying amounts of *P. aeruginosa* were added into the samples (4, 20, 40, 400 million cells/mL). Two control experiments were also included: one with an initial *P. aeruginosa* concentration of 4 million cells/mL without additional amino acid and

the second with the amino acids added but no bacteria. Electrochemical scans were taken every two hours over the course of ten hours. Fig. 5 shows scans taken after eight hours of growth. There is a clear correlation between the increasing starting concentration of bacteria and the amount of pyocyanin produced over a constant amount of time. For initial cell concentrations above 4 million cells/mL, 16 mM tyrosine causes *P. aeruginosa* to produce more pyocyanin than 17.6 mM valine. The minimum incubation time needed to detect a *P. aeruginosa* infection increases with decreasing initial cell concentration (Fig. 6). From the data presented in Fig. 6A, a statistically significant increase in current output is obtained for the highest initial cell concentration (400 million cells/mL) between four and six hours after the start of the experiment. These results indicate that the amount of time necessary to detect a current change due to pyocyanin production can be used to quantify the number of initial cells present in the sample.

The theoretical cellular limit of detection for our approach, without sample pre-concentration, is 0.1 cells/mL, since a 10 mL sample volume is used in the analysis and thus there would be 1 cell in the sample in this case. We expect it would take approximately one day to observe a pyocyanin peak for this minimal concentration. In previous work, where fresh growth media was inoculated with only a few cells from a *P. aeruginosa* colony on a plate, showed that it took approximately 24 hours to produce a 5 μM concentration of pyocyanin,[10] which supports our analysis.

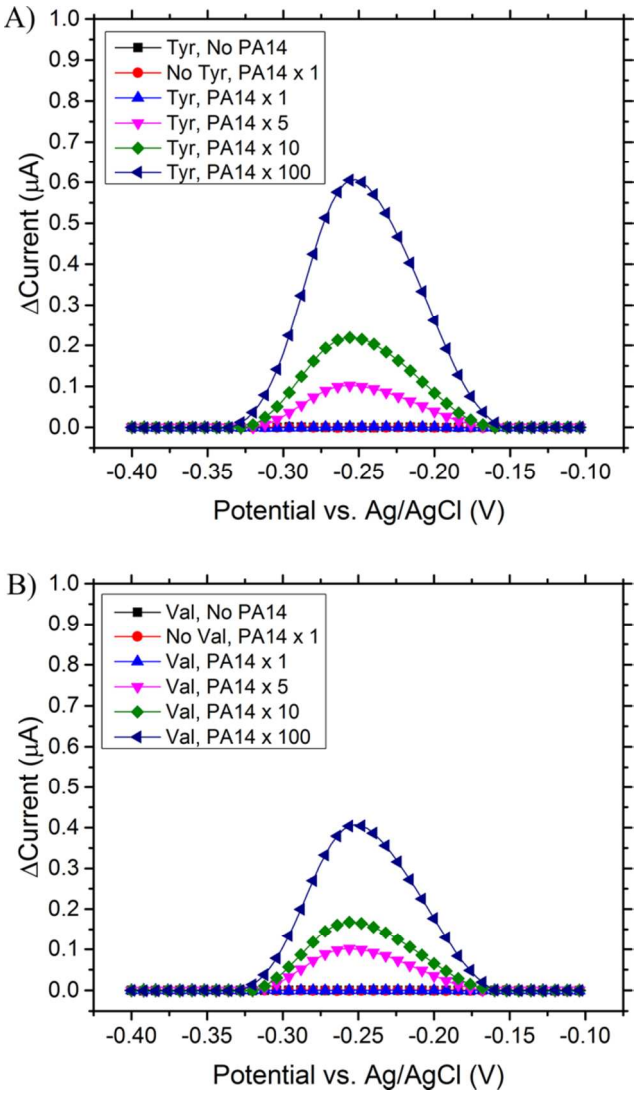


**Fig. 5.** Square-wave voltammograms of *P. aeruginosa* grown in TSB for eight hours in A) tyrosine (16 mM) and B) valine (17.6 mM). *P. aeruginosa* was used at the following concentrations (million cells/mL): 4, 20, 40, and 400 respectively. Control experiments contained 4 million cells/mL without additional amino acids added and sterile growth media with added amino acids.

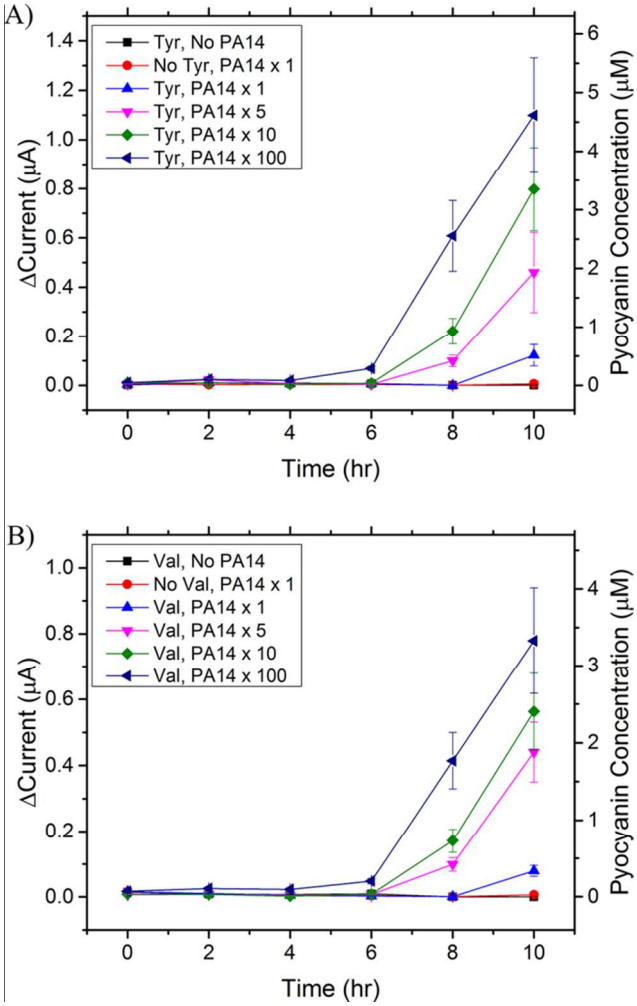


**Fig. 6.** Monitoring *P. aeruginosa's* production of pyocyanin over the course of ten hours in TSB cultures containing A) tyrosine (16 mM) and B) valine (17.6 mM). *P. aeruginosa* was used at the following concentrations (million cells/mL): 4, 20, 40, and 400 respectively. Control experiments contained 4 million cells/mL without additional amino acids added and sterile growth media with added amino acids.

## 4. Conclusions

Due to its natural resistance and increasing tolerance to antibiotics, *P. aeruginosa* infections are difficult to treat and, treatment options will require faster infection identification methods as multidrug-resistant strains continue to to become more prevalent worldwide.[2, 16]

Current diagnostic techniques rely on culture-based approaches that are time-consuming (one to several weeks) and require subjective interpretation.[23] While PCR techniques have recently emerged as a rapid molecular diagnostic, they require expensive hardware and have significant reagent costs (consumables can cost hundreds of dollars per run).[24] Therefore, a lower-cost alternative diagnostic for rapid pathogen identification is needed. To this end, six amino acids were evaluated as regulatory molecules in promoting the production of pyocyanin in *P. aeruginosa*. Liquid samples were prepared and spotted onto commercially available electrodes, and square-wave voltammetry was used to observe the electrochemical response due to pyocyanin. Comparing M63 and TSB growth media showed that components in TSB significantly decreased the amount of time needed to detect a pyocyanin signal. The addition of tyrosine and valine had the largest up-regulatory effect on the production of pyocyanin for the six amino acids tested. However, the amount of time needed to detect production of pyocyanin electrochemically did not noticeably decrease for a set initial concentration of cells in TSB. We showed that increasing the initial concentration of *P. aeruginosa* decreased the amount of time necessary to detect pyocyanin. Electrochemical detection has been shown recently to work in samples containing various opportunistic pathogens as well as in complex human biofluids,[14] demonstrating the potential application of this approach in real-world detection scenarios. Expanding our previous work and combining it with the results shown here, we envision an approach where patient samples are obtained and mixed with a liquid media containing an optimized mixture of amino acids and other compounds that up-regulate pyocyanin production. For early infection detection, where the real samples contain a small amount of bacteria, cells can be pre-concentrated and resuspended in a small volume of the optimized growth media.[25] In addition, employing a more sensitive electrochemical sensor, in combination with the results

shown here, may lead to faster detection of the bacterium in infected patients. Finally, the general concept of up-regulating and detecting target small molecules that are produced by cells, as a means of increasing sensitivity in diagnostic applications, is an interesting approach that can potentially be extended to other pathogens. In the case of electrochemical detection, the sensing surface can be modified with recognition elements to detect the unique target molecules if they are not themselves electro-active.

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## NOTES AND REFERENCES

Electronic Supplementary Information (ESI) available: Additional electrochemical experimental results. See DOI: 10.1039/b000000x/

1. H. Fazeli, R. Akbari, S. Moghim, T. Narimani, M. R. Arabestani and A. R. Ghoddousi, *J Res Med Sci*, 2012, **17**, 332-337.

2. V. H. Tam, K. T. Chang, K. Abdelraouf, C. G. Brioso, M. Ameka, L. A. McCaskey, J. S. Weston, J. P. Caeiro and K. W. Garey, *Antimicrob Agents Chemother*, 2010, **54**, 1160-1164.

3. T. W. Lee, K. G. Brownlee, M. Denton, J. M. Littlewood and S. P. Conway, *Pediatr Pulmonol*, 2004, **37**, 104-110.

4. J. B. Lyczak, C. L. Cannon and G. B. Pier, *Clin Microbiol Rev*, 2002, **15**, 194-222.

5. F. Ratjen and G. Doring, *Lancet*, 2003, **361**, 681-689.

6. J. B. Lyczak, C. L. Cannon and G. B. Pier, *Microbes Infect*, 2000, **2**, 1051-1060.

7. E. Kipnis, T. Sawa and J. Wiener-Kronish, *Med Mal Infect*, 2006, **36**, 78-91.

8. G. W. Lau, D. J. Hassett, H. Ran and F. Kong, *Trends Mol Med*, 2004, **10**, 599-606.

9. L. E. Dietrich, A. Price-Whelan, A. Petersen, M. Whiteley and D. K. Newman, *Mol Microbiol*, 2006, **61**, 1308-1321.

10. T. A. Webster and E. D. Goluch, *Lab Chip*, 2012, **12**, 5195-5201.

11. V. B. Wang, S. L. Chua, B. Cao, T. Seviour, V. J. Nesatyy, E. Marsili, S. Kjelleberg, M. Givskov, T. Tolker-Nielsen, H. Song, J. S. Loo and L. Yang, *PLoS One*, 2013, **8**, e63129.

12. D. Sharp, P. Gladstone, R. B. Smith, S. Forsythe and J. Davis, *Bioelectrochemistry*, 2010, **77**, 114-119.

13. T. A. Webster, H. J. Sismaet and E. D. Goluch, *Nano LIFE*, 2013, **03**, 1340011.

14. T. A. Webster, H. J. Sismaet, J. L. Conte, I. P. Chan and E. D. Goluch, *Biosens Bioelectron*, 2014, **60**, 265-270.

15. S. P. Bernier, D. G. Ha, W. Khan, J. H. Merritt and G. A. O'Toole, *Res Microbiol*, 2011, **162**, 680-688.

16. G. C. Palmer, K. L. Palmer, P. A. Jorth and M. Whiteley, *J Bacteriol*, 2010, **192**, 2722-2728.

17. E. Hellinger, *J Gen Microbiol*, 1951, **5**, 633-639.

18. N. Grossowicz, P. Hayat and Y. S. Halpern, *J Gen Microbiol*, 1957, **16**, 576-583.

19. W. He, C. Li and C. D. Lu, *J Bacteriol*, 2011, **193**, 2107-2115.

20. S. R. Thomas, A. Ray, M. E. Hodson and T. L. Pitt, *Thorax*, 2000, **55**, 795-797.

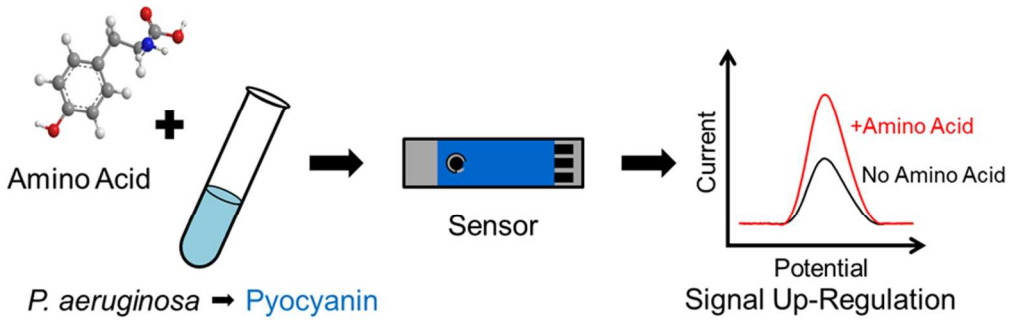
21. L. S. Pierson, 3rd and E. A. Pierson, *Appl Microbiol Biotechnol*, 2010, **86**, 1659-1670.

22. D. J. Musk, D. A. Banko and P. J. Hergenrother, *Chem Biol*, 2005, **12**, 789-796.

23. J. L. Fothergill, M. J. Ledson, M. J. Walshaw, P. S. McNamara, K. W. Southern and C. Winstanley, *J Cyst Fibros*, 2013, **12**, 675-681.

24. H. J. Choi, M. H. Kim, M. S. Cho, B. K. Kim, J. Y. Kim, C. Kim and D. S. Park, *Appl Microbiol Biotechnol*, 2013, **97**, 3643-3651.

25. R. T. Noble and S. B. Weisberg, *J Water Health*, 2005, **3**, 381-392.




A simple and rapid electrochemical detection method for Pseudomonas aeruginosa is demonstrated using amino acids as up-regulatory molecules for pyocyanin production.
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