Hydrogen Cyanide Production by *Pseudomonas uticensis*

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ABSTRACT

Pseudomonas uticensis is a novel bacteria species that was isolated from the cutaneous flora of red-backed salamanders in Central New York on the basis of its potent antifungal properties. We also have shown that this bacterium exhibits virulence against *Caenorhabditis elegans*, causing rapid loss of viability in early larval-stage worms. We have explored several potential virulence factors as the cause of mortality in *C. elegans*, including hemolysins and proteases, but these factors are repressed under the culture conditions in which we find the highest levels of virulence and antifungal activity. We recently searched the completed *P. uticensis* genomic DNA sequence for genes encoding putative antifungal and virulence factors, and discovered a homologue of the *hcn*ABC gene cluster *in Pseudomonas aeruginosa* that encodes hydrogen cyanide synthase. This enzyme produces toxic hydrogen cyanide (HCN) from glycine. Since HCN previously was identified as a virulence factor in *P. aeruginosa* with *C. elegans*, and may also be an antifungal agent, we examined whether *P. uticensis* has a functional hydrogen cyanide synthase that produces HCN, and if the toxic compound is produced at biologically significant levels. *P. uticensis* was grown in lawns on tryptic soy agar in 35 mm plates for 24 h, then placed in sealed containers with a dish containing 4M NaOH to trap HCN gas. HCN in alkaline solution was quantified using a colorimetric assay with 1,2-dinitrobenzene and 4-nitrobenzaldehyde. HCN was produced by the bacteria, and production was found to be proportional to the number of plates sealed in the containers. *P. uticensis* was also cultured in several types of liquid media, and bacterial supernatants were assayed for HCN content. Glycine stimulated HCN production in a concentration-dependent manner. HCN production in nutrient-rich tryptic soy – yeast extract broth was approx. 85 nmol/g wet cell mass and 100 mM concentration in broth, which was 10-fold greater than produced in minimal medium. These values are in the same order of magnitude as those reported for toxicity in adult *C. elegans* by *P. aeruginosa*. In agar plate bioassays with *Candida albicans*, fungal growth was not inhibited at the concentration of HCN produced by *P. uticensis*. We conclude that HCN is produced by *P. uticensis*, and it is not an antifungal factor for the bacterium, but may be a virulence factor.

BACKGROUND

Pseudomonas uticensis is a novel species isolated from the skin if red-backed salamanders (van Kessel et al., 2003). Biochemical and molecular genetic evidence suggest that this isolate is the prototype of a previously undescribed species (Lawrence et al., 2017). *Pseudomonas uticensis* was isolated on the basis of its potent antifungal activity. Experiments were conducted to explore whether *P. uticensis* also produced virulence factors using the nematode, *Caenorabditis elegans* as our model. *Pseudomonas uticensis* did exhibit virulence under certain media conditions. We had hypothesized that intercellular melanin might be responsible for mortality in worms as they were consuming bacteria, but studies were inconclusive (Morreale et al., 2011). We explored the possibility that proteases were responsible for the death of L1 and L2 larvae worms, but the loss of viability was the greatest under conditions where protease production was suppressed (Klempic et al., 2016). The same thing was found to be true for both beta- and alpha-hemolytic activity (Safdar et al, 2017). In previous research, HCN was identified as a potential antifungal factor in *P. fluorescens* (Michelson and Stougaard, 2012), and as a virulence factor in *P. aeruginosa* with *C. elegans* (Gallagher and Manoil, 2001). Homologs of the *hcn*ABC genes, encoding hydrogen cyanide synthase, have been found in the *P. uticensis* genome. Therefore, in the present study, we have explored the possibility that HCN is produced by *P. uticensis,* and at levels that would be biologically significant as an antifungal agent and/or virulence factor.

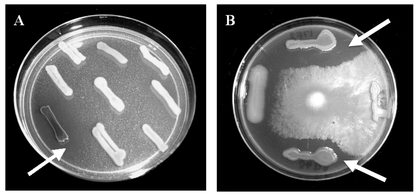

Fig. 1. Antifungal activity of *Pseudomonas uticensis*. Bacterial isolates from the cutaneous microflora of red-backed salamanders were streaked on TSYE agar plates containing *Candida albicans* blastospores.(A), or on Vogel's Complete Medium inoculated in the center with *Neurospora crassa* (B). Arrows indicate *P. uticensis* and the zone of inhibition of fungal growth around the bacterial streaks.

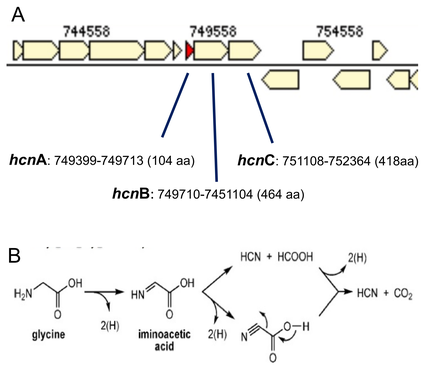

Fig. 2. Homologs of *hcn*ABC gene cluster found in *P. uticensis* genome. A. Location of putative *hcn*ABC genes in the *P. uticensis* genome, which encode subunits of hydrogen cyanide synthase. B. Reaction mechanism of hydrogen cyanide synthase, converting glycine to HCN.

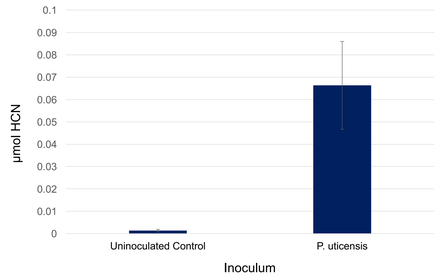

Fig 3. Production of HCN by *Pseudomonas uticensis*. P. uticensis was grown in lawns on 35 mm agar plates containing tryptic soy agar (TSA) at 30ºC for 24 hrs. Plates were then placed in sealed Ziploc bags with a dish containing 1 ml 4M NaOH to trap HCN gas and incubated for 4h at 30ºC. Production of HCN was assayed using the colorimetric method described by Gallagher and Manoil (2001), using KCN as a standard. Results indicate that the uninoculated plates did not produce HCN. Plates with *P. uticensis* did produce HCN.

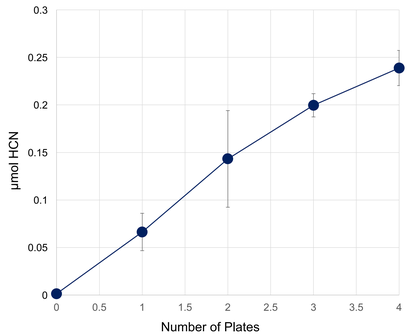

Fig 4. Production of HCN by *P. uticensis* is proportional to the biomass of bacterial growth. *P. uticensis* was grown on TSA plates as above, and Ziploc bags containing a dish of 4M NaOH were incubated with an uninoculated plate, or 1, 2, 3 or 4 plates containing 24 h bacterial lawns. HCN production was assayed as described above. Results indicate that HCN production increases in a linear manner with the number of number of plates (increasing biomass) in the assay chamber.

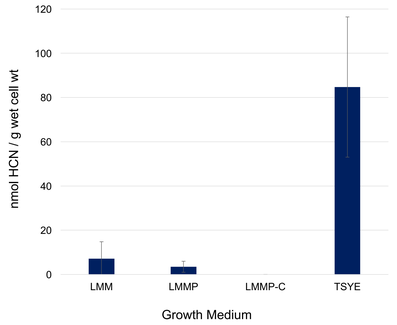

Fig. 5. Effect of media composition on production of HCN by *P. uticensis*. Plates of LMM, LMMP, LMMP-Citrate and TSYE were inoculated with lawns of *P. uticensis* and grown and assayed for HCN production as described previously. Results indicate that *P. uticensis* produced 10x more HCN in TSYE compared to LMM because LMM is minimal media, whereas TSYE is a highly enriched medium, containing more substrate for HCN synthase. Levels of HCN production on TSYE approximate those for *P. aeruginosa* required for killing *C. elegans* (Gallagher and Manoil, 2001)

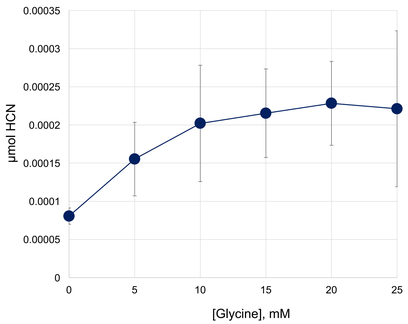

Fig. 6. HCN production is glycine dependent. Glycine is a precursor of HCN. *P. uticensis* was grown in LMM with varying concentrations of glycine, and assayed for HCN production. Results indicate that the production of HCN by *P. uticensis* increases proportionately with an increase in glycine concentration.

CONCLUSION

HCN is produced by *P. uticensis*. Agar plate assays with *Candida albicans* indicate that it is not an antifungal factor for the bacterium because the only concentration of HCN that inhibited *Candida albicans* was 1M, which is 100x more concentrated than the most HCN that *P. uticensis* produces (data not shown). This is an indication that it is not likely to be an antifungal compound, but it does not preclude it from being a virulence factor for *C. elegans*. In future studies, the larval *C. elegans* will be challenged with HCN at levels produced by *P. uticensis* to see if it is toxic. Furthermore, knockout mutants in HCN genes will be created in order to test whether mutant strains that do not produce HCN still kill the worms as quickly.

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ACKNOWLEDGEMENTS

The authors are grateful to the U.S. Department of Energy – Joint Genome Institute, Berkeley, CA for the *Pseudomonas uticensis* genome sequencing project. The project (JGI Project ID 1083821) was conducted courtesy of, and in collaboration with Dr. Joyce Loper, USDA, Corvallis, OR. This work was also supported by the Harold T. Clark, Jr. Endowed Professorship to L.R.A., and grants from Donald and Sally Majka, The Community Foundation of Herkimer and Oneida Counties, and The Alden Trust.

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