Challenges of using protein antibiotics for pathogen control

Ray Chai,a† William M Rooney,a,b† Joel J Milnerb* and Daniel Walkera* ©

Abstract

Bacterial phytopathogens represent a significant threat to many economically important crops. Current control measures often inflict harm on the environment and may ultimately impact on human health through the spread of antibiotic resistance. Antimicrobial proteins such as bacteriocins have been suggested as the next generation of disease control agents since they are able to specifically target the pathogen of interest with minimal impact on the wider microbial community and environment. However, substantial gaps in knowledge with regards to the efficacy and application of bacteriocins to combat phytopathogenic bacteria remain. Here we highlight the immediate challenges the community must address to ensure maximum exploitation of antimicrobial proteins in the field.

Keywords: bacteriocins; bacterial phytopathogens; Pseudomonas syringae; Pectobacterium

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1 INTRODUCTION

The global population is predicted to exceed 9 billion by 2050 and this will require a substantial increase in food availability. In 2020, it is estimated that 690 million people (8.9% of the world population) are hungry.1 This is expected to worsen due to the impacts of the COVID-19 pandemic, which is predicted to add a further 83 million to 132 million people that are undernourished.1 Furthermore, global warming and deteriorating economic growth in many countries has exacerbated food security issues. One of the ways in which food security can be improved is by preventing crops losses caused by pests such as bacterial phytopathogens. It is estimated that even with current biocontrol methods the losses caused to the five major crops by phytopathogens, i.e. wheat, rice, maize, potatoes and soybeans, are still as high as 10.2%, 10.8%, 8.5%, 14.5% and 8.9%, respectively.2 Moreover, measures to control bacterial plant diseases often have undesirable environmental side effects. For instance, some chemical sprays and seed treatments contain amounts of copper that are harmful to the environment and with copper resistance developing in target bacteria are becoming less effective.3 More disturbingly, control methods for some bacterial phytopathogens include using antibiotics, in particular streptomycin and oxytetracycline, that are also used as therapeutics in human medicine, potentially driving the spread of resistance in the wider microbial community and in human pathogens.

Bacteriocins are antimicrobial proteins produced by many bacteria that have evolved to kill closely related competitors. As a result, bacteriocins tend to have a highly specific killing range allowing the normal microflora to remain undisturbed. They represent a currently unexploited novel source of antimicrobials for use against bacterial phytopathogens. Economically important phytopathogenic bacteria (typically, gram-negative) are responsible for some of the most damaging diseases, including black leg and soft rot in potatoes caused by Pectobacterium and Dickeya species and more promiscuous bacteria such as Pseudomonas syringae and Xanthomonas spp. that infect many different types of plants.4 Several bacteriocins that have already been identified and characterized show killing activity against these bacterial genera, representing promising potential control agents. This review explores how bacteriocins can be exploited as biocontrol agents to treat bacterial infections of crops and the challenges that need to be overcome for this to happen.

2 IDENTIFICATION OF NOVEL BACTERIOCINS

Compared to conventional antibiotics, bacteriocins show much greater target specificity. Therefore, for bacteriocins to be practical antimicrobials, access to a very extensive armory of different bacteriocins will be essential. Fortunately, because bacteriocin production is almost ubiquitous amongst gram-negative bacteria, the potential for bacteriocin discovery is very great – what is
required are efficient strategies for identifying and characterizing novel bacteriocins.

Historically, bacteriocins were identified by testing filtrates/extracts of suspected bacteriocin producers for inhibitory activity against potentially sensitive target strains. For example, three bacteriocins from *Xanthomonas* were identified following the observation that in many Florida tomato fields infections by *X. euvesicatoria* were quickly outcompeted by *X. perforans*. This was subsequently attributed to the production of bacteriocins by *X. perforans*. Until recently, direct testing for activity was the only practical strategy for identifying bacteriocins from phytopathogens and several *Xanthomonas* bacteriocins,7,8 ulceracin 378 from *Corynebacterium ulcerans*,9 two bacteriocins from *Erwinia carotovora* CGE234-M40310 and several bacteriocins from pseudomonads11 were identified in this way. However, this approach is time-consuming and has a high rate of rediscovery so despite the widespread production of bacteriocins (and its historically important role in bacteriocin discovery) this approach does not provide an efficient strategy for the high-throughput programmes that will be required to identify bacteriocins in the numbers that will be needed for widespread uptake of the technology.

A much more efficient strategy for identifying bacteriocins is the identification of gene sequences encoding prospective bacteriocins by genome mining. The commercialization of next-generation sequencing has led to a boom in publicly available genome sequences, an immense resource from which bacteriocins can be identified using bioinformatic approaches. For example, at the end of 2010 there were 13 published *Xanthomonas* genomes in NCBL. Currently (December 2020) there are 1610 genomes available. Bacteriocin searches range from straightforward BLAST searches (using complete or partial sequences from different classes of known bacteriocins) to more complex algorithms and the use of machine learning to identify more diverse bacteriocins.12–14

BLAST searches have typically been performed by searching for homology to well characterized bacteriocins, e.g. colicins from *E. coli* and pyocins from *P. aeruginosa*. The colicin-like bacteriocins are typically modular and are organized into different domains based on their function (e.g. targeting specificity and catalytic/killing activity). The domains are interchangeable between different bacterial strains so closely related bacterial strains may produce bacteriocins that share one domain but differ in another. Often, matches to a single domain allow for the identification of novel bacteriocins, for example the *Pectobacterium* bacteriocins Pectocin M1 and M2. These share a cytotoxic domain with colicin M, but the N-terminal regions, comprising the translocation and receptor domains, are completely different and on subsequent investigation were shown to share a high level of similarity to plant ferredoxins.15,16 Importantly, this led directly to the identification of another putative pectobacteriocin, pectocin P, which also has an N-terminal ferredoxin domain but harbors a cytotoxic domain that shares structural homology with lysozyme. These results show the value of iteratively using homology searches to uncover novel bacteriocins from genome sequences.

Despite these promising results, relying on BLAST searches can be limited by an over-reliance on the use of well characterized classes of bacteriocins such as colicins and pyocins in searches. Consequently, novel classes of bacteriocins may be overlooked. For some genus of gram-negative phytopathogenic bacteria this leads to a paucity of hits – bacteriocins produced by them are either evolutionarily distant or have evolved completely independently. To address this problem there have been several attempts to develop new algorithms with less conserved and/or novel search parameters as a route to discovering new bacteriocins.16 This approach has enjoyed some success in identifying bacteriocins that may have been overlooked by the usual bioinformatics routes.

Whatever the strengths and weaknesses of the different approaches, given that most bacteria produce bacteriocins, with some producing several, the range and number of as-yet unidentified bacteriocins is clearly very great and represent a highly promising and currently underutilized source of antimicrobials.

### 3 BACTERIOCIN RESISTANCE

The development of bacterial resistance to conventional antibiotics is a well-documented and ongoing problem and bacteria similarly evolve resistance to bacteriocins. With conventional antibiotics, resistance typically involves the acquisition of genes, often plasmid borne, encoding enzymes that either break down or detoxify the antibiotic.17 Resistance to bacteriocins in contrast may evolve via a variety of different routes. Bacteriocins are important biological drivers underpinning the dynamics of complex bacterial communities; consequently, resistance is a common occurrence in nature.18 Many of the bacterial strains within these communities already carry bacteriocins and their cognate immunity genes. Also, because many members of a bacterial community may share a common bacteriocin receptor, mutations to that receptor that interfere with binding to one bacteriocin often lead to resistance to others that exploit the same receptor. For example, mutations in the vitamin B12 receptor BtuB confer resistance to all nine E coli E1–9.18,20 Furthermore, many colicin-like bacteriocins share a common translocation pathway, such as the Ton or Tol pathways. Mutations in either pathway can therefore lead to resistance against multiple bacteriocins. For example, mutations in *tonB* can lead to resistance against colicins Ia, Ib, B, D, G, H, M and V.21,22 Although superficially this might seem like a major disadvantage for bacteriocin use, Ton and Tol mutations incur significant fitness costs. Ton mutants lack the ability to efficiently acquire iron, a key determinant for a successful infection, and Tol mutants display highly permeable outer membranes, leading to hypersensitivity to a range of toxic compounds.23,24 Rooney et al. reported that although *P. syringae* mutants with high levels of tolerance to the lectin-like bacteriocin putidacin L1 were readily generated in *vivo*, the mutations all involved genes with putative roles in lipopolysaccharide production and conferred fitness costs.25

Although it is generally assumed that mutations to critical genes have a fitness cost, this may not necessarily be apparent under laboratory growth conditions.18 Resistance to nisin does not appear to confer significant fitness costs to *L. monocytogenes* grown in a rich broth media or in a meat model,26 but at lower temperatures and suboptimal NaCl concentrations mutants exhibited a more pronounced drop in growth.26 Fitness costs associated with resistance are of course in general dependent on the metabolic activity associated with the mutant gene product.18 The receptor for pyocin S2 is FpvA, which is involved in the uptake of iron-bound pyoverdine. Pyocin S2 is most active in iron-limiting conditions due to the requirement of an iron transporter (FpvA) and its efficacy is dependent on the iron concentration of the growth medium.26,27,28 Furthermore, compared to wild-type strains pyocin S2-resistant strains with FpvA mutations
perform poorly in iron limiting conditions. Since a common strategy of nutritional immunity against pathogenic bacteria is restriction of iron by the host, the fitness cost of bacteriocin resistance may be significant. The use of proteins involved in iron transport as receptors is a feature of many colicin-like bacteriocins, with targets including FepA, CirA and FhuA. Another common target among bacteriocins is BtuB, which is required to transport vitamin B12, an essential metabolite, into the cell.

Although information on the impact of bacteriocin resistance is poorly understood for phytopathogenic bacteria, for colicin-like bacteriocins the mechanisms of resistance are likely to follow a common modus operandi. As described above, mutations in the lipopolysaccharide (LPS) biosynthesis pathway in P. syringae conferred insensitivity to the lectin-like bacteriocin, putidacin L1, most likely because of the effect of the mutations on the ability of the bacteriocin to dock onto the outer membranes. Interestingly, these mutations facilitated increased sensitivity to reactive oxygen species and loss of bacterial motility. In A. tumefaciens strains that evolved resistance to the small nucleotide antibiotic Agrocin 84 (a structural mimic of a plant tumor-derived substrate) resulted in the bacteria becoming avirulent in planta. Of course, the plasticity of bacterial physiology may allow bacterial phytopathogens to temporally evade transient selective pressures (including protein antibiotics). In practice, for bacteriocins to become a robust strategy for plant protection, bacteriocins will likely need to be used in combinations (bacteriocin cocktails), reducing the ability of bacteria to evade killing by single-use protein antibiotics. Such cocktails are probably already common in nature, for example P. syringae pv. syringae B728a produces two bacteriocins that work together in vitro to eliminate competitors.

4 APPLICATIONS OF PROTEIN ANTIMICROBIALS IN THE FIELD

The diversity and specificity of bacteriocins make them promising biological agents for plant protection. Bacteriocins represent a safer and more environmentally friendly alternative to conventional antibiotics like streptomycin. Bacteriocins exhibit a narrow (highly specific) killing spectrum and where specific examples have been assessed they have been classified by the US Food and Drug Administration as ‘generally regarded as safe’. Genetic modification and prophylactic application of bacteriocins represent the two main routes for delivery in the context of plant protection. We recently tested the potential of expressing bacteriocins to improve plant health by genetically modifying model plants (Arabidopsis and Nicotiana bethamiana) to express the lectin-like bacteriocin putidacin. The expression of putidacin L1 in both model organisms provided robust resistance against a variety of field isolates of P. syringae. This approach shows great potential, in particular given the potential improvements that could be used to optimize the temporal and spatial expression in planta. Of course, despite the apparent attractive simplicity of GM-based approaches there are considerable practical hurdles to be overcome. GM crops are not accepted in many countries and even in those where they are the costs of meeting regulatory regulations can be considerable, meaning that the GM approach will only be financially viable in high-volume/high-value crops.

The possible off-target effects of engineered bacteriocin-expressing plants on the plant microbiome remain poorly understood. Bacteriocins tend to exhibit a narrow killing spectrum (typically restricted to a single genus) but exhaustive testing against a large panel of phylogenetically diverse strains would still be necessary to demonstrate biosafety. For PL1, testing has been heavily biased towards a few select bacterial genera that do not fully reflect the complex nature of the plant microbiome. Weinhold and colleagues attempted to address this deficiency in the literature by transforming wild tobacco plants (Nicotiana attenuata) with the antimicrobial peptide ICE, which is potent against a wide range of Bacillus strains. They found that the plant root microbiome remained largely unaffected by the expression of ICE in experimental field plots. A major caveat of this study was that they were unable to confirm sufficient antimicrobial activity of ICE in the roots. Thus, the results possibly reflect the technical difficulties of expressing and/or delivering antimicrobial peptides to their targets. Assessments of the diversity of the leaf microbiome of wild tobacco plants disclosed an extremely low level of sequence depth (<40) in the leaves, indicating the bacterial community has low complexity and species richness. Similar results have been found with agave plants. These species might not be ideal for assessing the impacts of expressing antimicrobial peptides in the phyllosphere. Overall, literature in this area remains sparse and further studies are key to establish the viability of bacteriocins. Unless these technicalities can be resolved engineering plants to express bacteriocins will not be a viable control strategy for disease caused from soil-dwelling pathogens like Ralstonia solanacearum, P. carotovorum and P. atrosepticum. Despite the skepticism behind the transgenic deployment of protein antibiotics, their inherent specificity could mitigate the concerns on their influence on the global microbiome.

Another anxiety about the genetic transformation of crop plants to express alien protein antibiotics is their potential to disrupt the finely balanced biochemical equilibrium within plant cells, possibly translating into significant yield penalties. Weinhold and colleagues did assess the holistic effects of expressing ICE in wild tobacco and showed that the expression has no negative fitness consequences in terms of plant growth performance, flower production and herbivory. This single study, however, is not representative of the sheer diversity of antibiotic proteins and if GM approaches involving in planta expression of bacteriocins are to gain traction an extensive effort to assess all potential off-target effects will be required, potentially on a crop-by-crop basis. Prophylactic (direct) application of bacteriocins to plants, seeds, tubers etc. represents an alternative route for plant protection that avoids many of the limitations of the GM-based approach. In particular, the regulatory implications are far simpler and end users have the flexibility to decide where, when and what crops they wish to treat. Glasshouse experiments have demonstrated that phage-like bacteriocins (tailocins) directly applied to tomato and tobacco are highly effective at protecting against important plant phytopathogenic bacteria. Tailocins are multicomponent bacteriocins, making them difficult to produce in large-scale in other model organisms. They can be produced in large-scale fermenters but their economic viability has not been assessed. A potential route for exploitation of tailocins could involve exploiting commensal strains of bacteria that naturally produce them. For example, the avirulent strain Pectobacterium carotovorum pv. atrosepticum CGE234-M403 harbors the tailocin CtvCGE and is already marketed as ‘Biokeeper’ in Japan to manage bacterial soft rot in potatoes.

Single-gene bacteriocins are considerably easier to produce in suitable quantities due to their solubility and low molecular weight (30–70 kDa). Furthermore, techno-economic analysis of
Indeed, plants make excellent potential platforms for production of useful proteins such as bacteriocins. Plants have been shown to produce active antimicrobial proteins with accumulations as high as 3 g kg$^{-1}$ of fresh weight.37,49–53 Economic forecasts using three different scenarios project the manufacturing cost of biopharming protein antibiotics from plants to be $3.00–6.88 g$^{-1}.48 These production models include both the upstream and downstream processes involved in the production and purification of bacteriocins in the commonly used production platform species N. benthamiana. The numbers suggest that plants themselves represent the ideal platform for producing bacteriocins in industrial quantities.

5 Conclusion

Protein antibiotics represent a very promising approach to crop protection that is desperately needed. The human population is constantly rising, leading to huge demands in food availability, with onset of the COVID-19 pandemic only exacerbating the crisis. The exponential increase in published bacteria genomes and constantly refined bacteriocin searches provides a continual source of novel bacteriocins. Furthermore, niche host killing, which would reduce the spread of bacteriocin resistance, and detrimental costs to bacterial fitness and virulence after evolving resistance augments their use as a long-lasting antimicrobial.

Current approaches to crop protection are far from ideal as they involve the use of chemical antimicrobials (e.g. copper-based chemicals) or conventional antibiotics (e.g. Streptomycin) and their widespread use often creates potentially serious environmental and ecological side effects. Bacteriocins are likely to be much more environmentally friendly because of their high target specificity and the consequent low probability of unintended effects such as adverse changes to the plant or soil microbiome or the transfer of resistance, potentially into human or animal as well as plant bacterial pathogens. Of course, the downside of this high degree of specificity is the likelihood that effective treatment regimens will require the simultaneous use of multiple different bacteriocins (formulated into crop-specific bacteriocin cocktails). Although this may appear to be a major drawback, the extraordinary diversity and range of bacteriocins as evidenced by bioinformatic analyses provides a roadmap for bacteriocin discovery, formulation and use.

For crops grown on very large scales the GM approach (expressing bacteriocins in planta) offers an innovative approach to crop protection against bacterial pathogens. The expression of BT toxin in GM crops such as cotton, maize etc. provides a direct example of protection against bacterial pathogens. The expression of BT toxin formulation and use.

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For crops grown on very large scales the GM approach (expressing bacteriocins in planta) offers an innovative approach to crop protection against bacterial pathogens. The expression of BT toxin in GM crops such as cotton, maize etc. provides a direct example of how such an approach can be both effective and economically viable (albeit to control specific pests rather than pathogens). The addition of genes expressing bacteriocins into transgene stacks provides a route for introduction into existing GM crops. For crops grown in lower volumes, or where the use of GM approaches is not indicated or viable, the direct application of bacteriocin cocktails promises an effective, safe and economically viable approach to crop protection.

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