Bacteriocins active against plant pathogenic bacteria

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Abstract

Gram-negative phytopathogens cause significant losses in a diverse range of economically important crop plants. The effectiveness of traditional countermeasures, such as the breeding and introduction of resistant cultivars, is often limited by the dearth of available sources of genetic resistance. An alternative strategy to reduce loss to specific bacterial phytopathogens is to use narrow-spectrum protein antibiotics such as colicin-like bacteriocins as biocontrol agents. A number of colicin-like bacteriocins active against phytopathogenic bacteria have been described previously as have strategies for their application to biocontrol. In the present paper, we discuss these strategies and our own recent work on the identification and characterization of candidate bacteriocins and how these potent and selective antimicrobial agents can be effectively applied to the control of economically important plant disease.

Gram-negative bacterial plant pathogens

Feeding a rapidly growing global population is one of this century’s key scientific challenges. In order to achieve this, humanity relies on just 14 crop plants to provide more than 90% of its calorie intake. This number is reduced further when it is considered that the big four, i.e. rice, wheat, maize and potato, constitute the vast majority of this 90% [1]. Modern crop plants are grown over a wide area, distant from their geographic origins, and have been bred to near genetic homogeneity through the selection of individuals producing the greatest yield [2]. The results of this homogeneity and the large-scale monocultures of modern agriculture make plant crops prone to disease, often with devastating consequences [3]. Additionally, the vulnerability of crop plants to disease in some regions is likely to be exacerbated by climate change, creating a further challenge to food security [4]. Bacteria are responsible for a significant proportion of the >£100 billion lost worldwide per annum to disease and spoilage of crops caused by plant pathogens [1]. Gram-negative bacterial pathogens account for much of these losses and cause disease in all of the major plant crops [5]. For example, Xanthomonas oryzae pv. oryzae, the causal agent of bacterial blight and bacterial leaf spot in rice, constrains the growth of this crop over much of Africa and Asia, with losses as high as 75% reported [6]. Xanthomonas species also cause diseases, with analogous symptoms in maize, sugar cane and plantain [7,8]. Pseudomonas syringae is another important plant pathogen and is divided into over 50 pathovars. This broad host range of Ps. syringae includes both major and minor crop plants. The disease symptoms resulting from infection of Ps. syringae are equally diverse and include bacterial speck on tomato, leaf spot of soya bean, and trunk cankers and blast diseases on stone fruit and olive [9]. The enterobacterial plant pathogens Pectobacterium atrosepticum and Dickeya solani cause black leg and soft rot disease in the stems and tubers of potato during growth and storage and are the most damaging bacterial pathogens of potato in Europe [10]. Additionally, the related species Pectobacterium carotovorum and Pectobacterium brasiliensis cause black leg or soft rot type symptoms on a range of crop plants, including okra and sugar beet [11,12]. Current strategies for the control of these and other pathogens are of limited effectiveness and, as such, the development of alternative strategies, such as the application of colicin-like bacteriocins, is important from both an economic and a humanitarian perspective [13,14].

Colicin-like bacteriocins

Owing to their highly selective killing spectrum and potent cytotoxicity, colicin-like bacteriocins show potential as targeted next-generation antibiotics for medical and agricultural use. The colicin-like bacteriocins are antibacterial protein toxins (30–100 kDa) produced by a variety of Gram-negative bacteria that are active against bacteria closely related to the producing strain [15]. Bacteriocins are almost universally produced across the bacterial kingdom and play an important role in bacterial population dynamics [16]. Plant pathogenic bacteria encode numerous genes for the production of bacteriocins, with the majority of those characterized to date possessing cytotoxic domains that are highly homologous with those of well-studied colicins from Escherichia coli and pyocins of Pseudomonas aeruginosa. The cytotoxic activity of these bacteriocins is housed in a C-terminal domain, with the N-terminal domains encoding receptor-binding and translocation functions. The cytotoxic domains of these bacteriocins commonly take the form of...
a specific nuclease domain that hydrolyses DNA, tRNA or 16S rRNA, a pore-forming domain that depolarizes the cytoplasmic membrane, or a domain which interferes with cell wall integrity [17]. The C-terminal domain is also the site of binding for a specific immunity protein that protects the producing cell from the lethal effects of the toxin [18]. To gain entry into target cells, colicins and pyocins initially bind to a specific outer membrane receptor and cross this membrane through recruitment of host proteins in the periplasmic space [19]. In contrast with the colicins, very little is known about the receptors and mechanisms of entry used by bacteriocins from plant pathogenic bacteria.

**Colicin-like bacteriocins active against phytopathogens**

Genes encoding bacteriocins can be readily identified in the genomes of a large number of Gram-negative bacteria, including economically important plant pathogens such as *Ps. syringae*, *Pectobacterium* spp., *D. solani* and *Xanthomonas* spp. (R. Grinter and D. Walker, unpublished work). Our unpublished experimental work and previously published work indicates that bacteriocin production is widespread in these species as well as other Gram-negative phytopathogens such as *Agrobacterium* and *Bremneria* spp. [20,21]. The bacteriocins identified in these species are either colicin and S-type pyocin-like bacteriocins or phage-like particles resembling the F- and R-type pyocins of *P. aeruginosa*.

Three bacteriocins from *Pectobacterium* spp. with catalytic domains homologous with colicin-like bacteriocins have been characterized. These were proteins designated ‘carocins’ on the basis of the identity of the producing species, *P. carotovorum*. All three proteins have catalytic domains with nuclease activity, with the first protein identified, carocin S1, possessing a DNase domain homologous with the cytotoxyc domain of pyocin S3 [22]. Similarly, carocin S2 possesses a tRNase domain homologous with the cytotoxyc domain of colicin D, whereas carocin D possesses DNase activity [23,24]. Additionally, our group has recently identified and characterized two related bacteriocins from *Pectobacterium* spp., termed pectocin M1 and pectocin M2 [25]. The C-terminal catalytic domains of these proteins are homologous with the colicin M catalytic domain which kills cells through cleavage of lipid II, thus preventing cell wall synthesis [26]. Interestingly, the N-terminal domain of this protein which replaces the domains associated with translocation and receptor binding in colicin M exhibits approximately 60% sequence identity with a [2Fe–2S] plant ferredoxin and contains an intact iron–sulfur cluster (Figure 1A). As the primary role of plant ferredoxin is the transfer of electrons during photorespiration [27], this domain represents an extremely unusual receptor-binding domain. Additional experimental work, showing ferredoxin-dependent growth under iron-limiting conditions, suggests that this ferredoxin-like receptor-binding domain parasites an existing receptor which specifically binds plant ferredoxin. The normal physiological role of this receptor is presumably to acquire iron from this host protein during infection, although this remains to be proved [25].

As iron is frequently the limiting nutrient during bacterial infection of plants, it seems likely that *Pectobacterium* spp. would possess specific mechanisms to acquire iron from the host, although such systems are generally not that well characterized in phytopathogenic bacteria [28]. However, acquisition systems for obtaining iron from host proteins have been identified in a number of Gram-negative pathogens with mammalian hosts [29,30]. We have produced diffraction-quality crystals of pectocin M2, collected data and are currently working towards determining the structure of this unusual bacteriocin (Figure 1B). The structure of pectocin M2 and the identification of its receptor will facilitate the creation of a range of ferredoxin–cytotoxic domain hybrids and assist in the dissection of the mechanism and specificity of the ferredoxin-uptake system in *Pectobacterium* spp.

Previous experimental and bioinformatic analysis shows that *Ps. syringae* also possesses a number of homologues of the canonical colicin/S-type pyocin class; however, characterization of these proteins has been limited [21,31]. In a study by Barreteau et al. [32], a protein from *Ps. syringae* pv. *syringae* DC3000 with a catalytic domain homologous with colicin M was purified and shown to have catalytic activity against the substrate of colicin M, lipid II; however, no cytotoxic activity was observed against a limited number of *Pseudomonas* species [32]. Other studies on bacteriocin production in *Ps. syringae* and closely related plant-associated pseudomonads have identified high-molecular-mass phage tail-type bacteriocins and unusual bacteriocins analogous to rhs (recombination hotspot) proteins or possessing domains similar to mannose-binding lectins of plant origin [33,34].

Lectin-like bacteriocins have been identified in a range of Gram-negative bacterial species and form a structurally distinct group of bacteriocins [34]. The initial member of this class putidacin L1 (Llp1) was isolated from a rhizosphere-dwelling *Pseudomonas* isolate from the *Pseudomonas putida* cluster and was found to have cytotoxic activity against a number of phytopathogenic pseudomonad species [35]. The structure of Llp1 was solved and shown to consist of two β-prism domains linked by a curved two-strand sheet [36]. In the related plant lectins, the β-prism domain contains mannose-binding pockets; however, the mechanism of action of LlpA and thus how it utilizes these domains is yet to be elucidated [37]. Further studies have expanded this class, identifying homologous proteins in strains of *Pseudomonas fluorescens*, *Ps. syringae* and *Xanthomonas citri*, which kill with genus-level specificity [34,38,39]. Genes encoding additional members of this class containing tandem β-prism domains are also present in the genomes of a number of *Burkholderia* strains. Interestingly the Gram-positive ruminal bacteria *Ruminococcus albus* 7, produces a bacteriocin containing a single β-prism domain fused to a putative peptidase that inhibits other *Ruminococcus* species [40].
Figure 1  | (A) Cartoon representation of crystal structures of spinach ferredoxin (PDB code 1A70) and the catalytic domain of colicin M (residues 122–271) (PDB code 2XMX), and chart showing regions of pectocin M1 that have identity with these proteins [50,51]. (B) Crystals of pectocin M2 with the space group P3$_2$1 diffracting to 1.83 Å (1 Å = 0.1 nm).

Application of bacteriocins to prevent or limit the effects of bacterial disease in plants are limited; however, this is predominantly due to a lack of research into the diversity and mechanisms of action of these proteins in phytopathogenic bacteria, rather than their potential [41]. A number of studies have shown bacteriocins to be effective agents in the treatment of bacterial disease. Lavermicocca et al. [42] report the use of an uncharacterized bacteriocin from Ps. syringae pv. ciccaronei in the prevention of olive knot disease. This study showed a 60–80% reduction in knot formation when stem wounds were pre-treated with crude bacteriocin before infection with the causal agent of the disease, Ps. syringae pv. savastanoi. Additionally, a 350–400-fold decrease in the epiphytic numbers of the pathogen was observed when unwounded olive plants were treated with a crude preparation of the bacteriocin [42]. A study by Kerr and Htay [43] showed that co-inoculation of a non-pathogenic bacteriocin-producing strain Agrobacterium radiobacter strain 84, with pathogenic Agrobacterium strains led to attenuation of gall formation observed during inoculation of only pathogenic strain. This attenuation was shown to be due to bacteriocin production, with only one bacteriocin-sensitive pathogenic strain not subject to biological control, which was in turn due to the production of a bacteriocin active against A. radiobacter 84 [43]. On the basis of this work, A. radiobacter strain 84...
Future prospects
An attractive strategy for the utilization of bacteriocins in disease control is the creation of transgenic plants which express multiple bacteriocin genes. Colicin-S-type pyocin-type bacteriocins are attractive targets for this approach for a number of reasons. First, bacteriocins of this class are unmodified protein molecules encoded by a single open reading frame, making transformation, even with a number of bacteriocins, a relatively simple procedure. Secondly, the narrow spectrum of these toxins allows for the targeting of specific pathogens without disturbing the wider microbial community. This narrow spectrum also means consumption of transgenic food crops expressing bacteriocins, are unlikely to negatively affect the heath of humans or their gut flora.

Focusing future work in this area on the identification and characterization of bacteriocins which target economically important plant pathogens will create a library of bacteriocins for the creation of transgenic plants. Delineating the functional domains and dissecting the catalytic mechanisms of these proteins will provide data required for protein engineering to create of novel bacteriocins with an expanded host range. Multiple bacteriocin genes could then be ‘stacked’ into host plants to provide robust resistance to a broad spectrum of pathogenic strains under field conditions.

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