The crystal structure of the lipid II-degrading bacteriocin syringacin M suggests unexpected evolutionary relationships between colicin M-like bacteriocins

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Background: Syringacin M is a colicin M-like bacteriocin from the plant-pathogenic species \textit{Pseudomonas syringae}.

Results: The receptor binding domain of syringacin M has unexpected structural homology to that of colicin M.

Conclusion: Syringacin M and colicin M appear to have evolved directly from a common ancestor.

Significance: Bacteriocins can evolve novel receptor specificities through diversifying selection.

\textbf{ABSTRACT}

Colicin-like bacteriocins show potential as next generation antibiotics with clinical and agricultural applications. Key to these potential applications is their high potency and species specificity that enables a single pathogenic species to be targeted with minimal disturbance of the wider microbial community. Here we present the structure and function of the colicin M-like bacteriocin, syringacin M from \textit{Pseudomonas syringae pv. tomato} DC3000. Syringacin M kills susceptible cells through a highly specific phosphatase activity that targets lipid II, ultimately inhibiting peptidoglycan synthesis. Comparison of the structures of syringacin M and colicin M reveal that in addition to the expected similarity between the homologous C-terminal catalytic domains, the receptor binding domains of these proteins, which share no discernible sequence homology, share a striking structural similarity. This indicates that the generation of the novel receptor binding and species specificities of these bacteriocins has been driven by diversifying selection rather than diversifying recombination as previously suggested. Additionally, the structure of syringacin M reveals the presence of an active site calcium ion that is coordinated by a conserved aspartic acid side chain and is essential for catalytic activity. We show that mutation of this residue to alanine inactivates syringacin M and that the metal ion is absent from the structure of the mutant protein. Consistent with the presence of Ca\textsuperscript{2+} in the active site, we show that syringacin M activity is supported by Ca\textsuperscript{2+}, along with Mg\textsuperscript{2+} and Mn\textsuperscript{2+} and the protein is catalytically inactive in the absence of these ions.

The colicin-like bacteriocins are high molecular weight protein antibiotics whose target is generally bacteria that are closely related to the producing strain (1). The best characterized members of this group are the plasmid-encoded colicins from \textit{Escherichia coli}. 

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coli and the chromosomally-encoded S-type pyocins from Pseudomonas aeruginosa (2,3). Colicins are active against strains of E. coli and some strains of other closely related bacteria such as Citrobacter spp. and Salmonella spp., whereas the S-type pyocins seem to specifically target only P. aeruginosa (3-5). Other related bacteriocins such as carocin S1-3 and pectocin M1-2 from the phytopathogenic Pectobacterium spp have also been characterized and shown to have a similarly restricted killing spectrum limited to bacteria closely related to the producing strain (6-8). Colicin-like bacteriocins from a variety of different species can be readily identified from genomic sequence data due to the high degree of homology between their well-characterized cytotoxic domains. These take the form of a nuclease domain that specifically targets DNA, tRNA or rRNA, or a pore-forming domain that targets the cytoplasmic membrane (9,10). In addition, colicin M and bacteriocins with homologous catalytic domains kill susceptible cells through a highly specific phosphatase activity that targets lipid II (11). Cleavage of lipid II at the phosphoester bond between the undecaprenyl and pyrophosphate moieties prevents recycling of undecaprenyl phosphate, thus preventing the translocation of peptidoglycan precursors across the inner membrane (12).

Entry of colicin-like bacteriocins into target cells is mediated by two functional domains responsible for receptor binding and translocation. The species-specificity of target bacteriocins is largely governed by binding to a specific outer membrane receptor. In the colicins, receptor-binding is associated with the central domain that is flanked by translocation and cytotoxic domains at the N- and C-termini respectively (13). For the S-type pyocins the order of the translocation and receptor binding domains is reversed (3). Passage across the outer membrane for the colicins is mediated by interaction with the Tol or Ton complexes that span the cell envelope and derive energy from the proton motive force (2).

To protect the producing strain from the lethal effects of bacteriocin production, a specific immunity protein is produced in tandem with the toxin (13). In the case of the nuclease type bacteriocins the immunity protein forms a 1:1 high affinity complex with the toxin and is exported from the cell as a heterodimeric complex. In the case of the pore forming and lipid II degrading bacteriocins complex formation with the immunity protein has not been demonstrated. These proteins are localized at the cytoplasmic membrane where they negate the lethal effects of the toxin by mechanisms that are yet to be clearly delineated (14,15). In general, full protection is afforded only by the cognate immunity protein.

The evolution of colicin-like bacteriocins has been proposed to occur through two major mechanisms: diversifying recombination and diversifying selection (16). In the former, novel killing specificities are generated through domain shuffling to give combinations of receptor binding, translocation and cytotoxic domains that allow the resulting bacteriocins to exploit different receptors on the surface of target cells and circumvent immunity protein based resistance (9). The results of evolution by recombination can be seen with the well-characterized colicins where, for example, colicins B and D share extensive sequence homology within the translocation and receptor binding domains, but carry unrelated cytotoxic domains with pore forming and tRNase activity, respectively (16). Similarly, bacteriocins from distantly related species frequently share homologous cytotoxic domains, but unrelated translocation and receptor binding domains. For example, colicin E9 and pyocin S2 share sequence homology within their C-terminal cytotoxic domains but sequences of the translocation and receptor binding domains appear to be unrelated (17). A variation on this mechanism of bacteriocin evolution is illustrated by the recently described pectocins M1 and M2. These bacteriocins, which are produced by strains of the phytopathogenic genus Pectobacterium possess a catalytic domain homologous to colicin M and a receptor binding domain derived from an iron-containing plant ferredoxin. In this case an ancestral bacteriocin appears to have recombined with a horizontally acquired host ferredoxin gene to give an active bacteriocin with a colicin M-like catalytic domain (8). Presumably, the acquisition and exploitation of ferredoxin as a bacteriocin receptor binding domain enables the parasitization of an existing iron acquisition pathway in
Pectobacterium spp (8). This example, perhaps illustrates a general mechanism for how a domain with receptor binding function is initially recruited.

Diversifying selection in colicin evolution is thought to play a more restricted role in driving the evolution of novel toxin-immunity specificities through strong positive selection (16). For example, the closely related DNase colicins E2, E7, E8 and E9 are neutralized by their cognate immunity proteins which bind the catalytic domain adjacent to the active site (13,18). The overall amino acid sequence identity of these proteins is high (>70%), however the region to which the immunity protein binds shows a high level of sequence divergence, which is mirrored by divergence in the complementary binding surface of the immunity protein.

In order to gain insight into the activity and evolution of colicin M-like bacteriocins, we have used X-ray crystallography to solve the three dimensional structure of the colicin M-like bacteriocin, syringacin M, from Pseudomonas syringae pv. tomato DC30001. Comparison of the structures of colicin M and syringacin M indicates that in addition to the expected structural conservation of their related catalytic domains, these bacteriocins show a striking and wholly unexpected similarity in their receptor binding and translocation domains. Given that there is no discernible sequence similarity in these regions, this result is surprising and suggests that these bacteriocins may have evolved from a common ancestor through diversifying selection without the requirement for a recombination event to facilitate a change in species and receptor binding specificity. In addition, the structure of syringacin M reveals insights into the catalytic mechanism of colicin M-like bacteriocins by revealing the presence of a metal ion binding site in the catalytic centre of the protein.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids* - Strains and plasmids utilized in this study are presented in Table S1.

*Bacteriocin Screening and Identification* - Eleven test strains of P. syringae were screened for bacteriocin production using the soft agar overlay test (19). Colonies of each test strain were grown on Kings B agar (20 g l−1 peptone, 10.8 mM glyceral, 8.6 mM K2HPO4, 5.4 mM MgSO4, 1.5% w/v agar, pH 7.5) with mitomycin C (200 μM) for 36 hours. Colonies were lysed with chloroform vapor and overlayed with 6 ml molten 0.6% w/v agar containing 200 μl of 0.6 OD600 culture of a test strain. Plates were incubated for 14 hours at 28 °C and bacteriocin activity of a strain scored based on zones of inhibition surrounding the lysed colony. Bacteriocin production was scored as R/F or S-type activity based on whether a large diffuse or small defined zone of inhibition was observed (19). Of the test strains Pto DC3000 and Pseudomonas syringae pv. syringae LMG1247 produce an S-type bacteriocin under these conditions. In order to identify the agent responsible for this activity strains were grown for 24-48 h in 2 × 625 ml of Kings B media with mitomycin C (200 μM). The culture was clarified by centrifugation and the supernatant concentrated by ammonium sulfate precipitation; 3 M ammonium sulfate was added to the supernatant and precipitation was allowed to proceed at 4 °C with stirring for 1 h. Precipitate was collected by centrifugation (3000 g for 20 minutes) and resuspended in sample buffer (50 mM Tris, 20 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) with protease inhibitor tablets (Complete EDTA free, Roche) and was dialyzed against sample buffer and tested for killing activity by spotting onto a KB agar plate overlaid with soft agar containing 200 μl of 0.6 OD600 culture of a test strain. Proteins were separated by native PAGE at 4 °C. Samples were run in duplicate, gels were halved and killing activity was tested via soft agar overlay of half the gel. The other half was stained with Coomassie Brilliant Blue 250, the band corresponding to zones of inhibition in the soft agar overlay assay were excised and identified via tandem mass spectrometry2, with trypsinization as the fragmentation step.

*Cloning and purification of syringacin M* - Plasmid pETMDC was produced to express syringacin M with a C-terminal His6-tag. The gene corresponding to syringacin M was amplified from Pto DC3000 using forward and

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1 Pto. DC3000

2 MS/MS
reverse primers (Forward: 5'-CAT ATG CCT ATT GAG CTT CCT CC-3', Reverse: 5'-CTC GAG GTT GCC ACT AAC CGT AAC CG-3'). The PCR product was digested with the NdeI and XhoI and ligated into the corresponding sites of pET21a. Syringacin M was over expressed from E. coli BL21 (DE3) carrying the plasmid pETMDC. 5 L of LB broth was inoculated (1:100) and overnight culture and cells were grown at 37 °C in a shaking incubator to an OD600 = 0.6. Protein production was induced by the addition of 1 mM IPTG, the cells were grown for a further 12 hours at 24 °C and harvested by centrifugation. The cell pellet was resuspended in 20 mM Tris-HCl and 100 mM NaCl containing 5 mM imidazole (pH 7.5). Cells were lysed using a MSE Soniprep 150 (Wolf Laboratories, UK) and the cell debris separated by centrifugation. The cell-free lysate was applied to a 5 ml His Trap™ HP column (GE Healthcare, USA) and the protein eluted over a 0-350 mM imidazole gradient. Syringacin containing fractions were dialyzed overnight into 20 mM Tris-HCl, 20 mM NaCl (pH 8.0) and the protein was further purified by gel filtration chromatography on a Superdex S75 26/60 column (GE Healthcare) equilibrated in the same buffer. Cytotoxicity assays - The cytotoxicity of purified syringacin M was tested using the soft agar overlay method described above. Five-fold serial dilutions of syringacin M were performed and spotted against the most sensitive strain P. syringae pv. lachrymans LMG 5456, against which it displays a minimum inhibitory concentration of 60 nM. To assess the ability of inactive syringacin M mutants to interfere with the cytotoxic activity of the wild-type protein, 4 µl of 100 µM wild-type and mutant protein were spotted 10 mm apart on a soft agar overlay plate.

Crystallization of syringacin M - Initial crystallization trials were performed using a Cartesian Honeybee 8+1 dispensing robot into 96 well plates via the sitting drop method (reservoir volume of 80 µl, drop size: 0.5 µl protein, 0.5 µl reservoir solution) by vapor diffusion with syringacin M at a final concentration of 200 µM. Hexagonal shaped crystals that diffracted to 6-7 Å at a home X-ray source were obtained in 10 % w/v PEG 8000, 20 % v/v ethylene glycol, 0.03 M CaCl2, 0.03 M MgCl2, 0.1 M Bicine/Trizma base pH 8.5 at 20 °C from the MORPHEUS crystallization screen (20). This crystal was of the space group P6322 and had a symmetry of the space group P6322 and an unusually high solvent content of 80%. Optimization was performed in sitting drop plates around this condition for both wild-type and D232A mutant protein. The best hexagonal crystals were obtained in 7% w/v PEG 8000, 30% v/v ethylene glycol 0.03 M CaCl2, 0.03 M MgCl2, 5% v/v dimethyl sulphoxide, 0.1 M Bicine/Tris base pH 8.5 at 20 °C, and used for data collection at Diamond Light Source, Oxfordshire with diffraction to 2.83 Å and 3.12 Å for wild-type and mutant proteins, respectively. All data sets collected at the DLS were processed with the programs XDS (21) and Scala (22). Phasing was
attempted by molecular replacement\textsuperscript{4} using PHASER (23), with the catalytic domain from colicin M as the search model. This proved unsuccessful and selenomethionine\textsuperscript{5} labeled protein was prepared and crystallized in the above conditions. A SeMet labeled crystal, producing diffraction to 3.1 Å, was used to collect multiple-wavelength anomalous dispersion\textsuperscript{6} data near the selenium absorption edge at the DLS. Phases were determined by MAD/SAD techniques using programs SHARP/autoSHARP (24,25), SHELXC/D/E (26), and a partial model was built using ARP/wARP (27) and BUCCANEER (28). Further modeling and refinement against the wild-type or mutant data was performed with programs COOT (29), REFMAC (30,31) and various other programs from the CCP4 suite (32). Quality control of the geometry of the model was performed using RAMPAGE (33).

A summary of crystal data and refinement statistics are provided in Table 1.

The limited resolution of data from the hexagonal crystal form resulted in difficulty in modeling of the β-strands and connecting loops and so \textit{in situ} trypsin digestion/crystallization was performed to change the crystal packing (34). Trypsin was added to syringacin M (500 µM) at a ratio of 1:200 on ice, immediately prior to addition to drop. Stacks of thin plates, diffracting to 2.1 Å at a home X-ray source, were grown in 8% w/v PEG 8000, 30% v/v ethylene glycol, 0.13 M CaCl\textsubscript{2}, 0.03 M MgCl\textsubscript{2}, 0.1 M Bicine/Tris base pH 8.5 at 4 °C. To obtain single crystals, crystals grown under these conditions were crushed in the above reservoir buffer and 3-fold serial dilutions of this solution were used to seed sitting drops of the same composition at 16 °C. Single rhombic crystals formed after two days. These crystals had symmetry of the \textit{P}2\textsubscript{1}\textit{1} space group and a solvent content of 58 %.

Diffraction data were collected for the crystal of this form at DLS, reaching a resolution of 1.46 Å. This data was phased by MR using PHASER (23) and the model previously built against the data for the hexagonal crystal form, excluding the N-terminal 38 amino acids. High resolution data allowed for improved structure modeling in COOT (29) and final refinement with the use of individual atom anisotropic thermal parameters performed by REFMAC (30). Model based on monoclinic data was subsequently used to improve the full-length wild-type and mutant models from the hexagonal form. The N-terminal residues 2-38 were also modeled using the hexagonal form data for both wild-type and the D232A mutant. Features of the N-terminal electron density suggested some degree of conformational disorder, which limited the quality of the modeling of this region. Despite this, numerous interactions between N-terminus and the rest of the protein were observed.

**Lipid II cleavage assay** - Lipid II hydrolysis assays were performed using a modified version of the assay described by Barreteau \textit{et al.} (35), with non-radiolabeled lipid II substrate obtained from the UK Bacterial Cell Wall Biosynthesis Network (36,37). Assays were carried out in 100 mM Tris, 150 mM NaCl, 0.2% w/v n-dodecyl-D-maltoside with 2.6 nmoles of lipid II and 0.33 nmoles of wild-type or mutant syringacin M. To test the metal dependence of syringacin M, assays were performed in the presence of MgCl\textsubscript{2}, CaCl\textsubscript{2}, MnCl\textsubscript{2}, CoCl\textsubscript{2}, ZnCl\textsubscript{2} or NiSO\textsubscript{4} at 20 mM or with 1 mM EGTA. Reactions were performed at 30 °C for 0-120 minutes and terminated by heating at 100 °C. Reaction products were separated by thin-layer chromatography on LK6D silica gel plates with 2-propanol: ammonium hydroxide: water (6:3:1) as the mobile phase and visualized by staining with 20% w/v phosphomolybdic acid solution in ethanol and heating. A band corresponding to lipid II was observed with an \textit{Rf} of 0.7 as reported previously (35).

**RESULTS**

\textit{Syringacin M is an active bacteriocin} - As part of a wider study into the potential use of bacteriocins we used a cell killing assay to screen a number of diverse pathovars of \textit{P. syringae} for the production of bacteriocins (Table S1). Of these potential producing strains, \textit{P. syringae pv. syringae} LMG1247 (the type strain for this pathovar) produced a large diffuse zone of inhibition, reminiscent of the S-type pyocins from \textit{P. aeruginosa}, against two indicator strains in soft agar overlay experiments when bacteriocin production was induced by mitomycin C. In order to identify

\textsuperscript{4} MR
\textsuperscript{5} SetMet
\textsuperscript{6} MAD
this bacteriocin we collected and concentrated the supernatant of a Pss. LMG1247 culture by ammonium sulfate precipitation and separated the precipitated proteins via native PAGE. This native gel was used in a soft agar overlay against test strains and a band which correlated with cytotoxicity was excised, trypsinised and subjected to LC-MS/MS (Fig. 1A). Analysis and database searching of MS/MS data using the MASCOT search engine matched the secreted bacteriocin through 5 peptides to a predicted colicin M-like bacteriocin, ZP_07266212 from Pss. 642 for which the genome sequence is available (Fig. 1B). We designed primers based on this sequence to amplify and identify the corresponding gene from Pss. LMG1247. Successful amplification and sequencing of this gene showed that Pss. LMG1247 harbors a bacteriocin-encoding gene that shares 99 and 94 % sequence identity at the amino acid level to ZP_07266212 from Pss. 642 and a close homologue Q88A25 from Pto DC3000 respectively (Fig. 1B). This homologue from Pto. DC3000 has been purified previously and shown to have lipid II degrading activity in vitro; the study however did not find any cytotoxic activity associated with the bacteriocin against 24 strains of P. syringae or isolates of P. aeruginosa and P. fluorescens (35). To support the idea that Q88A25 from Pto DC3000 and the trivially different homologous bacteriocin from Pss. LMG1247 are in fact active bacteriocins we cloned the Q88A25 gene from Pto DC3000 into an E. coli expression vector and produced and purified recombinant C-terminally His_{6}-tagged protein (Fig. 2B). The spectrum of activity of this recombinant protein was identical to the secreted bacteriocin from Pss. LMG1247, supporting the MS/MS identification data (Fig. 2A/C). To standardize the nomenclature for bacteriocins from P. syringae, we propose the name syringacin M for this bacteriocin. In addition to the 10 P. syringae test strains, we tested the activity of syringacin M against strains of P. aeruginosa, Pectobacterium spp, P. fluorescens and P. putida (Table S1). Killing of these bacterial species was not detected. Thus, syringacin M is an active bacteriocin, whose targets like other colicin-like bacteriocins are bacterial strains that are closely related to the producing strain.

Domain structure of syringacin M - For colicin M and other colicins the N-terminal domain of the protein, which is often disordered in crystal structures and lacks secondary structure, corresponds to the translocation domain, and the central domain is involved in receptor binding (9). However, for the closely related S-type pyocins of P. aeruginosa the order of these domains is reversed and the receptor binding functionality resides within the N-terminal domain. To unambiguously assign functionality to the N-terminal and central domains of syringacin M we created truncated syringacin M variants lacking the first 10 or 20 amino acids. Both deletion mutants lacked discernible bactericidal activity, indicating an essential functional role for the N-terminal domain of syringacin M. In order to determine whether these mutants possessed an intact receptor binding domain competition experiments were performed by spotting syringacin M Δ10 and Δ20 protein within diffusion zone of wild-type protein in a soft agar overlay experiment, both proteins inhibited killing by wild-type syringacin M (Fig. 2D). This inhibition is attributable to competition for binding to the cell surface receptor required for entry of syringacin M into the target cell and indicates that the mutants possess a functional receptor binding domain. The N-terminal domain is therefore a translocation domain, while the globular central domain is the receptor binding domain. These data suggest that syringacin M displays a colicin-like arrangement of domains with an N-terminal translocation domain, a central receptor binding domain and a C-terminal cytotoxic domain.

Syringacin M crystal forms - Crystals were obtained and intensity data collected for two crystal forms of purified syringacin M, one of full length protein in the monoclinic form diffracting to 2.83 Å and another resulting from in situ trypsin digested protein, in the hexagonal form diffracting to 1.46 Å. Hexagonal crystal data produced electron density corresponding to residues 2-276, although the density corresponding to residues 15-22 and 26-34 was weak suggesting these sections have a high degree of conformational flexibility. Supplementary animation S4 shows omit map density for this region contoured from 1.0-4.8 σ. This region was built but the
Ramachandran geometry (33) is imperfect (Fig. 3A). The monoclinic crystal contains two molecules per asymmetric unit related by a non-crystallographic 2-fold symmetry with only minor differences due to crystal packing. Electron density from this data allowed unambiguous modeling of residues 38-276, which correlates well with the presence of a predicted trypsin cleavage site between R37 and G38. Fig. 3B presents a cartoon representation of molecule A. Alignment of residues 38-276 from hexagonal wild-type and monoclinic trypsin-digested models gave an overall root mean square deviation of 0.47 Å and showed that the conformation of the protein is the same. As such, the high resolution model will be used in the further discussion except for the N-terminus.

**Overall structure of syringacin M** – The structure of syringacin M reveals a protein with a compact mixed sheet/helix structure and an unstructured N-terminus consisting of 39 amino acids. Inherently unstructured translocation domains are also observed for colicins E3 and M and like the N-terminal 39 amino acids of syringacin M they are rich in Pro, Ala, Ser and Gly residues. In colicin E3, where the first 83 amino acids of the protein are disordered, an unstructured N-terminal domain is required to enable threading of this region of the protein through the lumen of the OmpF porin to deliver the TolB interacting region of the protein into the periplasm (2,38). Fig 3C shows that regions of the N-terminal domain of syringacin M display high temperature factors relative to most other regions of the protein. High temperature factors are also observed in some loop regions of the catalytic domain in structures of both the full-length and truncated forms of syringacin M (Fig 3C). Similarly, in colicin M, the N-terminal 35 residues, although visible in the crystal structure display markedly higher temperature factors than other regions of the protein and lack regular secondary structure (39). Thus syringacin M possesses an N-terminal translocation domain that is typical of colicin-like bacteriocins.

As with colicin M the receptor binding domains and catalytic domains of syringacin M do not form obviously distinct structural entities. However, on the basis of truncation experiments Barreteau et al reported that the minimum catalytically active region of colicin M corresponded to amino acids 122-271 and that helix 1 (37-46) is required for receptor binding. This delineates the receptor binding domain as a compact bundle of 5 helices spanning residues 37-122 (40-127 in syringacin M) (40). As expected from the extensive sequence similarity (Fig. 3D) the structures of the colicin M and syringacin M catalytic domains are structurally homologous (RMSD 2.6 Å). However, for syringacin M well defined electron density for a single metal ion is present in the active site. This density correlates well with the presence of Ca²⁺ which is present at high concentration in the crystallization buffer. In the syringacin M model, Ca²⁺ is coordinated by backbone carbonyl groups of residues L202, N204 and the side chain of a highly conserved aspartic acid, D232. The corresponding aspartic acid in colicin M (D226) is critical for catalytic activity in colicin M with mutation to a number of chemically similar residues (including glutamic acid) abolishing activity (41). The potential role of this metal ion in the binding and cleavage of the lipid II substrate is discussed below.

The receptor binding domains of colicin M and syringacin M show extensive structural similarity - Outside the C-terminal cytotoxic domain there is no clear sequence similarity between colicin M and syringacin M (Fig. 3D). However, using the DALI server (42) to search for proteins in the protein data bank that are structurally homologous to syringacin M revealed not only the expected structural homology between the cytotoxic domains of colicin M and syringacin M, but also extensive similarity within the respective receptor binding domains of these proteins. Indeed, structural homology extends from the C-terminus of the proteins and spans the entirety of the cytotoxic and receptor binding domains, with the exception of helix 1 in syringacin M (43-54) that adopts a more compact conformation. The RMSD for the alignment of the receptor binding and cytotoxic domains (residues 43-276 of syringacin M) with those of colicin M (entry 2XTR) is 3.3 Å with a Z score of 16.9. A structural alignment of the two proteins is illustrated in Fig. 4. In addition,
Z-scores of >16 were obtained from alignment of syringacin M with other wild-type and variant colicin M structures (entries 2XTR, 2XMX, 2XTQ, 3DA3 and 3DA4). Limited homology (Z-scores <5) over less than 80 amino acids was detected with streptavidin (for example entry 1SRF) and OmpF (1IQP), which after colicin M were the most similar structures to syringacin M according to the results from DALI. The superposition of colicin M and syringacin M structures shown in Fig. 4A and B is based on alignment of the catalytic domain half beta-barrels, while Fig. 4C shows the alignment based on helices 2-4 of the receptor binding domains. These alignments illustrate a difference in angle between the receptor binding and cytotoxic domains in colicin and syringacin M. Alignment of the core helices shared by colicin M and syringacin M are shown in Fig. 4D/E.

Given the lack of discernible sequence homology between the receptor binding domains of these proteins their structural similarity is wholly unexpected. Indeed, it has been previously postulated that this first 126 amino acids of syringacin M represent receptor binding and translocation domains that are specific to P. syringae and unrelated to those of colicin M (39). The striking similarity in tertiary structure suggests a hitherto undetected divergent evolutionary relationship between the receptor binding domains of colicin M and syringacin M and suggests that novel receptor binding function of these colicin M-like bacteriocins has been generated through diversifying selection and not through diversifying recombination as previously suggested (41).

**Comparison of the syringacin M and colicin M active sites** - The syringacin M cytotoxic domain consists of residues 129-276 and forms the mixed helix-sheet structure, incorporating a half β-barrel structure in an overall fold that is unique to colicin M type bacteriocins (39). Previous mutational studies on colicin M have identified the conserved residues D226 (D232), D229 (D235), H235 (H241) and R236 (R242) (syringacin M numbering in brackets) as essential for activity. These residues are surrounded by additional conserved residues and together these likely constitute the active site of these enzymes (Fig. 5). In the syringacin M structure a Ca$^{2+}$ ion is present, with distorted octahedral coordination, by the side chain of D232, backbone carbonyl groups from L202 and N204, two water molecules and an ethylene glycol$^9$ molecule (Fig. 5B).

Our assignment of Ca$^{2+}$ to this density as opposed to Mg$^{2+}$, which is also a cofactor for the enzyme and is present in the crystallization buffer is based on a number of factors. Firstly, the difference Fourier $F_o-F_c$ map shows insufficient electron density has been attributed to the metal ion when Mg$^{2+}$ is fitted but not when Ca$^{2+}$ is fitted (data not shown). Secondly, the B-factors obtained for the metal ion when Mg$^{2+}$ is fitted to this density (19.1 and 21.4 Å$^2$ for molecules A and B, respectively) are significantly lower than the surrounding environment (average B-factor for coordinating protein oxygens in molecules A and B are 23.6 and 25.1 Å$^2$, respectively) suggesting that an insufficient number of electrons have been attributed to the metal ion when Mg$^{2+}$ is modeled. However, with Ca$^{2+}$ modeled, the metal ion B-factors (25.0 and 27.5 Å$^2$ for molecules A and B, respectively) are in the expected range. Thirdly, the average bond length of the coordinating oxygen atoms to the metal ion is 2.34 Å, close to the average value of 2.39 Å calculated from consideration of 3818 high resolution Ca$^{2+}$ containing protein structures (43). The average bond length for magnesium-oxygen bonds from 2310 high resolution structures is 2.16 Å (43). Finally, the metal ion has a coordination number of eight. This coordination is common for Ca$^{2+}$ but is not favored by Mg$^{2+}$, which rarely has a coordination number higher than six (44,45). The presence of this active site metal ion, which is absent in the colicin M structure, likely orientates the side chain of D232 close to the side chains of the other putative active site residues. In the structure of syringacin M a break in helix 8 within the cytotoxic domain results in the side chain of R242 being orientated away from the putative active site, whereas in the colicin M structure the equivalent arginine lies closer to the active site pocket. The surface model representation of syringacin M 38-276 shown in Fig. 5C reveals a potential active site binding pocket, formed by the essential Ca$^{2+}$ and its...

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$^9$ EDO
coordinating residues on one side and key catalytic residues Y234, D235 and H241 on other. In contrast, in the colicin M structure 37-271 the unbroken conformation of helix 9 and the loop extended due to the conformation of D226 results in a pocket too narrow to accommodate the substrate (Fig. 5D).

Colicin M activity has previously been shown to be dependent on the presence of Mg\(^{2+}\) although it has not been reported whether Ca\(^{2+}\) and other metal ions are capable of supporting activity. To determine if the Ca\(^{2+}\) bound structure of syringacin M likely represents a physiologically relevant cofactor and to determine the ability of other metal ions to support activity we tested the ability of syringacin M to cleave lipid II in the presence of a range of metal ions. In addition to activity in the presence of Mg\(^{2+}\), both Ca\(^{2+}\) and Mn\(^{2+}\) ions were also able to support activity, while other divalent cations tested, Zn\(^{2+}\), Ni\(^{2+}\) and Co\(^{2+}\) were not (Fig. 6A). Semi-quantitative time course experiments show that syringacin M displays comparable catalytic activity in the presence of either Ca\(^{2+}\) or Mg\(^{2+}\) (Fig. 6B). The syringacin M D232A mutant which displays no bactericidal activity was also found to be catalytically inactive under all conditions (Fig. 6A). The structure of the D232A mutant was also determined and shown to lack the Ca\(^{2+}\) ion found in the active site of the wild-type protein, demonstrating that this side chain is critical to Ca\(^{2+}\) binding. However, despite the loss of Ca\(^{2+}\) from the active site of syringacin M D232A the crystal structure and circular dichroism spectra of the mutant show the overall secondary structure and conformation of active-site side chains were analogous (data not shown). These data indicate that the lack of activity of the D232A mutant is likely due to its role in the coordination of a catalytically essential metal ion.

**DISCUSSION**

The overall structure of syringacin M shows that this bacteriocin from *P. syringae* shares an analogous arrangement of structural domains to colicin M with an unstructured N-terminal translocation domain that is a characteristic of colicins. Colicin M binds to the FhuA receptor and utilizes TonB in order to gain entry into target cells. The translocation domain of colicin M spans the N-terminal 37 amino acids, with binding to TonB mediated by the first 7 amino acids that constitute the TonB box. Mutation or deletion of the TonB box results in a protein that does not possess cytotoxicity, but is not deficient in receptor binding or catalytic activity (46). A similar loss of cytotoxicity is also observed for Δ10 syringacin M and so it is reasonable to postulate that this region of syringacin M contains a binding box which is equivalent in binding the TonB orthologue of *P. syringae*. Adding further weight to this hypothesis, a putative colicin M homologue from *P. syringae pv. morsprunorum* str. M302280 (Fig. 7), has overall sequence identity with syringacin M of only 18%, but shares a nearly identical N-terminal 8 amino acids (MPIELPPTY/MPVELPPTY).

Diversifying recombination is a major evolutionary mechanism in generating novel bacteriocins that are able to exploit novel receptors or that possess novel cytotoxic activities, and clear examples of the results of recombination events can be observed through comparative sequence analysis (9). For example, colicin E3 and E9 share almost identical translocation and receptor binding domains but their cytotoxic domains share no sequence, structural or functional similarities, with the former possessing an rRNase domain and the latter a DNase domain. Similarly, pectocin M and colicin M both share homologous lipid II cleaving catalytic domains but there is no sequence or structural similarity between their receptor binding domains, with the pectocin receptor binding domain closely related to 2Fe-2S plant ferredoxins and in fact possessing an intact iron-sulfur cluster (8). Given that diversifying recombination has been shown to be the major mechanism for the acquisition of novel receptor binding, translocation and cytotoxic functionalities and that the receptor binding domains of colicin M and syringacin M share no obvious sequence identity, the observed structural similarity between these proteins within their receptor binding domains is unexpected. However, as protein structure is conserved over much longer evolutionary distances than amino acid identity (47), the observed structural similarity, suggests that colicin M and syringacin M have evolved divergently from a common ancestor. This hypothesis is supported by the fact that the receptor binding
groups of proteins, direct structural domains of these bacteriocins. As with other relationship between the receptor binding a reliable indicator of the evolutionary comparative sequence analysis is therefore not documented evolutionary pathways of colicin-colicin-like bacteriocins (50,51). The main unexpected, considering the data presented like bacteriocins are summarized in Fig. 8. evolutionary mechanism in the genesis of colicin or syringacin M may have evolved through convergent evolution, it seems unlikely that such structural homology would have arisen independently given the structural diversity of receptor binding domains from other colicins, which essentially share the same function (38,48,49). Secondly, structural conservation is unlikely to have evolved in order to stabilize the catalytic domain or overall structure of colicin M-like bacteriocins, as the receptor binding domains of pectocin M1 and 2 consists of a ferredoxin-like protein with no structural similarity to the receptor binding domains of colicin or syringacin M (8).

While it is worth considering that the structurally analogous receptor binding domains of colicin or syringacin M may have evolved through convergent evolution, it seems unlikely that such structural homology would have arisen independently given the structural diversity of receptor binding domains from other colicins, which essentially share the same function (38,48,49). Secondly, structural conservation is unlikely to have evolved in order to stabilize the catalytic domain or overall structure of colicin M-like bacteriocins, as the receptor binding domains of pectocin M1 and 2 consists of a ferredoxin-like protein with no structural similarity to the receptor binding domains of colicin or syringacin M (8).

Therefore, we suggest that diversifying selection, in addition to its role in the generation of novel cytotoxic domain-immunity protein pairs, may be an important evolutionary mechanism in the genesis of novel receptor-binding function in colicin-like bacteriocins. Interestingly and perhaps not unexpectedly, considering the data presented here, positive selection has also been detected in the outer membrane receptors of some colicin-like bacteriocins (50,51). The main documented evolutionary pathways of colicin-like bacteriocins are summarized in Fig. 8.

In the case of colicin M and syringacin M comparative sequence analysis is therefore not a reliable indicator of the evolutionary relationship between the receptor binding domains of these bacteriocins. As with other groups of proteins, direct structural comparison is required to detect this relationship (52). Bioinformatic analysis based on conservation of key residues in the cytotoxic domain of colicin M reveals a growing family of colicin M like proteins (Fig. 7). The previously discussed pectocin M1 and M2 have clearly evolved through diversifying recombination, however secondary structure prediction of other homologues suggests they also possess an un-structured N-terminus, followed by a receptor binding domain that like colicin M and syringacin M consists of multiple helices (53). In colicin M homologues from Burkholderia spp., the putative translocation domain is preceded by a predicted signal sequence, which may represent a means of bacteriocin export in this species (54).

While Ca$^{2+}$ was identified as the metal ion bound in the active site of syringacin M, both Mg$^{2+}$ and Ca$^{2+}$ have been shown to be relatively abundant in the periplasm of Gram-negative bacteria, at least under the conditions tested, with Ca$^{2+}$ ions concentrated therein relative to the cytoplasm or extra-cellular environment (55,56). As the ionic composition of the periplasm, where colicin M-like bacteriocins are active, is highly dependent on external conditions, flexibility in the use of metal ion cofactors could be advantageous and so this class of bacteriocins may have evolved to utilize both Mg$^{2+}$ and Ca$^{2+}$ as a cofactor. Interestingly, published work on colicin M and our observations with syringacin M, show that cytotoxicity in liquid culture is dependent on the presence of Ca$^{2+}$, suggesting that at least under some conditions Ca$^{2+}$ acts as the metal cofactor (57) (unpublished data).

The mechanism of lipid II cleavage by colicin M-like bacteriocins is currently unknown and although our data do not provide a clear picture of this mechanism some speculation is warranted. From mutational and structural studies of colicin M, two conserved aspartic acid residues D226 (D232), and D229 (D235), a histidine H235 (H241) and an arginine R236 (R242) residue, have been identified as key catalytic residues (40,41). In the structure of colicin M the D226 side chain lies some distance from the other catalytic residues, which led to the proposal that the active site of colicin M may be highly elongated as a consequence of the size of the large lipid II substrate. However, in our
structure of syringacin M, the D232 side chain coordinates an active site Ca\(^{2+}\) ion and as a consequence is brought close to other catalytic side chains (Fig. 5). Additionally, the relative temperature factors of Colicin M D226 are high compared to surrounding residues, while the opposite is true for the Ca\(^{2+}\) ligated D232 side chain of syringacin M. This suggests the conformational flexibility required for metal binding by this residue exists in colicin M. The putative active site of syringacin M also differs because of a break in helix 8-9 repositions R242 so that compared to the colicin M structure it is further from other catalytically important residues. These differences create a much more open pocket in syringacin M that is surrounded by key catalytic residues and may indicate that this class of enzyme possesses a highly flexible active site that undergoes conformational changes upon substrate and metal binding (Fig. 5).

Cleavage of lipid II by colicin M yields undecaprenol\(^{10}\) and 1-pyrophospho-MurNAc (pentapeptide)-GlcNAc. In most phosphatases, catalysis occurs in two steps with an initial attack by a nucleophilic side chain leading to the formation of a covalent enzyme-substrate intermediate and a subsequent phosphoryl-group transfer to a water molecule. The role of the metal cofactor is generally to bind the phosphate group of the substrate, correctly orientating the phosphorus atom for nucleophilic attack. In the phosphatases of the large haloacid dehydrogenase\(^{11}\) family attack by the Asp nucleophile leads to the formation of an aspartylphosphate intermediate, which is hydrolyzed by nucleophilic attack of a water molecule (58,59). If a similar mechanism were to operate in the colicin M-like bacteriocins, D235 which has been shown to be essential for catalytic activity, would be a likely candidate for the attacking nucleophile (41). The essential role of D232 in Ca\(^{2+}\) binding and the bidentate coordination of this metal ion likely preclude it from acting as a nucleophile. Also by analogy with the mechanism of the HAD phosphatases H241 may act as a general acid and A235 as a general acid/base. However, a clear view of the mechanism of lipid II cleavage will require structures of colicin M-like bacteriocins in complex with substrate or substrate analogues.

\(^{10}\) C\(_{55}\)-OH
\(^{11}\) HAD
References


The crystal structure lipid II-degrading bacteriocin syringacin M


Acknowledgements: We thank Diamond Light Source for access to beam-lines I03 and I04 (proposal number MX6638), Dr Nick Tucker (Strathclyde) for P. aeruginosa strains and Philip Younger for creation and characterization of Syringacin M D232A. The authors would also like to thank members of the Walker group for critical reading of the manuscript.

FIGURE LEGENDS

FIGURE 1. Identification of bacteriocin produced by Pss. LMG1247. A, Native-PAGE gel (8%) of DEAE anion exchange purification fraction from concentrated culture medium of Pss. LMG1247, coomassie stained and overlayed with soft agar seeded with test strain Psl. LMG 5456. Band corresponding to zone of growth clearing was excised and indentified using tandem mass
spectrometry. B, Trypsin derived fragments from band from A, matching sequence from Pss. 642 ZP_07266212 identified using the MASCOT server.

FIGURE 2. Purification and characterization of wild-type syringacin M and deletion mutants. A, susceptibility of P. syringae test strains to syringacin M, B, 15% v/v SDS-PAGE gel showing purified syringacin M (SyrM) and mutant proteins generated in this study, C, Light field image of five-fold serial dilutions (37.5 μM to 1 nM, MIC = 60 nM) of syringacin M spotted onto a soft agar overlay of susceptible strain P. syringae pv. lachrymans LMG 5456, D, Dark field image of interference of killing activity of syringacin M on soft agar overlay by adjacent spotting of inactive mutants (proteins spotted at 100 μM).

FIGURE 3. Sequence and structure of syringacin M. A, Full length structure of syringacin M showing AA 38-276 as a surface model and the unstructured N-terminus (AA 2-37) as a ribbon model (orange). The positive electron density from the Fo-Fc omit map calculated after omitting the N-terminus residues is shown as a chicken wire model at the 3 σ level. B, cartoon representation of high resolution structure of syringacin M (AA 39-276) showing receptor binding domain (green), cytotoxic domain (red) and Ca\(^{2+}\) ion (yellow). C, B-factor putty model of full length (left panel) and truncated (right panel) structures of syringacin M, cool colors/thin ribbon represents lower relative b-factors, to hot colors/fat ribbon represents higher relative B-factors. D, sequence alignment of syringacin M and colicin M, showing conserved (dark blue) and similar (light blue) residues, secondary structure (sheets and helices) and location of truncation for -10 and -20 deletion mutants of syringacin M generated in this study.

FIGURE 4. The receptor binding and cytotoxic domains of syringacin M and colicin M show structural homology. A, Structural alignment of syringacin M 57-276 (light green 57-126, green 127-276) and colicin M 48-271 (blue 48-120, sky blue 122-271) based on the catalytic domain beta barrel, residues 180-190, 204-216, 220-229, 269-275 (minus 6 for colicin M residue numbers) (RMSD 1.765 Å) B, Subsection of alignment from A, syringacin M (145-276) and colicin M (140-271) showing overlay of conserved catalytic region. C, Structural alignment as A, based on receptor binding domain helices 2-4, residues 58-70 (53-65), 73-92 (73-92), 95-101 (97-103) (colicin M residues in brackets) (RMSD 2.051 Å). D, Subsection of alignment from C, syringacin M (57-126) and colicin M (48-120) showing overlay of conserved core helices in wall-eyed stereo. E, Alternative orientation of alignment from D showing fit of two main helices of receptor binding domain. Terminal residues labeled for syringacin M (black) and colicin M (red).

FIGURE 5. Comparison of the syringacin M and colicin M active sites. A, superimposition of key conserved active site residues of syringacin M (green) and colicin M (blue) in stick representation (labels for colicin M = red, syringacin M = black) and Ca\(^{2+}\) ion from syringacin M (yellow). B, Coordination of Ca\(^{2+}\) ion in the active site of syringacin M, residues and the ethylene glycol (EDO) involved in coordination shown as a stick model, 2Fo-Fc electron density map shown as chicken wire model at 1.7 σ. C, Surface representation of syringacin M active site showing conserved residues essential (red) and important (orange) for activity in colicin M. D, Surface representation of colicin M active site showing conserved residues essential (red) and important (orange) for cytotoxicity.

FIGURE 6. Dependence of syringacin M activity on divalent metal ions. A, Thin layer chromatography (TLC) visualization of reaction of syringacin M (wildtype top panel or D232A mutant bottom panel) and lipid II in the presence and absence of various divalent cations, all reactions identical except for labeled component (buffer = no additional reagents added). B, TLC visualization of time course reaction of syringacin M and lipid II in the presence of CaCl\(_2\) or MgCl\(_2\).

FIGURE 7. Molecular phylogeny of catalytic A, and receptor binding and translocation domains B, of colicin M homologues. Tree constructed using nearest neighbour joining method, values at nodes are percent bootstrap values (1000 rounds) >55%. Tip labels correspond to the following
FIGURE 8. Graphical representation of mechanisms of diversification of bacteriocins. Diversifying recombination for novel translocation (T), receptor binding (R) or cytotoxic domain (C) functions has been widely observed in colicin-like bacteriocins and diversifying selection is the mechanism through which novel cytotoxic-immunity protein (I) specificities are generated in the DNase and rRNase type colicins (16),(13). The structural similarity between the receptor binding domains of colicin M and syringacin M suggest diversifying selection may also be important in the generation of novel receptor binding specificities.

TABLE 1. Data collection and refinement statistics
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<td>( P 6_2 )</td>
<td>( P 6_2 )</td>
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<sup>a</sup> Values in parentheses refer to the highest resolution shell.

<sup>b</sup> \( R_{pim} = \sum_{hkl} \left[ \frac{1}{(N - 1)} \right]^{1/2} \sum_{i} \frac{I_i(hkl)}{I(hkl)} - \frac{<I(hkl)>}{\sum_{hkl} \sum_{i} I_i(hkl)} \)

<sup>c</sup> Estimated standard uncertainty, first calculated using the method of Cruickshank (31) and second based on maximum likelihood as implemented by REFMAC (30)

<sup>d</sup> Percentages of residues in favoured/allowed regions calculated by the program RAMPAGE (27)
## Figure 1

### A

![Image of Coomassie Strain Gel and Soft Agar Overlay of Gel with an Excised Band]

### B

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<th>Mr (calc)</th>
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<tr>
<td><em>P. syringae</em></td>
<td>10</td>
<td>2</td>
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**Figure 2**

**A**

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**B**

- SyrM
- SyrM D232A
- SyrM -10
- SyrM -20

**C**

**D**

- D232A -10
- D232A -20
Figure 3

Cytotoxic Domain
Receptor Binding Domain
Translocation Domain

α1
α2
α3
α4
α5
α6
α7
α8
α9

β1
β2
β3
β4
β5
β6
β7
β8
β9

Met39
Pro2
Arg25
Ser34

N-term
C-term
Cytotoxic Domain
Receptor Binding Domain
Translocation Domain

Ca²⁺

Syringacin M
Colicin M

Protein Sequence:
Syringacin M
Colicin M

Protein Sequence:
Syringacin M
Colicin M

Protein Sequence:
Syringacin M
Colicin M

Protein Sequence:
Syringacin M
Colicin M

Protein Sequence:
Syringacin M
Colicin M
Figure 6

A

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<td>20 mM CaCl$_2$</td>
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<tr>
<td>20 mM MgCl$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mM MnCl$_2$</td>
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<td>20 mM ZnCl$_2$</td>
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Lipid II reaction results:

B

Reaction time (minutes)

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<td>20 mM MgCl$_2$</td>
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A  Catalytic Domains

B  Receptor Binding and Translocation Domains
Ancestral Bacteriocin

Diversifying Recombination for novel T/R or C

Diversifying Selection for novel immunity protein specificity

Diversifying Selection for Novel Receptor Binding Function

X = amino acid substitution
The crystal structure of the lipid II-degrading bacteriocin syringacin M suggests unexpected evolutionary relationships between colicin M-like bacteriocins
Rhys Grinter, Aleksander W. Roszak, Richard J. Cogdell, Joel J. Milner and Daniel Walker

*J. Biol. Chem.* *published online September 20, 2012*

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