A New Protein Antimicrobial Isolated from the Genome of an Uncharacterized Soil Streptomycete

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Abstract

The genomes of uncultivated bacteria represent a fertile reservoir of molecular diversity. *Streptomyces*, a medically and industrially important genus of soil bacteria, produce many useful antibiotics and enzymes, contain large prokaryotic genomes, and produce secondary metabolites with exceptional functionality. Among these, protein antimicrobials have received attention as natural food preservatives, agricultural biocontrol agents, and pharmaceutical alternatives to antibiotics.

In this study, the previously uncharacterized *Streptomyces* sp. isolate 212 bacterium was cultivated from rich organic soil samples collected from a woodland bluff environment in Alabama. Production of protein antimicrobials from the isolate was evaluated using a standard line inoculum assay and zymogram analyses of concentrated growth supernatant. Whole genome extracts from the isolate were analyzed by *de novo* GS/FLX 454 pyrosequencing, and putative genes within the draft quality genome were annotated. The recombinant gene coding for the Mitrecin A protein was identified, synthesized, and heterologously over-expressed in *E. coli*. Mitrecin A was purified by affinity and size exclusion chromatography. Well-diffusion and dye-release assays were used to determine the tolerances of purified Mitrecin A activity against ranges of temperature, pH, and salinity.

Streptomyces sp. isolate 212, a novel soil bacterium, produces multiple species of bacteriolytic enzymes as determined by zymogram analyses of growth supernatant. The genome of the streptomycete, estimated by pyrosequencing, is approximately 10 Mbp with a GC content of 68%. Annotation of the draft quality genome identified a suite of putative bacteriolytic genes, including the gene for Mitrecin A. Synthesis and over-expression of the gene followed by multi-step purification of the gene product resulted in a 14.3 kDa cationic protein with bacteriolytic activity against the medically important genera of *Salmonella*, *Vibrio*, and *Yersinia*.

Introduction

Bacteriocin-like inhibitory substances (BLIS) are ribosomally synthesized proteins produced by bacteria that kill or inhibit other microorganisms in response to resource competition with surrounding microorganisms (13). Once thought to target only bacterial species closely related to the producing organism (13), BLIS were initially described as tailored enzymes displaying narrow activity ranges. With the characterization of several BLIS exhibiting a broad spectrum of activity (3, 4, 7), the definition has been expanded beyond closely related species (6). The role of BLIS in the microbial community has been suggested as one of mediator of population dynamics among soil microbial species providing a

competitive advantage to the producer strain over the nonproducing sensitive strains in stressful, nutrient-poor environments (10).

As more BLIS have been identified and characterized, the varied structural and functional diversity of these enzymes have become apparent. Most BLIS are cationic and amphiphilic, which allow them to target and permeabilize susceptible bacterial cell membranes as the preferred mode of killing (7). While bacteriocin activities from *Streptomyces* species have been previously reported (14, 15), relatively few bacteriocins from this metabolically rich genus have been characterized. The large genomes found in the representatives of the genus contain molecular diversity and expanded secondary metabolic capabilities as displayed by their production of a variety of antibiotic compounds (2, 9). The genetic reservoir of the streptomycete genome represents a wealth of under explored biochemical diversity. The rapid advances in pyrosequencing technologies and *de novo* bacterial genome assembly and annotation techniques allow access to these enzymes once hidden within the genome.

BLIS are considered an emergent source of alternative antimicrobial agents as small molecule antibiotic resistant microorganisms continue to become a more prevalent health concern. However, they have found utility in other applications beyond medicine including natural food preservatives, biohazard decontamination, pathogen control for agricultural crops, and dietary supplements for humans and agricultural livestock health (1, 5, 8, 11, 12, 15).

Materials and Methods

Bacterial strains, media, and bacteriocin production. *Streptomyces* sp. strain 212 was isolated on environmental extract medium from a soil sample of Rainbow Bluff, a woodland bluff of Lynn, Alabama. The strain was one of 45 isolates demonstrating lytic activity against heat-killed bacterial substrate. Of the lytic enzyme producers, strain 212 resembled the genus *Streptomyces* in colony morphology, carbohydrate utilization, and 16S rRNA gene sequence. The total bacteriocin activity of strain 212 was measured against both Gram-negative and Gram-positive bacteria using the line inoculum assay and zymogram (renaturing SDS-PAGE) analyses.

Phylogenetic analysis of *Streptomyces* sp. strain 212 16S rRNA sequence. Phylogenetic relatedness of Strain 212 to other closely related bacteria was assessed using partial 16S rRNA gene sequences identified with BLASTn. The reference sequences and strain 212 sequence were aligned in BioEdit Sequence Alignment Editor using CLUSTAL W. The neighbor-joining algorithm of PAUP* version 4.0 was used to infer the phylogenetic relatedness of the sequences. Tree topologies were calculated by bootstrap analyses based on 1000 resamplings.

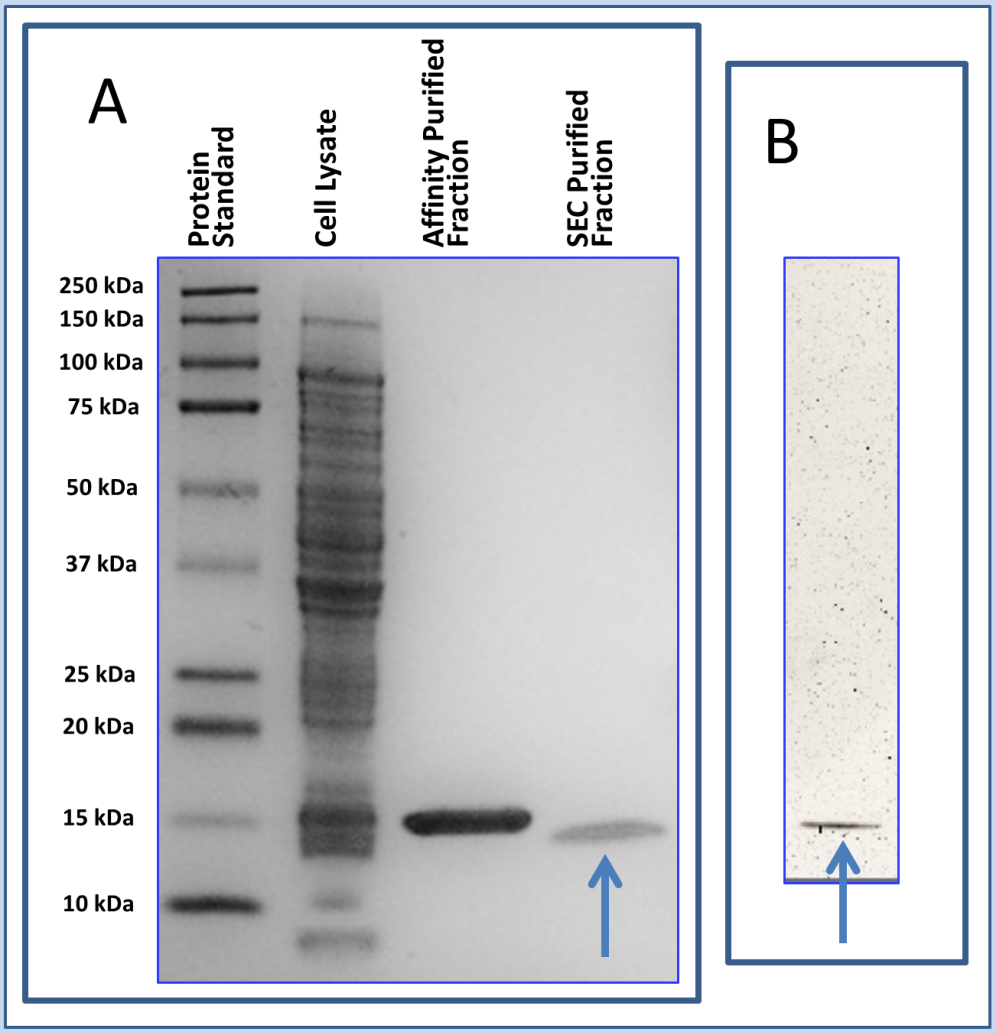
Genome sequencing and annotation. Genomic DNA from Strain 212 was subjected to *de novo* genome sequencing and assembly at The Institute for Genome Sciences (IGS) Genomics Resource Center at the University of Maryland using the 454 GS/FLX pyrosequencing platform and GS *de novo* sequence assembly software, Newbler. Genome annotation was performed by the IGS Annotation Engine. Open reading frames (ORFs) were identified by Glimmer 3 algorithm, while tRNA and rRNA genes were detected by tRNAscan-SE and RNAmmer, respectively.

Gene synthesis, expression, and Mitrecin A purification. The gene for Mitrecin A fused with a C-terminal 6-histidine tag sequence was synthesized, expressed, and partially purified by one-step immobilized metal ion affinity chromatography by GenScript, Corp. to a purity of 90% as estimated using the 2100 Bioanalyzer platform. Partially purified protein was stored at -80°C in 50 mM Tris (pH 9.0), 0.5 mM L-arginine, and 10% glycerol. Mitrecin A was further purified using an HPLC system fitted with a Superdex 75 size exclusion column. Mitrecin A was determined to be isolated from contaminates using 12% SDS-PAGE, western blot, and Bioanalyzer analyses. The N-terminus of the purified enzyme was sequenced using Edman degradation at the Iowa State University Protein Facility.

Effects of temperature, salinity, and pH on Mitrecin A activity. For each stressor, residual enzyme activity was assessed using a modified version of the quantitative dye-release assay described by Zhou *et al.* (16) using *Y. pseudotuberculosis* as cell substrate. Thermal stability of Mitrecin A was tested after challenge against various temperatures (4 – 95°C) for 30 min. Stability of the enzyme in various pH and saline conditions was assessed in dye-release assays (16) over 16h incubations at 37°C.

Functional assays of Mitrecin A activity. The spectrum of bacteriolytic activity against Gram-positive and Gram-negative bacteria was assessed using slide diffusion assays. The residual viability of the *Y. pseudotuberculosis* indicator organism in the presence of various concentrations of Mitrecin A was also assayed by serial dilution and colony forming unit assays after 16h incubation at 37°C.

Results

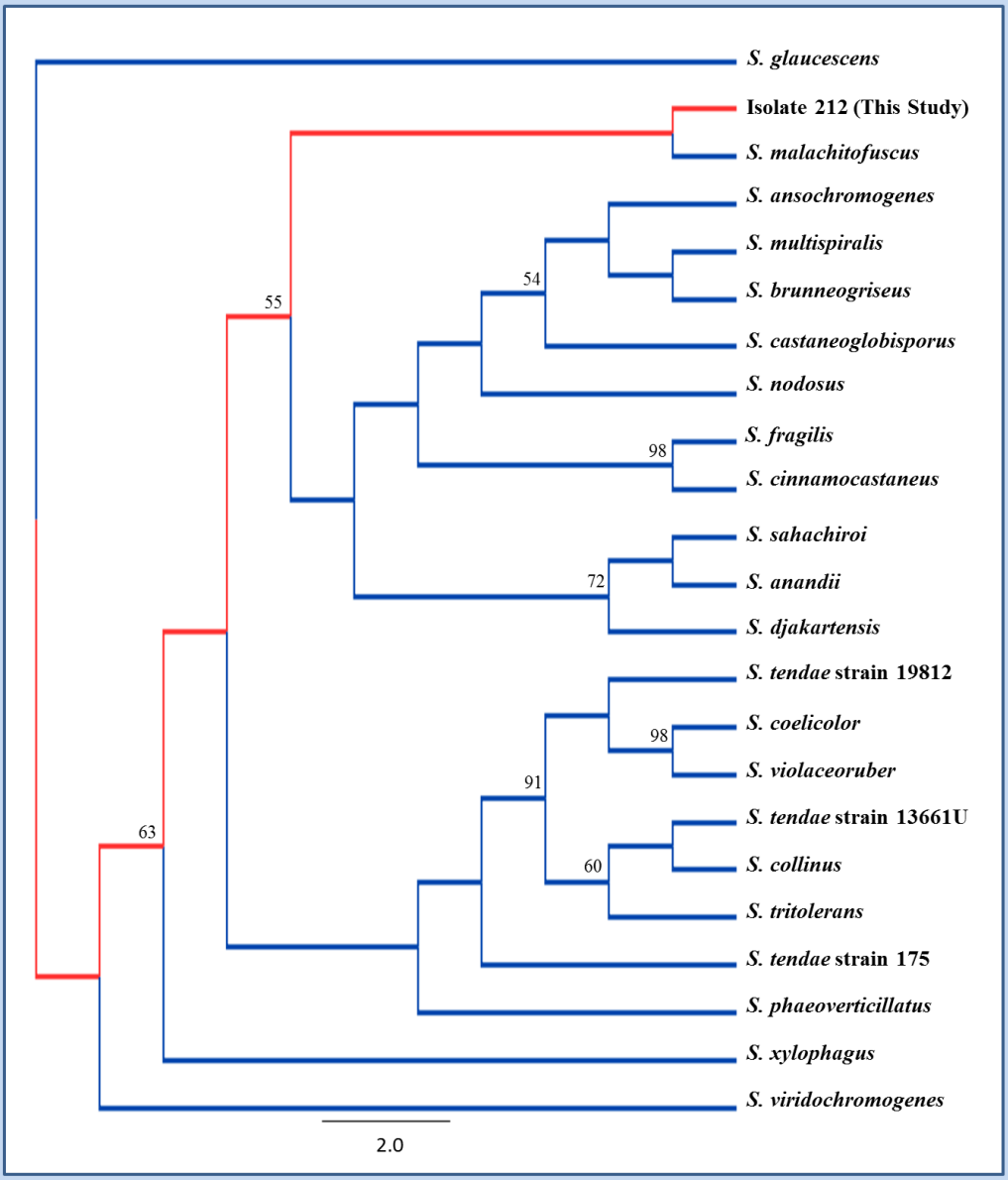


A. Stepwise purification of the Mitrecin A 6-histidine fusion protein as shown in SDS-PAGE separation. Lane labeled Protein Standard contains molecular mass standards. Lane labeled Cell Lysate contains whole cell lysate of the heterologous expression host *E. coli* BL21 (DE3)pLysS; lane labeled Affinity Purified Fraction contains metal affinity purified Mitrecin A fusion protein; and lane labeled SEC Purified Fraction contains Mitrecin A as purified by size exclusion chromatography.

B. Western Blot Analysis of purified Mitrecin A 6-histidine fusion protein.

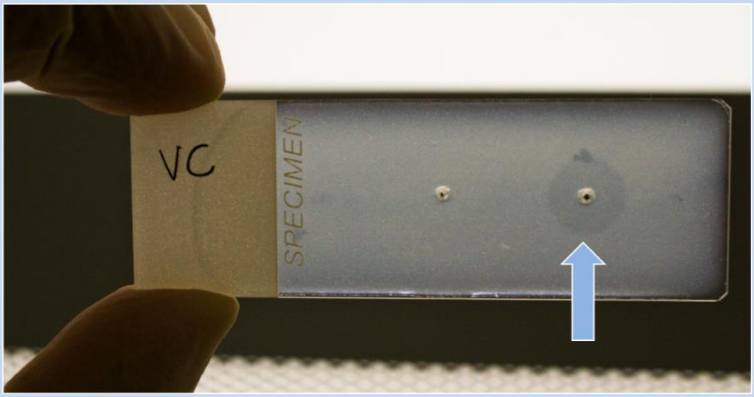
Arrows indicate purified Mitrecin A.

Phylogenetic Relatedness of *Streptomyces* sp. strain 212 to Other *Streptomyces*



Neighbor-joining cladogram based on nearly complete 16S rRNA gene sequences showing the phylogenetic relationship between *Streptomyces* sp. strain 212 and related *Streptomyces* species. Percentages of bootstrap support (>50%) based on 1000 replications are shown at the nodes.

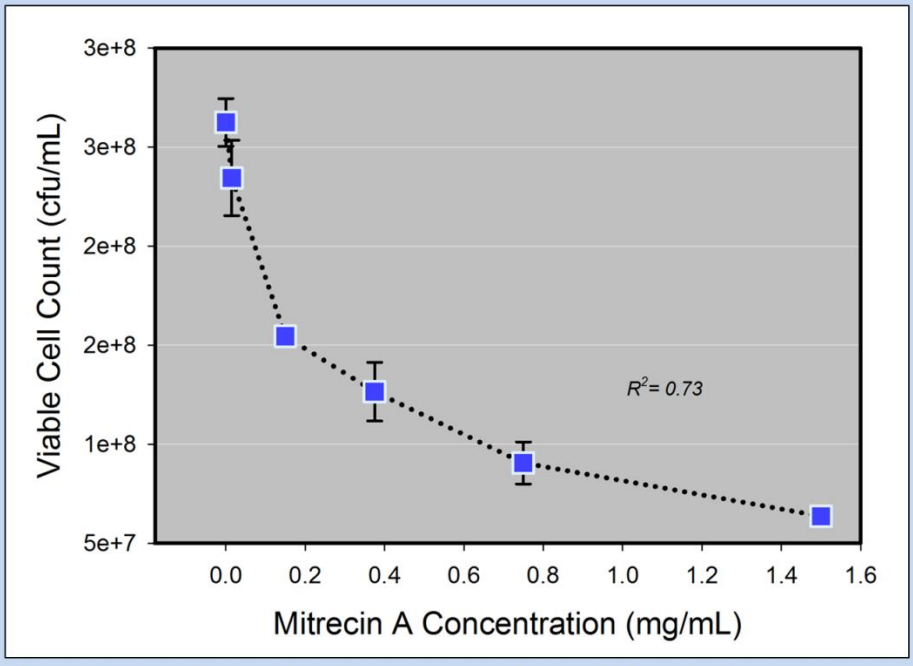
Mitrecin A Functional Assays



Slide diffusion assay measuring the bacteriolytic effects of Mitrecin A on indicator organisms. Right well contains 1 µg of Mitrecin A suspended in 10 µl Tris-HCl 50mM NaCl buffer. Left well contains buffer with no enzyme. The slide was custom constructed with an overlay of agarose containing *Vibrio cholerae* cells. Slides were incubated at 37°C.

Bacteriolytic Activity of Mitrecin A

against *Y. pseudotuberculosis*



Bacteriolytic effects of various concentrations of Mitrecin A against *Yersinia pseudotuberculosis*. Viable cell count assays were conducted after 16h incubation at 37°C in the presence of enzyme.

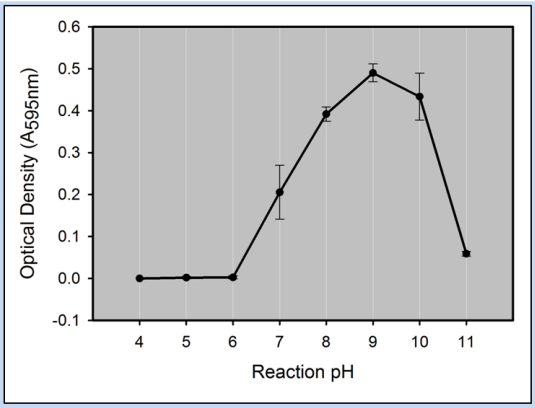
Inhibitory Spectrum of Mitrecin A

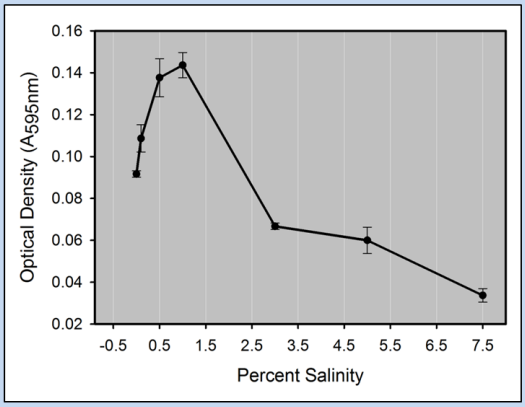
Gram-Negative Bacteria	Lysis	Gram-Positive Bacteria	Lysis
Francisella philomiragia	Negative	*Bacillus cereus*	Negative
Salmonella enterica	Positive	*Bacillus thuringiensis*	Negative
Vibrio cholerae	Positive	*Bacillus subtilis*	Negative
Yersinia pseudotuberculosis	Positive	*Staphylococcus aureus*	Negative

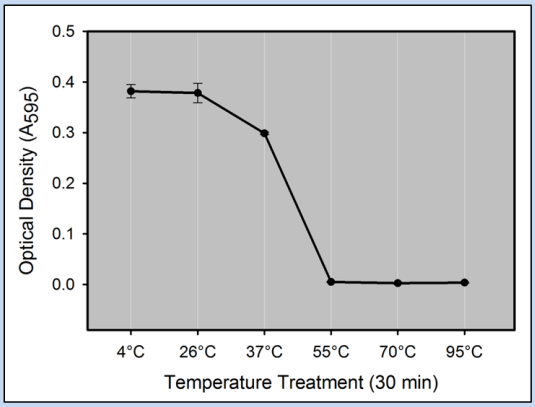
Inhibitory spectrum of Mitrecin A as measured in slide diffusion assays. Heat-killed cultures of the respective Gram-negative and Gram-positive bacteria were incorporated into custom agarose slide diffusion assays for challenge against 1µg of Mitrecin A.

Characteristics of Mitrecin A

Factors affecting Mitrecin A activity. Using a quantitative dye-release assay, residual bacteriolytic activity of Mitrecin A (1µg) was measured at various pH conditions (right), various saline concentrations (below), and after various 30-minute temperature treatments (below, right).







Conclusions

- The genome of the *Streptomyces* sp. strain 212, estimated by pyrosequencing, is approximately 10 Mbp with a GC content of 68%. Annotation of the genome identified a suite of putative bacteriolytic genes, including the gene for Mitrecin A.
- Mitrecin A is a novel lytic enzyme, 14.3 kDa in size, with activity against select Gram-negative bacteria of medical importance – *Salmonella*, *Vibrio*, and *Yersinia*.
- A decrease in viable cell count of *Yersinia pseudotuberculosis* after incubated exposure indicates that the enzyme has bacteriocidal activity.
- Mitrecin A displays the greatest bacteriolytic activity at elevated pH values ranging from 7 to 11, in saline conditions of 1.5%, and at ambient temperatures.
- Comparison of the Mitrecin A gene sequence to other known genes using BLASTn indicates similarity to bacteriophage endolysin genes.
- The Mitrecin A-histidine fusion protein retains activity when expressed and purified from a heterologous host.
- *Streptomyces* sp. strain 212 demonstrates characteristics of a unique species of *Streptomyces* as indicated by 16S rRNA gene relatedness as well as morphological and chemotaxonomic data, which are not shown.

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