Bacteriocin Gene Isolation and Physical Characterization in *E. faecalis*

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Abstract

Enterococci are a part of the normal flora of the human intestine and can reach concentrations of up to 10^8 CFU/gram in feces. As an opportunistic pathogen *enterococci* can cause nosocomial infections such as urinary tract infections, bacteremia, and infective endocarditis. Vancomycin (Vm) was one of the antibiotics that exhibited reliable inhibitory effects on *Enterococcus faecalis* until 1989 when the first vancomycin - resistant *Enterococcus* (VRE) strain was isolated. The strain of *E. faecalis* used in this project is a VRE clinical isolate which contains a plasmid that encodes production of a bacteriocin. Bacteriocins are toxic proteins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. As the food industry and medical institutions face a growing problem of resistant strains of pathogenic bacteria—ones against which antibiotics have become less or even completely ineffective--bacteriocins provide a promising alternative to controlling microbial growth. Some have already found use in the food industry, and this project aims to provide support for their use as a replacement to antibiotics. In this project we are using molecular cloning techniques to isolate the bacteriocin gene from plasmid pAM369 in *E. faecalis* as well as physically characterize the bacteriocin protein

INTRODUCTION

Enterococci are a part of the normal flora of the human intestine and can reach concentrations of up to 10^8 CFU/gram in feces. As an opportunistic pathogen *enterococci* can cause nosocomial infections such as tract infections, bacteremia, and infective endocarditis. Vancomycin (Vm) was one of the antibiotics that exhibited reliable inhibitory effects on *Enterococcus faecalis* until 1989 when the first vancomycin - resistant *Enterococcus* (VRE) strain was isolated. The number of VRE isolates has increased steadily since then causing major problems in the treatment of nosocomial infections. In the United States, VRE isolates carry primarily the VanA or VanB type of resistance. A contributing factor in the rise in VRE isolates may relate to the fact that *enterococci* posses elaborate mechanisms for genetic exchange via conjugation. Conjugative plasmids and conjugative transposons, both of which contribute to the dissemination of antibiotic resistance are ubiquitous in these organisms.

S. aureus resistant to high levels of Vm have been reported recently and under laboratory conditions, it has been reported that Vm^r can be transferred from *E. faecalis* to *S. aureus*. It is noteworthy that the *E. faecalis* plasmid pAM373 encodes a response to the pheromone cAM373 that is also produced by *S. aureus* although this plasmid does not appear to replicate in staphylococci.

Herein, we report the characterization of a strain of *E. faecalis* which contains two conjugative resistance plasmids (pAM368 and pAM369), one of which (pAM368) encodes Vm^r ,and the other production of bacteriocin. Both plasmids respond to sex-pheromones and transfer to *E. faecalis*.

MATERIALS AND METHODS

Plasmid DNA Isolation and Characterization: Plasmid DNA was prepared using the Qantum Plasmid Midiprep Kit as described by the manufacturer(Bio-Rad Laboratories, CA). Restriction endonucleases were used to digest plasmid DNA as directed by the manufacturer (Promega, Madison, WI). The digests were analyzed by agarose gel electrophoresis [(1.0%) in TAE buffer (0.04M Tris-acetate, 0.001M EDTA (pH=8)].

Assay for Bacteriocin Activity: Bacteriocin-producing cells were spotted onto a lawn of non-producing cells to assay for bacteriocin activity. A zone of inhibition indicated bacteriocin production (Fig.3).

Purification of Bacteriocin: An overnight culture of bacteriocin producing *Enterococcus faecalis,* grown in Todd Hewitt Broth (THB) was centrifuged (10,000Xg for 30 min). The supernatant was passed through a Millipore filter (0.22mm). The resulting cell-free fractions were assayed for bacteriocin activity as described (Fig. 3). Next the cell free crude extract was concentrated using ammonium sulfate precipitation and dialysis against 50 mM sodium phosphate. This was then run through a CM sepharose column and varying concentrations of sodium chloride were used to elute the extract. Two fractions showed activity and ammonium sulfate concentration was performed. This was then run on a size exclusion column on the HPLC for further purification.

Determination of MW: The cell-free supernatant was spun in a Centricon Microconcentrator as described by the manufacturer (Amicon). First through a 100,000Da then a 50,000Da microconcentrator. The resulting fractions (>100KDa,100KDa - 50KDa and <50KDa) were assayed for bacteriocin activity. Fractions with bacteriocin activity were run on a SDS-PAGE gel (12.5% acrylamide) (Fig. 2).

Bacteriocin Activity Under Various Environmental Conditions: *E. faecalis* SAS58 cell-free extracts were subjected to various temperatures, pH and enzymes for 60 min. After the 60 min incubation the extracts were normalized to the original pH (pH=7) and temperature (room temperature) before they were used for the bacteriocin assay (Table 1).

Molecular Cloning: We used the pBlueScript(pBS) plasmid as a vector into which partially digested fragments of the *E.faecalis* plasmid pAM 369 were inserted. The plasmid pAM369 was partially digested with *Bam*HI and *Hind*III to produce fragments of appropriate size. The fragments were then ligated into the pBS vector to isolate the bacteriocin gene found on pAM 369. The recombinant plasmids were inserted into *E.coli*(1) (Fig. 5). Blue-White Screening was performed by plating the transformed cells on to Ampicillin and X-Gal treated plates (Fig. 4). The white colonies were selected and the recombinant plasmids were extracted from the transformed *E.coli* cells. Extracted plasmids were digested with *Hind*III, *Bam*HI, or both *Hind*III and *Bam*HI to isolate the pAM 369 inserts. Gel electrophoresis was performed, on the digested plasmids ,using a 1.5% agarose gel, to determine if the bacteriocin gene was isolated (Fig. 6).

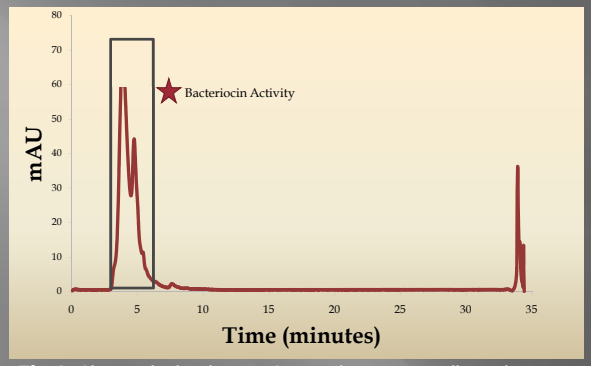

Fig 1. Size exclusion by HPLC. Fractions were collected at each peak seen. Activity of each fraction was tested and the first elution showed activity. Further analysis using mass spectrometry will ensue.

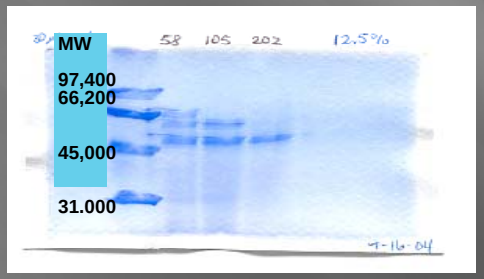

Fig. 2. SDS-PAGE gel

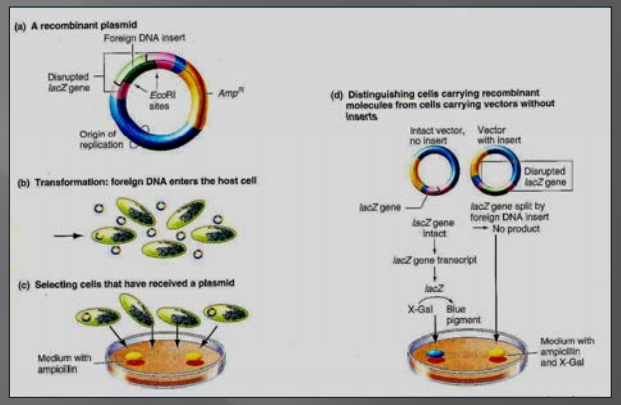

Fig 5. Molecular cloning procedure.

Environmental condition/treatment	Zone of Inhibition on *E. faecalis* FA2-2 lawn
pH = 3	-
pH = 4	-
pH = 5	-
pH = 6	+
pH = 8	+
pH = 9	+
pH = 10	+
pH = 11	+
Temperature = 4˚C	+
Temperature = 20˚C	+
Temperature = 30˚C	+
Temperature = 37˚C	+
Temperature = 40˚C	-
Temperature = 50˚C	-
Temperature = 60˚C	-
RNAse A	+
Proteinase K	-

[a] Cell free extracts containing bacteriocin were subjected to various treatments then tested against a lawn of *E. faecalis* FA2-2.

Table 1. Environmental effects on bacteriocin activity

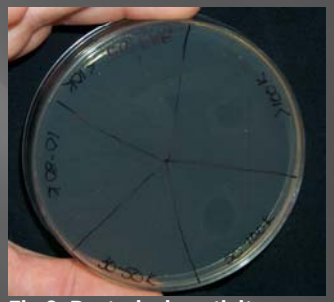

Fig 3. Bacteriocin activity assay

Fig 4. Blue-White screening

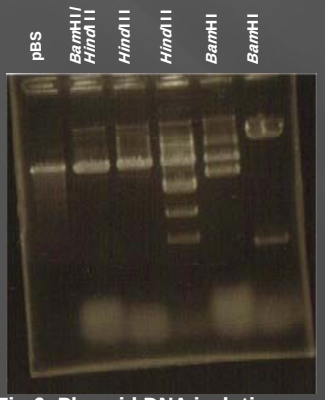

Fig 6. Plasmid DNA isolation and digest with restriction enzymes.

Results

Purification of Bacteriocin and MW Determination: Cell free fractions containing protein between 100KDa - 50KDa exhibited bacteriocin activity (Fig. 3). The >50KDa MW fraction collected from the microconcentrator showed bacteriocin activity while fractions <50KDa MW fraction had no bacteriocin activity(Fig. 2). SDS-PAGE analysis demonstrates that *E. faecalis* SAS58 contains an additional band at 66KDa that is not shared by either SAS200 or SAS105 strains (both are non-producers of bacteriocin) (Fig. 2).

Analysis of Bacteriocin Activity Under Different Conditions: Analysis of bacteriocin activity demonstrated that the bacteriocin was inactivated at temperatures above 40˚C, has a pH spectrum between pH 6 and pH11 and was inhibited by proteinase K but not by RNAse (Table 1).

Bacteriocin Purification: CM sepharose column elution fractions with activity were run on HPLC size exclusion. Two peaks with poor resolution at about 3.2-4.5 minutes show activity (Fig 1). Further purification with anion exchange may be possible.

Molecular Cloning: Many *E.coli* were successfully transformed with recombinant pBS plasmids (Fig. 4). Through gel electrophoresis it was determined that when the recombinant plasmids were cut using *Bam*HI, the fragments of pAM 369 that were created are of appropriate size to allow for isolation of the bacteriocin gene. When the recombinant plasmids were cut using*Hind*III, there were many fragments of smaller size created. The *Bam*HI and *Hind*III sites on the pAM369 plasmid are far enough apart to cut around the bacteriocin gene. When the recombinant plasmid is cut with both *Bam*HI and *Hind*III, the fragments created may be to small to contain the whole bacteriocin gene (Fig. 6).

CONCLUSIONS

- The crude extract has been purified to either two proteins or two isoforms of the bacteriocin protein. Once the protein is purified further by anion exchange, mass spectrometry can be performed.
- Once the bacteriocin gene is isolated it will be placed into plasmid pAM 401, which lacks a bacteriocin gene, and inserted into E.faecalis to amplify the gene and assay for bacteriocin activity of the cells.
- The bacteriocin's molecular weight is about 66 KDa (Fig 2). The bacteriocin was inactivated at temperatures above 40˚C and has a pH spectrum between pH 6 and pH 11 (Table 1).
- *Bam*HI and*Hind*III provide fragments of the correct size for ligation into the pBS vector, but the *E.faecalis* bacteriocin gene has not yet been isolated (Fig 6). Once isolated, reverse genetics techniques can be used to sequence the bacteriocin gene.

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Resources:

1. Hartwell, L., Hood, L., Goldberg, M. (2008). Genetics, Third Edition. McGraw-Hill, New York.