

Discovery of novel microorganisms involved in ergot alkaloid detoxification: An approach

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Abstract

Fescue toxicosis and ergot alkaloids are estimated by the United States Department of Agriculture (USDA) to cost the U.S. ranchers \$860 million per year. The endophyte fungi *Neotyphodium coenophialum* is present in tall fescue (*Festuca arundinacea*). This fungus produces toxic alkaloids (principally ergovaline) that cause deleterious effects in cattle and other livestock when infected grasses are eaten. Previous studies in sheep and cattle have yielded no culturable microbes able to detoxify the ergot molecules, leading to the search for novel sources of anaerobic organisms capable of degrading ergot alkaloids. Anaerobic incubation of earthworm (*Eisenia fetida*) homogenate with pure ergovaline has exhibited a 60% decrease in ergovaline concentration. Digestive tracks were dissected from worms obtained from an endophyte-fed digester. Cloning and partial sequencing of bacterial 16S rDNA obtained from these samples revealed a diverse microbiota, including bacteria sequences which classified into the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Planctomycetes*. Future work is focused on refining our understanding of the contribution towards possible ergot alkaloids detoxification made by the bacteria of the worm-gut microbiota.

Keywords: ergovaline, microbial degradation, endophyte, tall fescue, perennial ryegrass, *Eisenia fetida*

Introduction

Ergotism caused by the consumption of ergot alkaloids has been known for many centuries. Specific chemical composition and concentrations of ergot alkaloids vary with the symbiotic combination between the grass and the fungus. In tall fescue (*Festuca arundinacea*) infected with *Neotyphodium coenophialum* (*N. coenophialum*), the fungus forms a symbiotic relation and produces ergovaline as its predominant ergot alkaloid (Arechavaleta *et al.* 1992). *Neotyphodium lolii* (*N. lolii*) associates with perennial ryegrass (*Lolium perenne*) and also produces ergovaline.

Tall fescue grows on about 14 million hectares in the United States and is the most widely grown pasture grass in humid areas of the southeastern and to a lesser extent in the northwestern United States (Buckner *et al.* 1979). Unfortunately, the alkaloids produced by the endophyte cause deleterious effects in cattle and other herbivore species when endophyte-infected grasses are grazed or fed as hay. In mammals, ergot alkaloids affect the central and sympathetic nervous systems, as well as immune and reproductive systems, resulting in symptoms such as muscle contractions, changes in blood pressure, lowered immune response, reduced lactation and reproductive capability, disturbances in sleep/wake cycles, hallucinations, and gangrene of the extremities (Porter & Thompson Jr. 1992). Ergot alkaloids also affect other organisms including bacteria, nematodes, and insects.

The economic loss to the U.S. beef cattle industry of tall fescue toxicosis is estimated at about \$860 million. Fescue foot or summer syndrome, ryegrass staggers, and reproductive problems caused by these plant toxins cause \$1 billion in livestock losses annually in the United States alone (Browning Jr. 2004). Research laboratories across the country are working on different strategies to address the endophyte problem. To date, none have provided the ultimate solution to this problem. Three scientific solutions have been proposed to solve the dilemma of ergovaline. The first involves genetic alteration of endophyte in tall fescue or perennial ryegrass seed to produce ergot alkaloids selective against insects rather than herbivores. Second alternative was to develop a vaccine that would allow the animal to systemically bind the toxic alkaloids. The third alternative would be to isolate anaerobic bacteria that possess ability to degrade ergot alkaloids before systemic absorption. Microorganisms are versatile in mineralising numerous toxic substances.

Previous studies in sheep and cattle have yielded no cultivable microbes able to detoxify ergot molecules, leading to the search for novel sources of anaerobic organisms such as in the gut of "red wiggler" earthworm (*Eisenia fetida*), capable of degrading ergot alkaloids. We incubated ergovaline spiked media with earthworm gut contents. Further, DNA from the earthworm gut was isolated and a 16S rDNA library constructed to identify native bacteria of the earthworm.

Materials and Methods

Sample collection and media

Earthworms (*Eisenia fetida*) were randomly collected from a compost bin which was previously seeded with ergovaline infected seed and grass clippings. Earthworms weighing 21.8 g were dipped/washed in 50% ethanol. The surface sterilised earthworms were transferred to an anaerobic glove box (Coy, Grass Lake, MI). In the anaerobic glove box, the earthworms were ground using a tissue homogeniser and 50 mL of McDougall's buffer (McDougall 1948). One mL of worm slurry was incubated with 10 mL media E in Balch tubes. Ergovaline crystals, obtained from Forest Smith (Dept. Pharmacal Sciences, Auburn University, AL), was added to each tube at a concentration of 150 ppb. Balch tubes were incubated at 38°C and samples were drawn anaerobically at 0 h, 2 day, 4, and 7 days using disposable syringes. The samples were then transferred to glass tubes and extraction was carried out by HPLC. All experiments were performed in duplicate.

Isolation and Extraction of DNA from earthworm gut

Two worms were soaked in 50% ethanol solution for 10 min before dissection. Using a No.20 scalpel blade the crop/gizzard and anus section of the worm were removed. Then a lateral incision was made to remove the outer covering of worm and to isolate the gut. The worm gut was then placed into a microfuge tube, and sterile phosphate buffered saline (PBS) added to

homogenise the worm gut. DNA was then isolated from worm gut homogenate using a DNA extraction kit (Mobio, Carlsbad, CA). The final purified DNA was visualised by running samples on a 1% agarose gel.

PCR amplification and construction of 16S rDNA clone library

The DNA samples from the worm gut were subjected to PCR amplification using primers fD1 and rP2 (Weisburg *et al.* 1991). The PCR reaction was carried out using the TripleMaster PCR system (Eppendorf, Hamburg, Germany) in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA). PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). The purified DNA was cloned into the TOPO[®] 4 vector system (Invitrogen, Carlsbad, CA) and transformation of One Shot[®] TOP10 (Invitrogen) chemically competent *E. coli* cells were done according to the manufacturer's protocol. The LB plates were incubated at 37 °C overnight and transformants picked and transferred to 96 well plates containing 1.6 mL of 2x LB broth amended with 100 µg mL⁻¹ of ampicillin and shaken (250 rpm) at 37 °C overnight. A Montage Plasmid Miniprep96 kit (Millipore, Billerica, MA) was used for the isolation of plasmid DNA.

Restriction fragment length polymorphism (RFLP) analysis

The PCR reaction was carried out in 96 well plates using the TripleMaster PCR system (Eppendorf, Hamburg, Germany) in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA). Plasmid DNA (200 ng) was digested with 10 U each of *Bsa*RI and *Mbo*II (Fermentas, Hanover, MD) in a final reaction mixture volume of 30 µL. Gels were stained with ethidium bromide and images obtained using a Kodak photo image system. The clones were then grouped manually based on their RFLP patterns.

High performance liquid chromatography (HPLC) analysis

Extraction and analysis of ergovaline from samples were done according to as described previously (Craig *et al.* 1994). A Jordi Gel DVB RP 500A HPLC column (Alltech, Deerfield, IL) was used for the analysis. This was attached to an Elmer Perkin series 200 HPLC system. The isocratic mobile phase comprised of acetonitrile/ ammonium carbonate (0.2 mg mL⁻¹) at a ratio of (70:30) run at a flow rate of 1.0 mL min⁻¹ attached a flourometer (Elmer Perkin, series 200). The excitation and emission of the flourometer was set at 250 and 420 nm, respectively.

Sequencing and phylogentic tree construction

Cycle sequencing was performed using the ABI Big Dye Terminator system (Applied Biosystems, Foster City, CA). The SDSC Biology Workbench website (<http://workbench.sdsc.edu>) was used to manage and analyse sequence data. Partial clone sequences were compared to sequences in published databases using NCBI BLAST and the Biology Workbench which extracted information from the GenBank bacterial sequence database. Only reference sequences of named organisms from the databases with the highest homology scores to the query sequences were chosen for phylogenetic comparison. GenBank reference sequences and clone library sequences 'Align 2' tool found at Oregon State University Center for Gene Research and Bioinformatic. Partial 16S rDNA clone and reference sequences were aligned using the Clustal W multiple sequence alignment algorithm. Bootstrapping of the alignment was performed 100 times using the ClustalTree.

Results

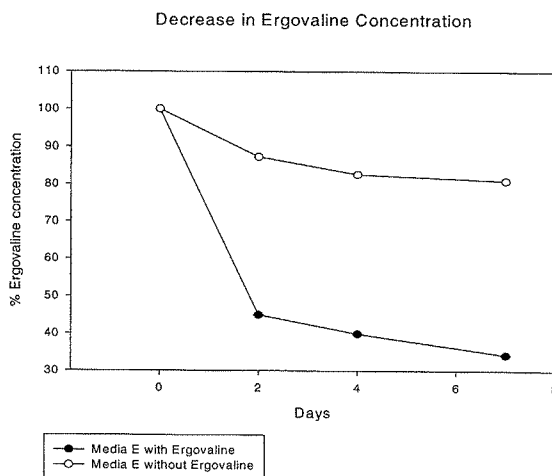
Degradation of ergovaline by earthworm gut microbiota

Fig. 1 shows the percentage loss of ergovaline after 7 days incubation with and without earthworm gut contents. In the control (treatment with no earthworm gut slurry added) background loss of ergovaline was 20% from the initial concentration over the 7 days. Most of this back loss of ergovaline took place on the second day with only slight reduction thereafter. In treatments inoculated with earthworm gut, there was a 60% decrease in the concentration of ergovaline from the initial concentration of 150 ppb by day 2. This trend continued and there was further reduction of ergovaline with losses up to 65%.

Earthworm gut clone library analysis

Fig. 2 represents partial 16S sequences and respective operational taxonomic units (OTU's) of the clones represented four major phyla. These phyla are *Plantomyces*, *Chloroflexi*, *Bacteroides*, and *Proteobacteria*. Clone WGC-A10 showed only 90.2% similarity to *Burkholderia sp.* PYX3 (β-Proteobacteria). Clone WGC-B01 had 86.1% similarity to *Amaricoccus macauensis* (α-Proteobacteria). Clone WGC-B02 had 99.2% similarity to *Algoriphagus dokdonsis* (Bacteroides). Clone WGC-B03 had 94.7% similarity to *Uncultured Chloroflexi* (Chloroflexi). Clone WGC-B05 had only 91.1% similarity to *Myxobacterium ATI-1* (δ-Proteobacteria). Clone WGC-B08 had 93.9% similarity to *Pirellula staleyii* (Planctomyces). Clone WGC-B10 had 96.6% similarity to *Ideonella sp.* B511 (β-Proteobacteria). Clone WGC-C03 had 97.0% similarity to *Amaricoccus macauensis* (α-Proteobacteria). Clone WGC-C05 had only 91.9% similarity to Flexibacteriaceae bacteria (Bacteroides). Clone WGC-D01 had 96.4% similarity to *ideonella sp.* B511 (β-Proteobacteria).

Figure 1 Decrease of ergovaline concentration over 7 days. Tubes were spiked with 150 ppb of ergovaline and inoculated for 7 days. Samples were drawn out on day 0, 2, 4, and 7 and ergovaline was extracted. HPLC analysis was performed on the extracted material. ● represents controls without the addition of earthworm gut. ○ represents treatments with earthworm gut. All treatments were performed in duplicate.



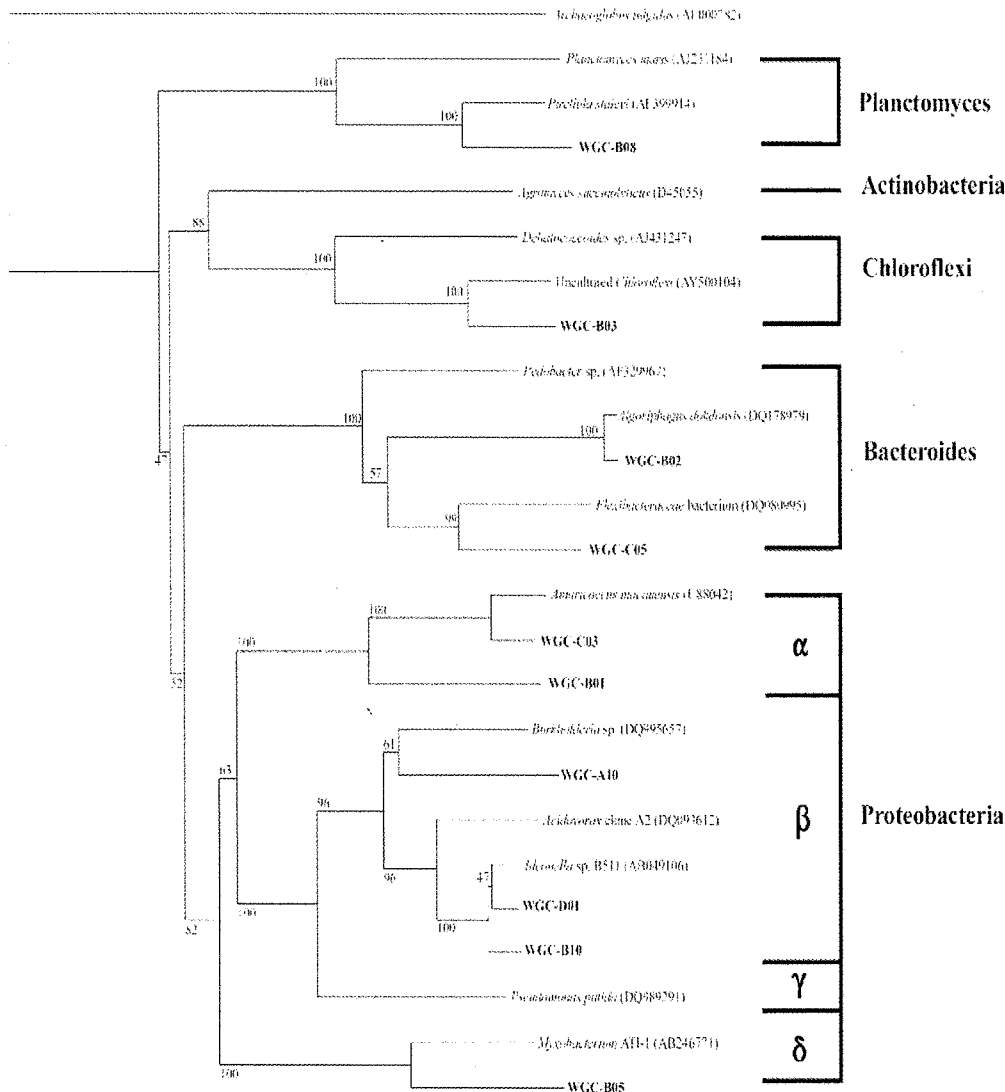
Discussion

Our novel approach to the ergovaline problem used anaerobic microorganisms obtained from the gut of the "red wiggler" earthworm (*Eisenia fetida*). There was over 60% reduction in ergovaline concentration in the worm gut experiments. We did not take epimerization into account due to the inability of the present method to completely resolve the epimer formation as described (Smith & Shappell 2002). The clonal library was created in an effort to elucidate species that are native to this earthworm involved in detoxifying ergovaline. We obtained only ten OTU's from our clone library construction and analysis. Moreover the number of clones that were picked (144) would not

be sufficient for estimating the entire diversity. Though we got PCR products of 1 500 base pair, we sequenced only one side of the primer. The 580 nucleotide reading frames we obtained due to this single side sequencing could be reason for obtaining less percentage homology with other bacteria in Genbank. Presently, we are constructing a much more complete clonal library of the earthworm gut flora and sequencing the whole 1 500 base pair inserts. Other reasons for such low diversity could also be attributed to factors such as PCR bias in amplification of 16S rDNA, extraction of DNA and the selective loss of few species.

This paper provides encouraging preliminary results by identifying the potential use of the earthworm gut flora to detoxify

Figure 2 Phylogenetic analysis of partial 16S sequences from worm gut clones. Neighbor-joining tree of a Clustal W (100 bootstraps performed). Scale bar represents evolutionary distance of 0.02 substitutions per base. Clones are displayed in bold type. Reference sequences are displayed with their GenBank accession numbers.



ergovaline. We are also utilising techniques such as LC-MS/MS/MS for metabolite identification for a better understanding of this complex process.

Conclusions

There is potential for earthworm gut microorganisms to detoxify ergovaline. The chemistry and molecular biology of this process has not yet been elucidated.

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