RESEARCH REPORT

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PORK SAFETY

Title: Application of a novel inhibition mechanism to control *E. coli* O157:H7 and other non-O157:H7 pathogenic *E. coli*. **NPB Project #10-086.**

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Industry Summary:

Enterohemorrhagic and exotoxigenic *E. coli* (EHEC and ETEC) pose a variety of animal health and food safety challenges for the food animal industry. *E. coli* F4 is an ETEC pathogen that is an important cause of post-weaning diarrhea and death in piglets. *E. coli* O157:H7 is an EHEC strain that may cause illness in young animals, but is more widely recognized as a food adulterant (primarily from cattle). Successful control measures for both EHEC and ETEC strains would yield significant animal and public health benefits as well as financial benefits to producers and consumers.

We previously identified several strains of *E. coli* from cattle that are capable of inhibiting growth of EHEC and ETEC strains. This inhibition phenotype was called "proximity-dependent inhibition" (PDI) because of the apparent necessity for the inhibitor strain to be in close proximity or direct contact with the susceptible strain for the mechanism to function. The goal of the current project was to identify candidate genes that are responsible for this phenotype and to determine their functional contribution to the PDI phenotype.

For this project we identified two strains of *E. coli*, one that produces the PDI phenotype and one that does not, but that were otherwise genetically identical using a conventional typing system (PFGE). We then generated whole-genome sequence data for both strains and compared them with the goal of identifying unique regions of DNA sequence that might be responsible for the PDI phenotype. This effort identified a region of DNA sequence that was unique to the inhibitor strain.

This PDI region appears to encode a novel microcin. Microcins are a subclass of bacteriocin antimicrobial peptides. They tend to be small in molecular mass and secreted in very low concentrations. Bacteria use bacteriocins to limit competition from other bacteria, but these peptides are usually specific to the same species of bacteria and do not affect other species or genera. In our case specificity includes both EHEC and ETEC strains. We completed a series of gene knockouts and other experiments that demonstrated that the putative microcin is indeed responsible for the PDI phenotype. We also demonstrated that the microcin limits *E. coli* O157:H7 by an unidentified killing mechanism.

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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This discovery is important to the industry because microcins could be used to inhibit growth of EHEC and ETEC bacteria in food animals as they are already being applied in the food industry. For example, a probiotic strain that induces PDI could be fed to food animals either to prevent colonization or to limit the population size of pathogenic strains. It might also be possible to deliver the antimicrobial peptide directly to the animal. The first step in development of this type of tool is to identify the genetic mechanisms responsible for PDI and funding from the National Pork Checkoff made this possible. The next steps include identifying the receptor that is required for bacteria to be inhibited by the microcin and determining if our PDI positive strains can be used as probiotics to control EHEC and ETEC in food animals.

Keywords:

Bacteriocin, microcin, colicin, EHEC, ETEC, *E. coli* F4, *E. coli* O157:H7

Scientific Abstract:

A novel phenotype was recently identified whereby specific strains of *Escherichia coli* inhibit competing *E. coli* via a mechanism that was designated “proximity-dependent inhibition” (PDI). PDI-expressing *E. coli* (PDI+) inhibit a broad range of susceptible *E. coli* strains (PDI-), including enterohemorrhagic (EHEC) and enterotoxigenic pathogens. In this study every strain from a genetically diverse panel of *E. coli* O157:H7 (n=8) and additional EHEC strains (*E. coli* O26, O103, O111, O121, O145) were susceptible to the PDI phenotype with an average 7 log decrease in population size when co-cultured with a microcin-producing strain. Live-dead staining was consistent with inhibition by death of susceptible cells. Comparative genome analysis identified the genetic component of PDI, which is a plasmid-borne operon that encodes a novel microcin. The plasmid was fully sequenced and is currently being annotated. The microcin operon includes genes presumptively required for secretion, immunity, microcin synthesis, and microcin activation. Transfer of the plasmid to a PDI- strain resulted in transfer of the PDI phenotype and deletion of different components of the operon resulted in loss of the inhibition phenotype. Deletion of *tolC* also resulted in loss of the PDI+ phenotype and this confirmed that the putative microcin is most likely secreted via a type I secretion pathway. Deletion of an unrelated plasmid gene (*traM*) had no effect on the PDI+ phenotype indicating that our gene deletion methods do not confound the experimental results. Quantitative RT-PCR experiments are currently underway to determine the kinetics of expression, but initial work is consistent with expression being maximized during rapid population growth. The ability to inhibit a diversity of *E. coli* strains indicates this microcin, called Microcin-E25, may influence community composition of the gut flora and it may be useful for control of important enteric pathogens.

Introduction:

Sawant et al. (9) recently described a novel inhibition phenotype by which defined strains of *Escherichia coli* from cattle are able to inhibit growth of other *E. coli* strains including enterohemorrhagic *E. coli* (EHEC) and exotoxigenic *E. coli* (ETEC). During *in vitro* competition assays, susceptible strains declined an average 4-6 log in population number relative to their expected population density when grown as monocultures. The inhibition phenotype was called “proximity-dependent inhibition” (PDI) because of the apparent need for inhibitor and susceptible to be located in close physical proximity or in direct contact for the phenotype to be observed. Two different *E. coli* strains were described as expressing this trait (PDI^{+}); multidrug resistant *E. coli*-25 and antibiotic susceptible *E. coli*-264. *E.* coli-25 and *E. coli*-264 do not affect the growth of each other, indicating that immunity is either conferred actively through the presence of an immunity mechanism, or passively through the absence of a receptor ligand found on susceptible cells.

Cell-cell inhibition mechanisms have been well documented in the literature and range from contact-dependent inhibition to production of narrow-spectrum antimicrobial peptides called

bacteriocins. Bacteriocins typically restrict the growth of closely related bacteria and their effects do not extend beyond the genus level (8). *E. coli* produce numerous bacteriocins that are classified as either colicins or microcins (1). Colicins are high-molecular weight, whereas microcins are very small, less than 10 kDa. Microcins can be either chromosomally or plasmid encoded, and colicins have only been found on plasmids. Colicin production is generally regulated along with the SOS response to stress and colicins are typically released during cell lysis whereas microcins are secreted from intact cells. Bacteriocins have been identified that kill competitors through pore formation, nuclease activity, or by inhibiting protein synthesis.

Certain characteristics of PDI resemble that of microcin production. For example, inhibition is particularly effective when strains are cultured in defined media where glucose is the only carbon source. Furthermore, expression of the PDI phenotype does not appear to harm PDI+ cells, consistent with secretion rather than release by lysis. Nevertheless, microcins are soluble proteins and when Sawant et al. employed a split-well experiment they demonstrated that close cell-cell proximity is required for the PDI phenotype to function. These findings suggested that the inhibition mechanism was not due to a soluble molecule unless the concentration of the inhibitor is so low as to require close proximity to be effective.

While the initial report of PDI provided a detailed description of the phenotype and a similar phenotype has been described between *Bibersteinia trehalosi* and *Mannheimia haemolytica* (2), the exact mechanism of inhibition and requisite genes for inhibition and immunity have not been identified.

Objectives:

1) Identify candidate genes that could be responsible for producing the PDI phenotype. This involves comparative genomic analysis of inhibitor and genetically similar non-inhibitor strains to identify gene candidates responsible for the inhibition phenotype.

2) Identify the functional role of candidate genes in the PDI phenotype. This involves analysis of the expression kinetics of the candidate genes and development of gene knockouts and complementation strains to confirm the contribution of the genes to the PDI phenotype

Materials & Methods:

Bacterial propagation and broth competition methods in defined media (M9) followed the methods of Sawant et al. (9). We included a survey of defined genetic lineages from *E. coli* O157:H7 to better determine if specificity of PDI activity. Live/dead staining was accomplished using a commercial kit and the WSU flow-cytometry core. DNA from three strains (*E. coli*-25, *E. coli*-82, and *E. coli*-264) was prepared using a commercial kit and sequenced using a Roche 454 FLX Titanium Genome Sequencer at the WSU Genomics Core. Alignment and BLAST algorithms were used to identify unique regions of DNA from the PDI+ strains, but absent for PDI- strains. Once a region of interest was identified, we used PCR to verify that this segment was missing from a panel of susceptible strains and we used a Southern blot with a plasmid profile to verify that the region of interest is harbored on a plasmid (the full plasmid sequence was also recovered from the 454 data, closed and is currently being annotated). Homologous exchange methods were employed to make genetic deletions (3). This included ABC transporter genes thought to be involved with secretion, the putative microcin, activator, and immunity genes. The immunity gene was tested by first making a microcin gene deletion strain followed by deleting the immunity gene and determining if the resulting double-knockout was susceptible to PDI. We included a negative control knockout from an unrelated gene and we constructed a knockout of the *tolC* locus from the bacterial chromosome to determine if this was required for the secretion system. We tagged the PDI plasmid with a kanamycin resistance gene and demonstrated that transferring the plasmid to a PDI- strain converted it into a PDI+ strain. Kinetics

experiments are in progress to determine when the putative microcin is being expressed during cell culture.

Results:

Objective 1. We tested the PDI against a panel of O157:H7 strains representing human-biased and bovine-biased genomes (10, 11). We also tested a small panel of other STEC strains (O26, O103, O111, O121, O145) and three *Shigella* strains; all were susceptible to PDI with an average 7.1 log reduction in population density after 24 hours co-culture with *E. coli*-25. The only exception was a single strain of O111 that was not appreciably affected (~1 log reduction) and we are currently confirming the serotype of this strain. It is clear that the PDI phenotype has a very broad spectrum of activity, which is an important attribute for practical applications.

Live-dead stain and flow-cytometry was used to assess mono- and co-cultures of *E. coli*-25 and a strain of *E. coli* O157:H7. The percentage of dead cells detected from the two mono-cultures was 0.5% and 0.3%, respectively. When co-cultured for six hours the percent of dead cells increased to 6.1% consistent with killing of *E. coli* O157:H7. When this susceptible strain was co-cultured with another PDI- strain (*E. coli*-6), the percentage of dead cells was 0.4%. These results indicate that PDI most likely functions by killing susceptible cells, which is an important attribute for practical applications.

Analysis of the 454 data identified a 5 kilobase region that included two ABC transporters and what appears to be, based on limited homology, a microcin with associated immunity and activator genes. Gene homology is limited, but gene synteny, size of the predicted proteins, and characteristics of the amino acid sequence are consistent with this conclusion. PCR testing demonstrated that this region was only present in known PDI+ strains, and absent from PDI- strains. Sequence analysis showed that the region was encoded on a plasmid and this was subsequently confirmed by Southern blot.

Objective 2. Transferring the putative PDI plasmid to a PDI- strain (*E. coli*-4) converted this strain into a PDI+ strain. We are currently determining if the plasmid is self-transmissible, but if it is, we have completed the plasmid sequence and thus we can quickly engineer a version that is not transmissible or mobilizable as would be needed for practical applications.

All gene knockouts from the putative microcin-encoding region produced PDI- strains as expected. Both the microcin and double microcin-immunity gene knockouts were susceptible to PDI (the former knockout probably causing a polar effect on the immunity gene). We are currently attempting to express the immunity gene in *trans* to convert a PDI- strain into one that is not susceptible to PDI. If this works, there will be no question that the putative immunity gene is serving this function.

A negative control deletion (*traM*) produced no change in PDI thereby confirming loss of PDI was not a function of our gene knockout procedures. We also deleted *tolC* from the chromosome and this resulted in loss of PDI. This result demonstrated that the ABC transporters most likely function with TolC to form a type I secretion system that is needed to secrete the microcin from the PDI+ cells.

We have constructed a quantitative PCR assay to study the kinetics of the microcin expression and preliminary results (as of 4 Jan 2012) are consistent with exponential accumulation of transcript during exponential growth phase with a peak near 12 hrs followed by a subsequent decline

Discussion:

We successfully completed both objectives 1 and 2 of this project and this work provided definitive evidence that the PDI phenotype is explained by secretion of a novel microcin that kills susceptible strains of *E. coli* including EHEC and ETEC strains. To capitalize on this system, we want to deliver

either a microcin-producing strain to animals in the form of a probiotic, or deliver the peptide itself to block or limit pathogen colonization. Our findings further this goal for several reasons.

The fact that the microcin encoding region is found on a plasmid makes it simple to transfer the trait to different strains of *E. coli*. This will be important if we need to use niche-specific strains to gain better access to the target pathogens. An empirical study is needed to determine if this will be necessary, but because we have the full plasmid sequence we can also engineer the plasmid so that it cannot move to native strains of *E. coli* while in the animal.

As we learn more about the kinetics of expression, we will also determine how the microcin is regulated and it may be possible to decouple expression from growth phase (e.g., make microcin production constitutive assuming this is not lethal and does not cause a high fitness cost). We should note, however, that we have ancillary data suggesting that it is pretty simple to cause cyclic rapid growth of *E. coli*-25 *in vivo* and this should result in repeated cycles of microcin production in the gut. Addition of a simple milk supplement (dried milk, vitamin A, and vitamin D) to the diet of pre-weaned calves was sufficient to allow our *E. coli*-25 strain to dominate the fecal *E. coli* population (4-7). If this dominance is explained by expression of the microcin, this simple dietary supplement may be sufficient to manipulate the system in favor our microcin-producing strains.

We hypothesize that the microcin interacts with a cell surface receptor of some kind that is expressed on the PDI- cells. For practical applications it is clearly important that these receptors also be expressed *in vivo*. To date we have not been able to identify the microcin receptor from the susceptible cells, although we have employed several sophisticated molecular strategies in this effort. Recently we produced a recombinant version of the inactive microcin and we are currently generating polyclonal antibodies. With these antibodies we plan to isolate the microcin receptor by using either far western or immunoprecipitation methods followed by mass spectrometry (all methods currently employed in my lab). Once we identify the receptor we can ascertain its expression kinetics. We will also attempt to chemically activate our recombinant protein as an alternative approach to enabling application of the microcin to fight enteric infections.

In summary, the support from the National Pork Checkoff made it possible to identify a novel microcin that is very effective at killing EHEC and ETEC pathogens. We will complete the expression kinetics work in January 2012 and a draft manuscript describing our findings (with partial credit given to the NPC) should be submitted in the next two months as part of a dissertation project. We are also planning to present our findings at the general meeting of the American Society for Microbiology in May.

Our next goal is to complete a series of studies to determine if the microcin is expressed *in vivo*, if it can affect target populations, and determine where our strain resides in the gut of treated animals. We will also continue efforts to identify the receptor on the PDI- strains as described above. Unfortunately, our USDA-AFRI proposal was rejected in 2011 in part because our *in vivo* work was considered too risky. I hope to have a preliminary study completed for the next submission and I am applying to two additional intramural research programs in hopes of securing support for this work. If I am unsuccessful with these options, I plan to apply for NPC support again in the fall of 2012.

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