Annual Project Report/ Research Update to Minnesota Department of Agriculture
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Development of a DNA Marker Gene System for *E. coli* from Cows, Pigs, and Turkeys and Use of a Small Watershed to Monitor Bacteria Loadings and Effects of Mitigation Practices

Clean water Legacy Funds

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This project report covers part 1, objective 1a of the overall project. In this part of the project we will extend previous studies and use subtraction suppressive hybridization (SSH) to identify DNAs that are specific for *E. coli* originating from cows, pigs and turkeys. During the initial project period we hired two graduate students, Charlie Sawdey (Water Recourses Graduate Program) and Daniel Norat (Microbial Engineering Graduate Program) to work on the project. Both students are now fully involved in all experimental aspects of the research plan.

Two general approaches are being used to achieve the project goals. In the first we are using subtraction suppressive hybridization (SSH) to identify DNAs that are specific for *E. coli* originating from cows, pigs and turkeys. Secondly, and as a back-up plan in case the SSH experiments do not prove fruitful in identifying novel marker genes, we are evaluating and using DNA PCR primers that are specific for fecal bacteria (members of the Baceteroidales) originating from humans and cows. While the PCR primers will necessitate isolation of DNA from samples, they have proven useful in determining human and cow inputs into waterways, and can be made quantitative by using alternate PCR strategies.

A: Subtraction suppressive hybridizations to isolate DNA markers specific for *E. coli* from cows, chickens, and pigs.

During the project period two SSH reactions were carried out. In the first case (cow subtraction 1), we used DNA from 20 *E. coli* cow strains as tester and 20 DNAs from non-cow strains as driver (*E. coli* from 5 humans, 5 pigs, 5 chickens, 2 horses, 2 sheep, and 1 goose). The cow, human, pig and chicken strains were selected by HFERP dendrogram analysis, and the horse, sheep and goose strains were selected randomly. Subtraction products were cloned and 576 clones were picked for the subtraction library. Of these, 288 clones were tested by dot blot hybridization for specificity for cow *E. coli* – 68 were found to be tester specific. Furthermore, 60 of 68 were also confirmed to be tester specific by southern blot hybridization analysis. Restriction enzyme analysis on the 60 confirmed cow specific clones showed that the clones contained 25 different insert DNAs. Colony hybridizations were done using 14 of the 25 different inserts against an array of *E. coli* from 13 animal hosts and humans. These arrays included *E. coli* strains from the following sources, with the number of strains in parentheses, cat (48), chicken (96), cow (189), deer (96), dog (96), duck (81), goose (135), goat (42), horse (78), human (210), pig (218), sheep (60), and turkey (96). However, none of the insert DNAs tested were specific and results showed that inserts cross hybridized with isolates from many different animal hosts. Inserts hybridized with considerable numbers (>15%) of isolates from source groups not represented or poorly represented in the driver sample. The remaining 11 inserts were not tested, since the isolated probes appeared not be specific for cows.

To potentially increase the specificity of the probes, we chose to modify the subtraction reaction – by both increasing the diversity of strains present in the driver sample and increasing the time of the primary hybridization. To do this, DNAs from 25 cow *E. coli* strains were used as tester and DNAs from 40 non-cow strains used as driver in SSH reactions. The driver sample consisted of 5 strains from each of the following source groups: chickens, goats, geese, horses, humans, pigs, sheep, and turkeys. All strains used in the subtraction were selected by HFERP DNA fingerprint dendrogram analysis. Subtraction products were cloned and 384 clones were initially picked for the subtraction library. We initially analyzed 192 clones by dot blot

hybridization to tester and driver DNAs and 35 were found to be tester specific (for cows). Of these, 26 of the 35 were confirmed as tester specific by southern hybridization analysis. Gel electrophoresis analysis of cloned DNA fragments suggested that 10 of the 26 clones contained the same DNA insert. In total, it appears that there are 12 different fragments. It is possible that these inserts have different sequences and nearly the same size. Further analysis through restriction analysis and/or hybridization will be necessary to determine the exact number of different inserts. This is currently ongoing. We also tested 6 cloned insert DNAs by hybridization to a panel of *E. coli* strains from 12 animal hosts and humans. Results of these analyses indicated that 5 of the inserts had a significant percentage of cross-hybridization with non-cow strains, approx 15-25% of all isolates (including cow isolates) hybridize with these inserts. However, 1 insert hybridized only with cow strains, although it recognized 11 of 189 strains tested. This insert was sequenced and found to be nearly identical to the colicin-N gene of *E. coli*. The remaining 6 inserts (plus any found to be unique after restriction analysis/hybridization) will be tested in the coming weeks.

We also performed SSH reactions to isolate DNA clones specific for pigs. The DNAs from 21 *E. coli* strains from pigs were chosen as tester and 30 non-pig strains (10 cows, 10 humans, 5 chickens, 1 dog, 1 cat, 1 goat, 1 goose, 1 turkey) were chosen as driver DNAs. All strains used in the subtraction were selected by HFERP DNA fingerprint dendrogram analysis. Subtraction products were cloned and 576 clones were picked for the subtraction library. All 576 clones were tested by dot blot hybridization and 50 were found to be tester (Pig) specific. Of these, 12 of the 50 clones were confirmed as tester specific by Southern blot hybridization to genomic DNAs. Seven of the cloned insert DNAs were tested by colony hybridization. However, none of the inserts were specific to pig isolates, although one insert hybridized predominately with pigs (~40%) and turkeys (~30%). Isolates from other host species cross-hybridized with the probes at <15%, suggesting that this insert may be useful to identify Pig contamination in waterways not impacted by turkeys. We are currently examining this probe in more detail to determine if the specificity can be increased. The remaining 11 inserts were not tested, instead we chose to modify the subtraction as described above – by both increasing the diversity of strains present in the driver sample and increasing the time of the primary hybridization. To do this, DNAs from 21 pig strains were chosen as the tester and DNAs from 40 non-pig strains (chickens, cows, goats, geese, horses, humans, sheep, and turkeys) were used as driver DNAs. All strains used in the subtraction were selected by HFERP dendrogram analysis. Subtraction products were cloned and 192 clones were picked for the subtraction library. All 192 clones are currently being screened by dot-blot hybridization.

Lastly, we are currently performing SSH reactions to isolate DNA clones specific for chickens. DNAs from 25 chicken strains were chosen as the tester and DNAs from 40 non-chicken strains (cows, goats, geese, horses, humans, pigs, sheep, turkeys) are being used as driver for SSH. Subtraction products were cloned and 192 clones were picked for the subtraction library. All 192 clones are currently being screened by dot-blot hybridization fro specificity.

Since the last project report we have been continuing to evaluate potential probes for specificty for hybridization to Pigs and Chickens. However, since the robot broke down at the end of November, and is only now up and running, we had a slight delay in analyzing samples. Nevertheless, we currently have defined 8 hybridization probes designed to detect swine *E. coli*

and together they detect ~ 65% of the tested swine *E. coli*. However, they cross-react with ~ 8% of *E. coli* from Turkeys. One of the isolated probes proved to be very interesting, it reacted with Deer, Pigs, Sheep and Goat *E. coli*, but with very few (~1%) of human isolates. This may be useful as a more general animal probe. More over, one of the probes reacted with the following percentage of tested isolates as follows: Humans -1.4%, Horses - 16%, Goats - 2.4%, Sheep - 8.2%, Pigs -10%, and turkeys 0.08%. Since this probe reacted mostly with horses, it may have use for a general probe for non-human related contamination.

B: Evaluation and use of PCR primers for microbial source tracking.

As discussed, previously published microbial source tracking papers have indicated that PCR primers targeting members of the genus *Bacteroides* may be useful for microbial source tracking purposes. As a back-up plan for phase 2 of the project, in case the SSH experiments do not prove fruitful in identifying novel marker genes, we are evaluating and using DNA PCR primers that are specific for fecal bacteria (members of the Baceteroidales, *Bacteroides*) originating from humans and cows. As a positive control for the specificity of any environmental sample, a general probe for the presence of *Bacteroides* is being used (Bernhard 2000a). Human specific samples were tested against genomic DNA extracted from sewage influent along with extracted DNA from Lake Como and combinations of previously collected fecal isolates from various animal species. Lake Como was chosen because it has a low amount of recreational human use and is frequently contaminated by feces from ducks and geese. In initial studies, of the human-specific primer tested, hum163 appeared to be the most consistent for finding human feces from water and soil isolates. The primer reacted with human sewage samples and only minimally with a DNA from a water sample from Lake Como.

We have now completed evaluating a large number of cow- and human-specific PCR primers for specificity and sensitivity to detect *Bacteroides* in the environment. The following primers were evaluated: Human primers - HuBac566, B. theta, Hu336, Hum163Fa, HF 813; Cow primers BoBac367, BAC3F; and primers reacting with all *Bacteroides* strains: Allbac296f, and Bac32F.

Of these, the following primers will be used in our studies: Allbac296f, cow BAC3F, human HF 183, and Hum163Fa, which reacted with all Bacteroides, and those specific for cows and humans, respectively. These primers gave maximum specificity and sensitivity in tested reactions.

Using these primers we have been able to show that multiplex PCR (PCR simultaneously done using two primer sets) can be used to determine *Bacteroides* from different sources (see figure 1 below). This allows unambiguous assignment of sources of fecal bacteria in watersheds.

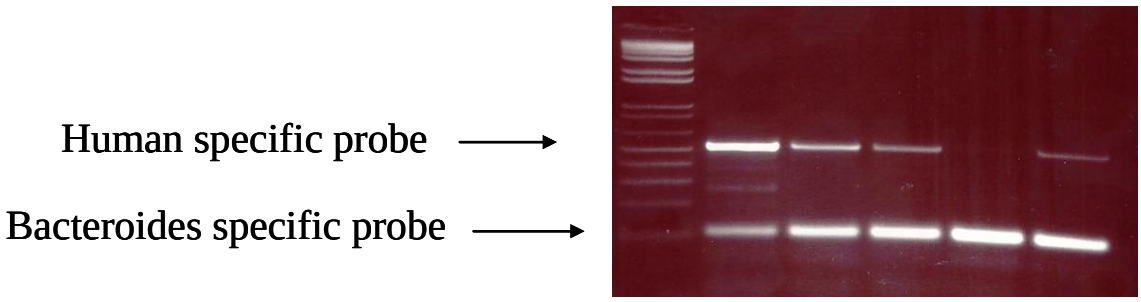

Figure 1. Multiplex polymerase chain reaction done to determine the presence of total *Bacteroides* (lower band) and human-specific *Bacteroides* (upper band) in water samples using the Allbac296f and B.theta probes.

C. Development of a high throughput, quantitative, platform to detect human- and animal-specific *Bacteroides* in water, sand, and sediment samples.

In order to aid in TMDL determinations and to develop abatement strategies it is necessary to determine the actual concentration of fecal bacteria (*Bacteroides*) in water, sands, and sediments. This is now being done using quantitative, real-time PCR (qPCR) technologies. Current qPCR technologies, however, are expensive, time consuming and requires specialized equipment and training. To overcome these limitations, we have been developing a high-throughput, qPCR system that utilizes a conventional PCR machine and inexpensive reagents and equipment. The technique relies on the use of SYBR green dye- incorporation and a fixed time point (endpoint PCR) to quantify *Bacteroides* (and eventually *E. coli*) in water samples. In qPCR, a fluorescent dye (SYBR green) binds to the DNA when every a copy is made the sample's fluorescence intensity in directly proportional to the amount (or copy number) of the specific bacterium in the sample. If there is more DNA in the original sample from more *Bacteroides*, it will take fewer copying cycles for the sample to make fluorescence (Figure2).

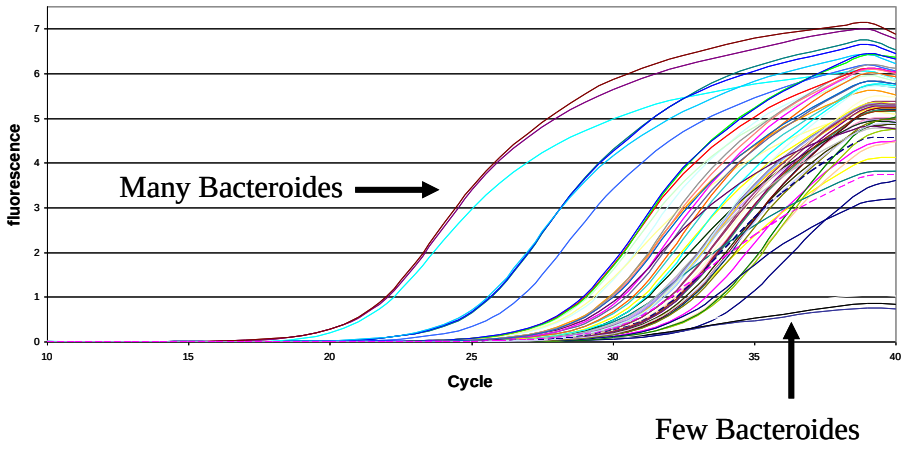

Figure 2. Relationship between fluorescence and cycle number required for fluorescence for different concentrations of *Bacteroides* using the HF183 primers.

By graphing the concentration of cells vs. how long it took to fluoresce you can use this graph to determine number of *Bacteroides* in your sample in a quantitative manner (Figure 3).

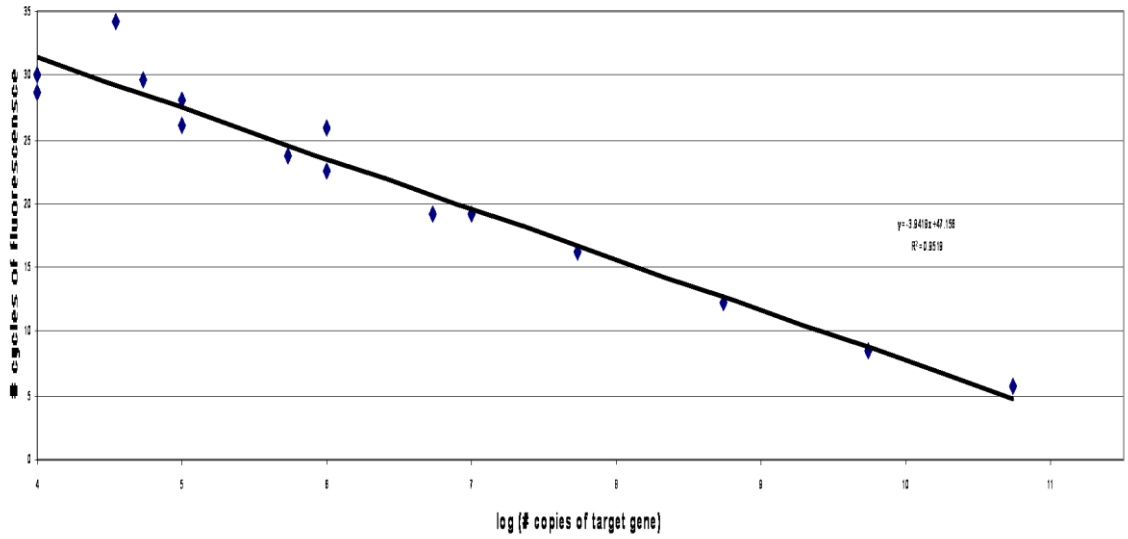

Figure 3. Quantitative relationship log number of *Bacteroides* in a sample cycle number required for fluorescence.

We are currently adapting this technology to be used with a standard fluorescence plate reader, which can quantify 96 PCR reactions in a matter of minutes. This will allow us to determine the quantity of animal-specific *Bacteroides* in water, sand, and sediment samples using a conventional PCR machine and inexpensive reagents and equipment. These studies are ongoing. When completed we will then adapt the developed methods for use with our Biomek FX liquid handling robot (Beckman/Coulter). The robot is equipped with 96-channel head that is capable of transferring reagents into 96-well microplates. DNA samples in the plates will be amplified by PCR using a 4-plate PCR Machine (Tetrad DNA Engine, MJ Research) which will allow the simulations analysis of 384 samples. Fluorescence in samples will be quantified by using Gemini XS plate reader (Molecular Devices Corp.) and the number of animal-specific Bacteroides in samples will be quantified via the values obtained from the above analyses.