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Identification of Lactic Acid Bacteria in Michigan Cherry Wines

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Identification of Lactic Acid Bacteria in Michigan Cherry Wines

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Abstract

Many factors contribute to the final flavor of wine. One factor is malolactic fermentation, during which lactic acid bacteria (LAB) transform the harsh tasting malic acid into a more drinkable lactic acid in grape wine. The role of LAB in the production of cherry wine is completely unknown. The goal of this study is to identify the species of LAB in cherry wine and compare them to those found in grape wines. Bacteria from cherry wine were grown on general media plates and plates fortified with malic acid, which may provide optimal growing conditions for the LAB. To identify the bacteria, we use PCR to isolate 16S ribosomal DNA sequences, which encode a general gene found in all bacteria. We then use nested PCR to narrow our focus on a specific variable region of the gene; this differentiates the DNA of different LAB after sequencing. Sequences of the variable region will be entered into an online database, which will allow us to identify the bacteria. We were able to isolate 62 colonies from two different wine samples and 18 are ready for nested PCR. Preliminary sequence analysis of earlier colonies allowed the identification of the genus, but not the species. In order to properly identify the bacteria, other identification tests will be used: catalase testing, Gram staining, identification of shape. By identifying LAB in cherry wine, winemakers may be able to determine how to use LAB to enhance the final flavor of wine and discourage growth that contributes to spoilage of wine.

Introduction

The process of wine making utilizes a number of microorganisms and fermentations to enhance the quality and final flavor of wine. The most familiar microorganism involved in wine making is yeast, which converts sugar into ethanol and carbon dioxide during alcoholic fermentation. Another important transformation is the malolactic fermentation (Figure 1). It is an optional secondary fermentation that uses lactic acid bacteria (LAB) to metabolize harsh tasting malic acid into a softer more rounded lactic acid (Browning *et al.* 1997).

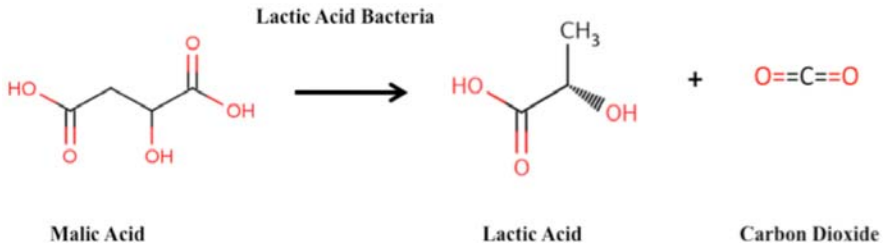

Figure 1. Metabolism of malic acid. Lactic acid bacteria transform malic acid into lactic acid and carbon dioxide. Images from http://www.bmrb.wisc.edu.

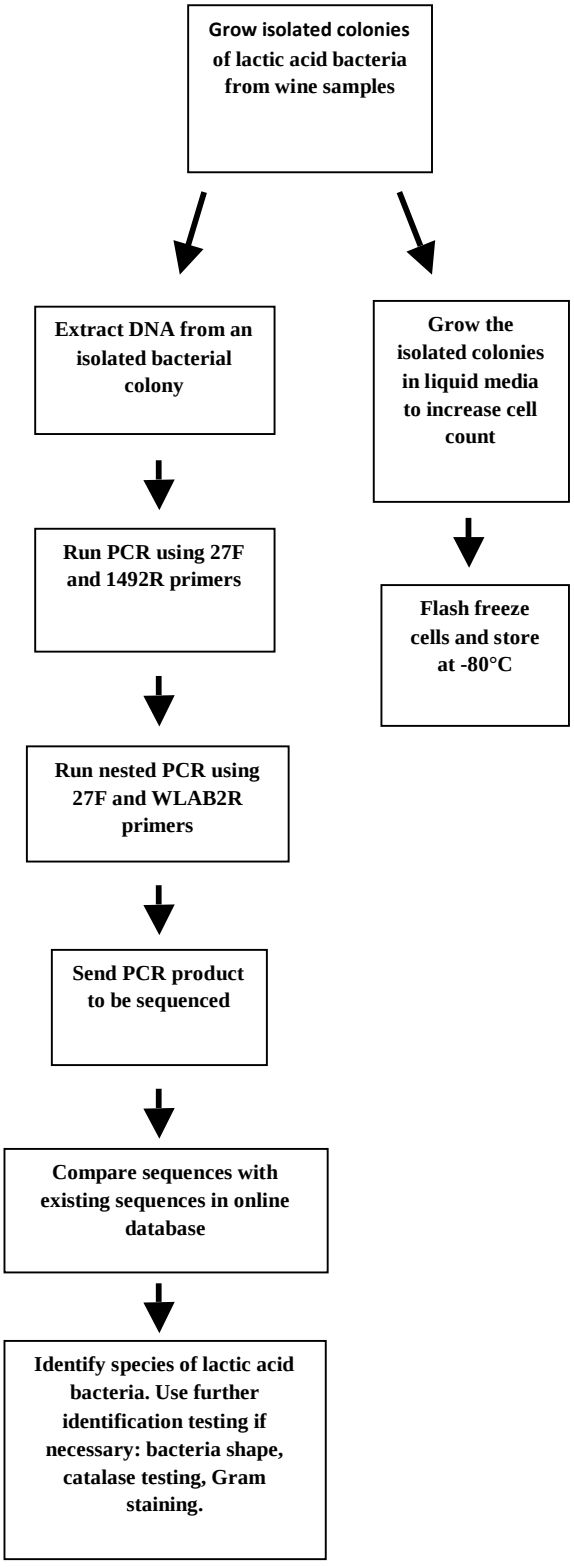

Figure 2. The experimental procedure for the identification of LAB in cherry wines.

The most frequently used LAB for this purpose are the *Oenococcus oeni* or *Leuconostoc oenos* (Rotter 2002). Although some LAB species improve the final flavor of wine by reducing the overall acidity, other LAB species have been known to cause wine spoilage (Bae et al.2006). Spoilage is caused by microorganisms producing excessive amounts of acetic acid, a volatile acid that hinders the overall quality of wine (Rotter 2002). Species that cause wine spoilage are usually from the *Lactobacillus* or *Pediococcus* genera (Rotter 2002). In addition to certain LAB species, acetic acid bacteria can also cause wine spoilage. Acetic acid bacteria, or vinegar bacteria, need oxygen to grow which can be easily limited by well-equipped wineries (Browning et al. 1997). By identifying the microorganisms in wine, winemakers will know what precautions they need to take in order to create the best quality wine.

Because LAB play a crucial role in wine making, the typical flora of grape wine is well-established and simple methods exist to monitor LAB present during all stages of the fermentation process (Lopez *et al.* 2003, Rodas *et al.* 2003, Moreno-Arribas and Polo 2005, Bae *et al.* 2006). However, the role of LAB in the production of wine from other types of fruit, such as cherry, is completely unknown. It is possible that similar LAB species may participate in the production of both grape and other fruit wines. Alternatively, the conditions may differ enough between grape wine and other fruit wines that a different suite of bacterial participants may be recruited in each type. In either case, LAB are likely to be as important in flavor development and stability in wines produced from other types of fruit as they are in grape wine, and a preliminary survey of LAB species present would be useful to fruit wine producers. Because of the importance of the tart cherry crop to Michigan's agricultural economy and the unique local production of cherry wines at Michigan wineries, we will survey the LAB present in tart cherry wine and compare the bacterial flora thus identified with that identified in grape wines.

In previous studies, sequencing the 16S ribosomal gene has helped distinguish LAB species in grape wine (Lopez *et al.* 2003). Here we use this gene sequence to characterize the LAB in the cherry wine samples (Figure 2). Amplification of the 16S ribosomal gene will follow standard PCR procedures using universal primers. Nested PCR narrows the region of the gene to a more variable section making it easier to sequence and analyze the DNA. Since the DNA between different LAB

species are so similar, our narrowed region will contain multiple variable regions as shown in Figure 3. Sequencing the LAB DNA might not be enough to differentiate between the different species. In this case, we may have to use other identification methods like catalase testing (Mills 2007), Gram staining, and shape (Thomas 2006).

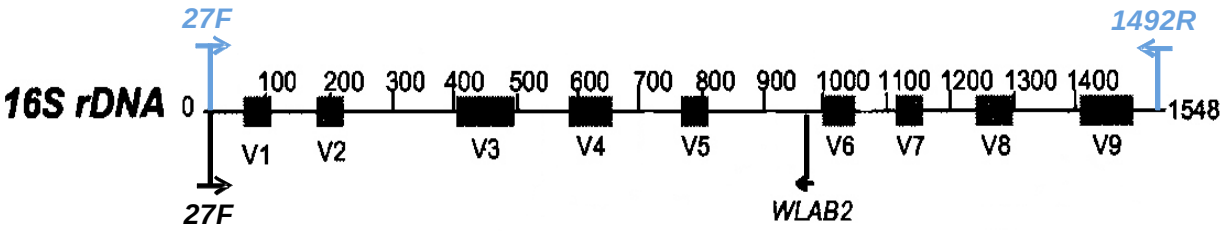

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Figure 3. Map of the 16S ribosomal gene for *Lactobacillus plantarum* (Gene Bank accession number AJ271852). Boxes signify variable regions between the different species of LAB. Our primers are highlighted with arrows. The universal primers 27F and 1492R are used for the initial amplification of the gene. Then primers 27F and WLAB2R are used for the nested PCR.

Methods and Materials

Sample acquisition and preparation

Cherry wine samples were obtained from Chateau Grand Traverse winery in Traverse City, Michigan in June 2008. Two different types of post-fermentation samples were collected: fresh pressed cherry wine (unfiltered) and cherry wine from frozen concentrate (filtered). All samples were placed directly into sterilized bottles and transported on ice to Grand Valley State University's Grand Rapids campus.

Wine samples were serially diluted and plated on MRS (Remel Inc., Lenexa, KS) and MLAB (MRS supplemented with 0.5% fructose, 0.1% malic acid, and 10.0% tomato juice) culture media. MRS is a general media for the growth of all bacteria and MLAB is an experimental media which may provide optimal growing conditions for lactic acid bacteria. Both media types were supplemented with 100 mg/l of cyclohexamide to discourage fungal growth. Samples were spread onto plates and incubated at room temperature until bacterial growth was observed. Plates with adequate bacterial growth were sub-cultured to insure isolated colonies. Selected colonies were then placed in their appropriate liquid media (MRS or MLAB) to increase cell count. Cells were placed in conical vials with freezer stock solution then placed in liquid nitrogen and stored at -80°C for future identification processes.

DNA extraction and PCR amplification

DNA for PCR amplification was extracted from the bacteria by placing a single colony from media plates into 40 µL of sterile distilled water and heat shocking the sample for 5 minutes at 95 C^{o} to lyse the cells, releasing the DNA into the water. The sample was then centrifuged to remove cellular debris. The supernatant was transferred to a new tube and stored at -20° C.

The gene of interest, the 16S ribosomal gene, is amplified using standard PCR protocol and the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Thomas 2004). Nested PCR (Delacroix-Buchet *et al.* 2004) using primers 27F and WLAB2R (5'-TCGAATTAAACCACATGCTCCA-3') (Lopez *et al.* 2003) amplifies a smaller, more variable region of the 16S gene (Figure 3). This is particularly helpful in distinguishing bacterial strains from one another. We have been successful using a PCR program consisting of 40 cycles that begin with an initial denaturation of 30 seconds at 95 C^o, 30 seconds of annealing at 52 C^o, and 60 seconds of extension at 72 C^o, followed by a final extension at 72 C^o for five minutes. The products of PCR reactions are visualized on 1.0% agarose gels to confirm fragment sizes.

DNA sequencing and bacterial identification

Fragments of interest will be purified using the GenElute PCR clean up kit (Sigma-Aldrich, St. Louis, MO). Sequencing will be conducted at the DNA Sequencing Core at University of Michigan. Traces will be proofread and edited before they are used to identify bacterial species.

The newly acquired DNA sequences will be compared to those in the public databases in order to identify the LAB species in the wine samples. The BLASTn search algorithm will be used to identify overall sequence similarity of all available 16S sequences in the National Center for Biotechnology Information (NCBI GenBank) and the Ribosomal Database Project public databases. Unique sequences will also be added to an alignment of all available LAB 16S sequences and analyzed using the parsimony criterion, as implemented using the PAUP*b10 software.

Results

We were able to isolate 62 bacterial colonies from our wine samples. The bacterial colonies had varying growth rates, which fell into three different categories (Figure 4). DNA templates and freezer stocks have been made from isolated colonies.

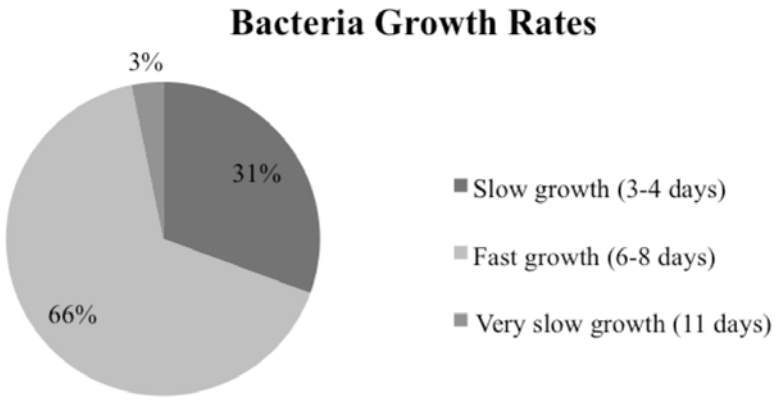

Figure 4. Growth rates of bacteria on MRS and MLAB media plates.

So far, we have been able to amplify 18 of the 62 bacterial colonies (Figure 5). They will proceed to the nested PCR. The other colonies are not amplifying as needed and require further investigation.

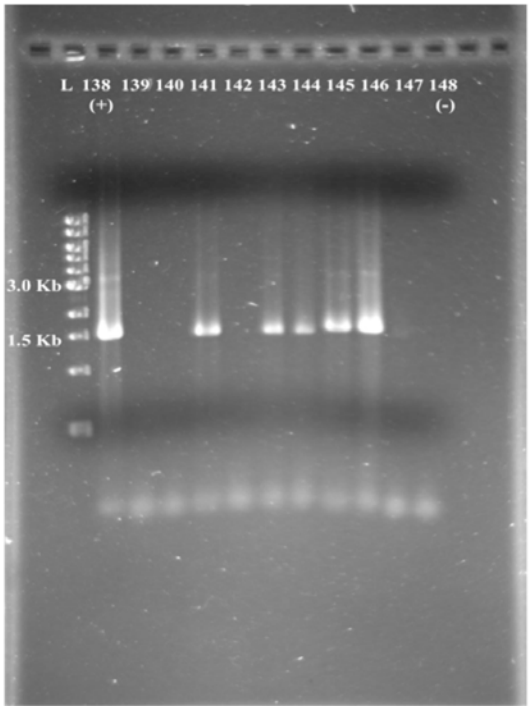

Figure 5. Gel electrophoresis of PCR products using universal primers 27F and 1492R and template DNA from colonies A1II, A7 II, D1, F1, G1, G3-4, H1, and H3.

Sequences were obtained from two PCR products amplified last summer. Preliminary sequence analysis has identified them as *Acetobacter*.

Discussion

Bacterial Growth Rates

The varying growth rates of the bacteria on the media plates are very interesting (Figure 4). These differences in time of growth could signify that these bacteria are from different genera or species. It could also signify that some of the bacteria are lactic acid bacteria and the others are acetic acid bacteria.

PCR

We have established a proper PCR protocol which has worked for 18 of our DNA samples. From our electrophoresis gels (Figure 5) we are able to confirm that the PCR product fragment sizes are accurate. PCR products using primers 27F and 1492R should be about 1500 base pairs long; products from primers 27F and WLAB2R should be about 900 base pairs (Figure 3).

Troubleshooting PCR

In order to make any kind of conclusive statement we are going to need more than 18 DNA samples. The DNA samples that are not amplifying are from the "slow growth" and "very slow growth" colonies. These colonies took more time to grow up, but grew so slow that they were able to sit out and grow up longer. Thus, the colonies from "slow growth" and "very slow growth" are larger. On the other hand, the colonies of the "fast growth" are smaller. They grew so fast we were afraid they would over grow the plates. For the "slow/very slow growth" templates, we are considering that there might be too much DNA in the templates and the templates need to be diluted. When we lyse the bacterial cells to make the template, the cells "explode" and release everything within the cell. Although DNA is released, the components that inhibit the duplication of DNA are released as well. We think that if there are too many cells present while making the template, too many of the inhibitors will be released as well. This would inhibit PCR from making amplifications of the 16S gene. We are in the process of diluting the templates. We set up three dilutions for 4 templates (1:10, 1:50 and 1:100) then continued with the standard PCR protocol. Once we confirm the fragment size, we can continue with the nested PCR and then sequence the DNA.

Sequence Analysis

Although the sequenced data from last summer did not end up being from LAB, finding two different species of *Acetobacter* in the cherry wine samples is very interesting. Acetic acid bacteria are known to cause spoilage in wine, if not properly controlled. *Acetobacter* and *Gluconobacter*, acetic acid bacteria genera, produce high levels of acetic acid when they come into contact with oxygen (Rotter 2002). It is important for a winemaker to know that these organisms are in their wine. This enables them to take the proper precautions to inhibit the growth of acetic acid bacteria and avoid wine spoilage.

Further Studies

Since the genetic differences between the species of LAB are so small, analyzing their DNA might not be enough. If their DNA results are not conclusive, we will continue the bacterial identification process using: catalase testing, Gram staining, and shape determination. All three techniques are common ways to identify bacteria. Catalase testing and Gram staining will help us to determine if our bacteria are LAB or acetic acid bacteria (www.awri.com.au/practical_solutions 2008). LAB are generally catalase negative, while acetic acid bacteria are catalase positive (www.awri.com.au/practical_solutions 2008). LAB *Lactobacillus*, *Pediococcus* and *Oenococcus* will stain Gram positive, while the acetic acid bacteria *Acetobacter* and *Gluconobacter* will stain Gram negative (www.awri.com.au/practical_solutions 2008). Identifying the shape of bacteria will help identify the genera. For example, if bacterial cells are spherical they could be from the genera *Pediococcus* or *Oenococcus*; if the cells are rod shaped they could be from the genera *Lactobacillus* (Rotter 2002).

In order to make a conclusive statement about the LAB in cherry wine, we need to troubleshoot the initial PCR amplifications. We also have to properly amplify the variable region of the 16S gene using nested PCR and sequence it. Once it is sequenced we can compare our data with database sequences to determine if the bacteria we have cultured are LAB and if so which ones. Other identification processes might be used in order to identify specific species.

Since the role of LAB is known in grape wines, we can take that information and relate it back to our work and determine if the LAB species found in cherry wine positively or negatively affect the overall taste of

wine. With this information, winemakers will have a better understanding of how to control the quality of their wine in order to create the best tasting wine with the highest quality.

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