5-2006

Electroporation of Pediococcus pentosaceus and the Curing Rate of Plasmids

Heidi E. Shields
Utah State University

ELECTROPORATION OF *PEDIOCOCCUS PENTOSACEUS* AND THE CURING RATE OF PLASMIDS

by

Heidi E. Shields

Thesis submitted in partial fulfillment of the requirements for the degree

of

HONORS IN UNIVERSITY STUDIES WITH DEPARTMENT HONORS

in

Nutrition and Food Science

Approved:

Thesis/Project Advisor	**Department Honors Advisor**
(Marie Walsh)	*(Jeffery R. Broadbent)*

Director of Honors Program

(Christie Fox)

UTAH STATE UNIVERSITY
Logan, UT

2006

Electroporation of *Pediococcus pentosaceus* and the Curing Rate of Plasmids

Heidi E. Shields
Undergraduate at Utah State University

1355 N. 800 E. 25 F
Logan, UT 84341
(801) 376-3483
hes@cc.usu.edu

Key words: *electroporation, genetic engineering, Pediococcus pentosaceus*
Abstract word count: 189
Text word count: 3,842

Abstract

Genetic engineering is a relatively new process and is a major focus of research in various fields, such as medicine, plant genetics, and food science. The potential applications of genetic engineering include designer drug production, mass protein production, and enhanced food processing. Finding an appropriate host organism to be used in genetic engineering is an important preliminary step. *Pediococcus pentosaceus* has been suggested as a bacterial species with such potential. Since plasmids are often the vector of choice in genetic recombination, this research tests the ability of *P. pentosaceus* to take up and incorporate plasmids into its genome. Two plasmids were chosen as test vectors based on genes they carry for antibiotic resistance. Electroporation was used to introduce the plasmids into the bacteria. It was found that *P. pentosaceus* possesses a rather high level of innate antibiotic resistance to both erythromycin and chloramphenicol. Natural levels of antibiotic resistance made it impossible to identify transformant colonies and, as a consequence, investigate plasmid stability in this host. There still may be possibilities for *P. pentosaceus* in genetic engineering, but a different method of selecting transformants will need to be developed.

Introduction

Obtaining the information necessary to determine the acceptability of a bacterium for use in genetic engineering can be a long process. The purpose of the research performed using the following procedures was to determine whether *Pediococcus pentosaceus* would be a suitable species for use in genetic engineering applications and research. A fair amount of background knowledge in molecular biology, including an understanding of DNA, plasmids, and bacteria in general, is necessary before endeavoring in actual experimentation.

DNA

The information for how a cell functions, grows, adapts, and divides is coded in its genetics. This genetic information is stored in the macromolecule known as deoxyribonucleic acid, or DNA (1). DNA is a polymer composed of units called nucleotides. Each nucleotide consists of a sugar with a phosphate group attached to it and a nucleotide base. There are four different bases: adenine, guanine, cytosine, and thymine. The sugar and phosphate groups link to form the backbone of DNA (1). In biological systems, DNA exists as a double-stranded molecule with the bases pairing to each other in a specific way. Due to the differences in size and structure of the bases, adenine will bind with thymine, and cytosine will bind with guanine. The complementary hydrogen bonding between bases dictates how new DNA is created, as well as how DNA is translated into actual gene products that carry out the necessary functions of a cell (1).

When a cell divides, in order for its daughter cells to be able to function, they must each have a copy of the original DNA so that correct gene products may be created. Thus, cells contain complex enzyme systems that replicate DNA. Enzymes accomplish replication by pulling apart the double-stranded DNA of the parent cell and using each of the individual strands

as templates to create new complimentary strands (1). Each daughter cell then receives a complete double-stranded DNA molecule that consists of one strand from the parent DNA and one newly synthesized strand (2).

Inside cells, DNA is coiled and twisted into a relatively small space. The entire collection of DNA in a cell, including both chromosomes and plasmids, is called a genome (2). Scientists have been working recently on sequencing the genomes of many different organisms such as bacteria, plants, and even humans. Sequencing involves the determination of the order of the nucleotide bases in a strand of DNA (1). Knowing this sequence allows scientists to gain understanding of how cells function individually and how multi-celled organisms function as a whole. Sequencing also enables researchers to be able to manipulate the DNA of cells in order to learn more about them by examining how cells react to the induced changes. DNA manipulation also permits scientists to be able to design cells with the desired genes so that the cells may be used in future applications (1).

Plasmids

Plasmids are generally small circular pieces of double-stranded DNA that are present in cells separately from the chromosome (3). They are often found naturally in bacteria, and replicate separately from the large bacterial chromosome (3). Plasmids can contain genes that will give bacteria valuable selective advantages. Such genes often code for specific antibiotic resistance or toxin production (3). Due to the independence of replication, it is possible for plasmids to either be passed on to every new cell formed or to only be passed on to a few. This will create colonies of cells with varying characteristics that will react differently to certain stimuli. Plasmids may also become incorporated into the bacterial chromosome, becoming part of that molecule and be passed on directly (4).

Bacteria

Bacteria are small single-celled organisms that exist in virtually every earthly environment (1). The DNA in bacteria usually exists as one large coiled chromosome (3). Some bacteria have the ability to take up DNA from their environment and add it to their chromosomal DNA in a process called transformation. Discovering this ability was extremely important in the development of genetic recombination because it allows researchers to introduce engineered DNA into bacteria and study its effects (3).

Most bacteria divide by a process known as binary fission. In this process, the parent cell enlarges and elongates, replicates its chromosome, and divides in half to produce two daughter cells (2). Due to their abundance and extremely short generation time – the time that it takes for one cell to produce two daughter cells (2) – bacteria are perfect vehicles for use in genetic experiments. The short generation time allows researchers to observe effects over many generations in a reasonable time period as well as being able to use selective media to monitor the uptake of DNA.

Genetic Engineering

Genetic engineering is the process of manipulating DNA with precision in a test tube or an organism (1), and it has opened the door to the study of cells in ways previously never imagined. One common way to manipulate the DNA of a cell is to use a plasmid vector. Plasmid vectors are plasmids that may have genes of interest inserted into them and are then introduced to the cell via transformation (1). Using plasmid vectors allows the gene products to be studied and the actual genetics of the cell to be altered. One type of vector that has proven to be useful in many instances is the temperature-sensitive vector. A plasmid is temperature-sensitive when it may be replicated at a certain incubation temperature, but not at another (4).

These incubation temperatures are termed "permissive" and "non-permissive" temperatures, respectively (5). When cells containing such temperature-sensitive vectors are grown at the non-permissive temperature, the plasmid is not passed on to every new cell and is eventually lost from the colony as a whole. The rate at which the plasmid is lost is called the "curing rate" (4). This temperature sensitivity can be used to the researcher's advantage. If a plasmid containing an antibiotic resistance gene is introduced to a group of cells that are then grown in the presence of antibiotic at the non-permissive temperature, the cells are forced to incorporate the plasmid into their chromosome in order to survive. Thus, any genes present on the plasmid will now also be a part of the cell's chromosome (4). Since it is known that any genes that were present on the plasmid are now part of the cell's chromosome, it is possible to observe the effect of the gene of interest in action. A bacterial strain in which experimental DNA has successfully been incorporated into the chromosomal DNA is termed recombinant.

Pediococcus pentosaceus

The specific bacterial species examined in this study for acceptability for use in genetic engineering is classified as *Pediococcus pentosaceus* ATCC 25745. In order to gain an understanding of why *P. pentosaceus*, in particular, was selected and how it will perform in the experiment, some background information regarding *Pediococcus* species in general and *P. pentosaceus* specifically is required. As the genus name suggests, *Pediococcus* are cocci shaped. The cocci grow in pairs and tetrads (6). *P. pentosaceus* is Gram positive – meaning the cell wall consists of a single thick layer of a compound

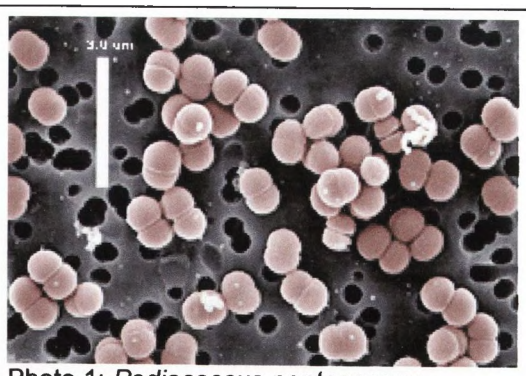

Photo 1: *Pediococcus pentosaceus* by J. Broadbent and B. McManus, USU (9)

known as peptidoglycan (7). It has been found that many *Lactobacillus* species as well as *Leuconostoc mesenteroides* are rather closely related to *P. pentosaceus* genetically (8). Being facultatively anaerobic, *P. pentosaceus* can grow with or without oxygen (9). Energy is obtained through fermentation – the breakdown of sugars without the use of oxygen. Lactic acid is the main byproduct of energy production (6). *Pediococcus* do not form spores and are non-motile (9). In addition, *P. pentosaceus* can grow in acidic environments and grow best at a temperature of 37°C (10).

Pediococcus can be found most often associated with plant matter, but can also be discovered in places such as the gastrointestinal tract, animal hides, and food utensils (6). They appear on fresh and processed meats, and may play a role in the spoilage of some pickles (6). Most species are saprophytes – that is, they live off the decomposition of decaying material – and are often present in fermented vegetable foods (11). *Pediococcus* organisms are an important part of the food industry because they are involved in many significant fermentations, such as that of pickles, green olives, sauerkraut, soy sauce, sausage products, and Cheddar cheese (11). The action of *Pediococcus* bacteria both preserves foods and adds significant flavor (11). *P. pentosaceus* itself has been isolated from several different plants and cheeses. It is a significant member of the non-starter bacteria microflora in most bacterial-riped cheeses (9).

Studies have found that there are native plasmids already present in *P. pentosaceus*. Such native plasmids may carry traits that play significant roles in the characteristic fermentation of *P. pentosaceus* (11). *P. pentosaceus* was selected for study based on its immediate possibilities for involvement in gene recombination for use in the food industry.

pGhost9 and pSA3

The two plasmids selected for study are named pGhost9 and pSA3. Both plasmids contain genes that provide a cell with resistance to erythromycin, and pSA3 also contains chloramphenicol resistance genes (see Fig. 1). The permissive temperature of pGhost9 had previously been determined to be 30°C with a non-permissive temperature of 37°C (14). pSA3 had permissive and non-permissive temperatures of 37°C and 42°C, respectively (12).

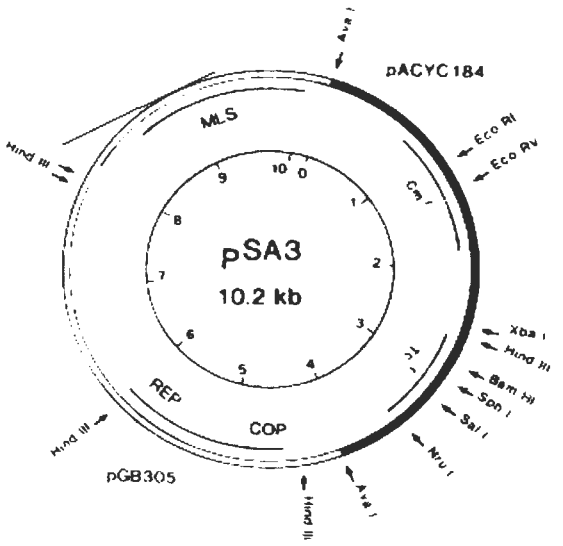

FIG. 1 Physical map of plasmid pSA3. Symbols and abbreviations: ■, pACYC184; ⊐, pGB305; MLS, macrolide linocosamide streptogramin B resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; REP, replication region; COP, copy control region

Figure 1 from: Dao and Ferretti (1985)

Experimental Design

In any experiment, it is vital to have procedures outlined well and results organized and documented. It is also important to consider how credible the findings will be and to make allowances that would increase the reproducibility and repeatability of the experiment. Since bacteria are so abundant and invasive, any procedure that involves bacteria must be designed to avoid any contamination while allowing the growth of the desired species. Avoiding contamination may be a difficult task at times. Aseptic techniques – including autoclaving or filter sterilizing of all media, flaming of bottle openings, and using sterile loops for culture transfers – are followed at all times and a mechanism to select for the desired bacteria are put in place in the procedure.

In the experiments outlined below, a procedure that had been used in the lab previously was followed. Using this procedure added credibility to the experiment because it had already been accepted as being adequate for accomplishing the desired purposes. Blanks were an important part of the study. A blank is a control sample that undergoes the procedure but does not contain one key factor, such as a plasmid. Blanks were run through the electroporation procedure and plated so that growth of the blanks could be compared to that of the transformants – cells that actually picked up the plasmid. By comparison, transformants could be selected for and their identity could be confirmed. Another way credibility was added was that a frozen stock culture – which had been previously isolated and identified in lab – provided the starting material. Using a stock culture ensured that the correct species was being used and as an extra caution, the culture was Gram stained and examined under a light microscope to confirm its identity. The plasmids used were also previously isolated and purified and were kept frozen until the time of use in order to guarantee credibility. Dilutions were made with proper and appropriate solutions in order to maintain the integrity of the delicate DNA samples and bacterial cultures. Duplicate samples were prepared for each step in the procedure so that results could be compared and a more accurate average could be reported rather than a single value. Consistency was maintained throughout the several trials of the experimental design so that results would be comparable. In essence, great care was taken so that the results of the experiment would be both reliable and repeatable.

Methods

Antibiotic Concentrations

In the following procedures, erythromycin was used at a concentration of 5 μg/ ml for experiments with pGhost9, and was later increased to 15 μg/ml (13). Experiments with pSA3

used erythromycin at a concentration of 15 μg/ml and chloramphenicol at a concentration of 7.5 μg/ml (13).

Competent Cell Preparation

Once the bacterial species of interest and appropriate plasmid vectors have been identified, isolated, and purified, the procedure of determining the acceptability of *P. pentosaceus* for use in genetic engineering may be carried out. The first step in the procedure is to prepare cultures of "competent cells." In order for cells to take up DNA from the environment in any significant amounts, they must become "competent" (4). In electroporation, cells are made competent by using electric shock to open pores in the cell walls. Pores cause the cell walls to be more permeable and allow DNA molecules to enter into the cell (1). Preparation of competent cells was accomplished by first allowing cells of *P. pentosaceus* to grow in specialized and previously tested conditions to obtain fresh cultures for use in further treatments. The following steps were performed to prepare cells for electroporation as has been described (10):

1. Prepare comeptent cells – *Pediococcus pentosaceus* ATCC25745 – by overnight growth in MRS-G with 0.5 M sorbitol. (Frozen culture had previously been used to inoculate MRS broth that was incubated at 37°C until used to create competent cells.)
2. Prepare 1.5% inoculation into 800 ml MRS-G containing 0.5 M sorbitol, 3% glycine, and 40 mM DL-threonine.
3. Incubate for 2 to 4 hours (or until $A_{600} = 0.4\text{-}0.6$) at 37°C.
4. Collect cells by centrifugation at 5K for 10 minutes (making sure that the centrifuge is extremely well balanced).
5. Wash twice in 25 ml of cold 0.5 M sorbitol-10% glycerol solution.

6. Suspend cells in 1 ml of cold electroporation buffer (0.5 M sorbitol, 1 mM K_2HPO_4, 1 mM $MgCl_2$, pH 7.0) in microfuge tubes (each tube containing 80 µl of suspension).

After Step #6, cells were frozen for later use, but they may also have been used immediately in the electroporation procedure.

Cell Transformation/Electroporation

After preparing cells for the procedure, the actual electroporation step may be carried out. Electroporation allows incorporation of the plasmid vector of interest into the cells. The prepared bacterial cells are subjected to an electric shock that opens temporary pores in the membrane and cell wall. The pores are large enough that the plasmid, which is present in the cell solution, may enter the cell (1). After being subjected to the electric shock, the cells are allowed to recover and the pores created in the membrane are closed again (1). The following steps were performed in order to carry out electroporation as has been described (10):

1. Mix 80 µl of the suspension (from the previous procedure) with 0.5-1.0 µg of the plasmid (1.0 µg of pGhost9 or pSA3, or an appropriate blank).
2. Transfer the mixture to a cold 0.1-cm electrode-gap electroporation cuvette.
3. Shock the cells using the following parameters for Bio-Rad Gene-Pulser apparatus: 200-Ω resistance, 25-µF capacitance, 1.8 kV field strength. The cells were submitted to one pulse.
4. Immediately after electric pulse, add 2 ml (or enough liquid to fill the cuvette) cold recovery medium – MRS with 0.5 M sorbitol, 20 mM $MgCl_2$ and 2 mM $CaCl_2$.
5. Keep cultures on ice for approximately 5 minutes.
6. Allow cells to recover for 2 hours at the permissive temperature of the plasmid.

Selection of Cells Containing Plasmid (Transformants)

When electroporation is carried out, not every cell will pick up plasmid molecules. The proportion of cells that do actually have plasmid present inside the membrane after electroporation is called the electrotransformation frequency (5). The proportion of transformants to non-transformants is specific to the cells and plasmid being used (5). Due to the fact that not all cells will be transformants and that other experimental errors are possible, it is necessary to identify the cells that contain the plasmid. Because the plasmids used contain antibiotic resistance genes, only cells that contain the plasmid should survive when grown in the presence of an appropriate antibiotic. Thus, recombinant cells were isolated, cultured, and their identity confirmed by the following steps (15):

1. After electroporation, plate cells (100 µl electroporated cells and recovery medium suspension, spread over plate with a plate spreader apparatus) on MRS agar that contains the appropriate antibiotic and incubate at the permissive temperature of the plasmid for 48 hours.
2. Isolate antibiotic-resistant colonies and confirm presence of pGhost9 or pSA3 in transformants by agarose gel electrophoresis – which identifies DNA pieces by their relative sizes.

Curing Rate Determination

Once cells containing the plasmid of choice are obtained, it is possible to determine the curing rate of the plasmid. The curing rate is found by accomplishing the following steps over time (5):

1. Propagate transformants in MRS broth without antibiotic at the permissive temperature of the plasmid and at the non-permissive temperature of the plasmid for 24 hours.

2. Each night, transfer cells from broth to fresh antibiotic-free MRS broth and return to respective incubation temperatures as well as plating cells on MRS agar with and without antibiotic to determine the fraction of the *Pediococcus* population that still contains plasmid (plates counted after being incubated at permissive temperature overnight).

Results (13)

Observations of the wild-type – unaltered – *Pediococcus pentosaceus* cells were as expected. They appeared as round cocci shaped cells attached to each other in somewhat cluster-like formations. The cells stained purple with a Gram stain test, meaning they are Gram positive. Observing the cells at the beginning of the experimentation allowed a mental image of the working species to be gained for future reference.

All the solutions and media used in the procedures were carefully weighed out, mixed, and autoclaved in advance in order to facilitate smooth and efficient carrying out of the experiments. No obvious visual problems such as clouding, which would indicate contamination, were observed with any of the media used.

Many of the results of the experimentation were not what they were expected to be. Some unanticipated reactions were observed and changes had to be made to the procedures in order to continue testing. To begin with, the plates used for growth of bacteria after electroporation were first incubated in a small incubator. The temperature in the incubator was controlled with the use of fans that circulated air. The fans resulted in the agar plates becoming extremely dried out and deformed, and the colony counts to be unreadable. To compensate for the dry conditions of the incubator, the plates were subsequently wrapped in a plastic bag in a stack to protect from desiccation.

Once electroporation had been performed using pGhost9 as the plasmid vector, it was found that the blanks were also able to grow on agar containing erythromycin, rather than just the transformants. The electorporation procedure was repeated with different cells to confirm that the cells without plasmid were actually growing in the presence of antibiotic. The concentration of antibiotic was increased by a factor of three and the procedure was repeated along with several transfers. However, the blank samples continued to be able to grow along with the transformants. Thus, the method of selection to determine the number of colonies that contained plasmid was unreliable.

Wild-type resistance to erythromycin was investigated by inoculation in MRS broth with increasing levels – 5 to 25 μg/ml – of erythromycin. The test tubes were incubated at 37°C overnight. Unfortunately, growth was found in all tubes, which demonstrated that *P. pentosaceus* has a relatively high innate resistance to erythromycin.

In order to test for the possibility of using a different plasmid for the same electroporation and selection experiment, the same procedure used for determining wild-type erythromycin resistance was performed to determine wild-type chloramphenicol resistance. These experiments used a similar dilution spectrum of chloramphenicol – using a 1 mg/ml Cm in 100% ethanol solution which was filter sterilized. Although there was a small amount of growth in all tubes, it was thought that using a combination of both erythromycin and chloramphenicol would be sufficient to select for transformants with a plasmid that contained resistance genes for both antibiotics. When electroporation was carried out using pSA3, however, there was still some growth apparent in the blanks. In fact, almost equal growth of the blanks and the transformants occurred. Also, growth did not appear as individual colonies as was expected, but appeared more as a smear or lawn.

Because *P. pentosaceus* does not usually grow in such a pattern, cultures were tested for contamination by staining and visualizing with a light microscope. A simple metabolic test – the presence of catalase – was also performed to determine whether there was contamination present. There did not appear to be any non-*Pediococcus* cells present. Once contamination was ruled out, the wild-type resistance to a combination of erythromycin and chloramphenicol was tested. Resistance was investigated by inoculation in MRS broth with increasing levels of chloramphenicol and constant levels of erythromycin. Erythromycin was kept at a concentration of 15 μg/ml while chloramphenicol was varied from 5 to 20 μg/ml. Test tubes were again incubated at 37°C overnight and, once again, growth was observed in all tubes.

Conclusions

The main conclusion drawn from the proceeding experiments and results was that *P. pentosaceus* is not a good candidate to be used for the transformant selection procedure using plasmids conferring erythromycin or chloramphenicol resistance (13). A different selective marker, however, may work. Finding the inadequacy of the procedure was a surprise because the sequencing of the genome of *Pediococcus pentosaceus* has not revealed anything that would indicate the kind of high resistance to antibiotic found upon further testing (13). Also, the same type of procedure has been used on other *Pediococci* and has been successful. In other words, in the past, recombinant colonies of *P. pentosaceus* have been selected based on their ability to grow in the presence of erythromycin (10).

It is possible that something is present in the genome that has simply not been discovered or identified that would give the preceding results. It may be more likely, however, that something has occurred with the particular frozen stock culture used that would cause it to react

the way it did. The stock culture may have been misidentified or altered in some way or some other human errors may have occurred during the procedure that would have caused the results to be what they were. Care was taken to try to minimize the effect of human error, but it is always a possibility.

Future Research/Possibilities

The goal of performing the procedures outlined above was to assess the possibility of using *P. pentosaceus* for genetic engineering. One thought was to give *P. pentosaceus* a better ability to ferment lactose so that it might be used as a starter culture in the dairy industry (10). From the results found, genetic recombination and engineering may still be possible with *P. pentosaceus*, but some other method of determining transformants will need to be developed and used. A different vector with a different antibiotic resistance gene may be used or other strains or species of *Pediococci* could work. Some investigation should be done as to why the results of the experiment to determine the acceptability of *P. pentosaceus* for use in genetic engineering turned out the way they did. There are definitely still many possibilities for *P. pentosaceus*, but more research needs to be conducted. The process of research continues to be a very dynamic study with endless future possibilities.

References

1. Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2002. Molecular Biology of the Cell. 4th ed. Garland Science. New York, NY.

2. Talaro, K. and A. 2. 1996. Foundations in Microbiology. 2nd ed. Wm. C. 7 Publishers. Dubuque, IA.

3. Black, J. G. 1999. Microbiology Principles and Explorations. 4th ed. Prentice-Hall, Inc. Upper Saddle River, NJ.

4. Prescott, L., J. Harley, and D. Klein. 2005. Microbiology. 6th ed. McGraw-Hill Companies, Inc. New York, NY.

5. Bhowmik, T., L. Fernandez, and J. Steele. 1993. Gene replacement in *Lactobacillus helveticus*. Journal of Bacteriology, 175(19): 6341-6344.

6. Jay, J. M. 2000. Modern Food Microbiology. 6th ed. Aspen Publishers, Inc. Gaithersburg, MD.

7. Brown, A. E. 2005. Selected Chapters from Benson's Microbiological Applications Complete Version. 9th ed. The McGraw-Hill Companies, Inc. San Fransisco, CA.

8. Stackebrandt, E., V. J. Fowler, and R. Woese. 1983. A Phylogenetic Analysis of *Lactobacilli*, *Pediococcus pentosaceus* and *Leuconostoc mesenteroides*. Systematic and Applied Microbiology, 4(3):326-337.

9. Anonymous. JGI Microbes: *Pediococcus pentosaceus*. http://genome.jgi-psf.org/draft_microbes/pedpe/pedpe.home.html. Date accessed: March 29, 2006. Source of Photo 1.

10. Caldwell, S. L., D. J. McMahon, C. J. Oberg, and J. R. Broadbent. 1996. Development and Characterization of Lactose-Positive *Pediococcus* Species for Milk Fermentation. Applied and Environmental Microbiology, 62(3):936-941.

11. Graham, D. C. and L. L. McKay. 1985. Plasmid DNA in Strains of *Pediococcus cerevisiae* and *Pediococcus pentosaceus*. Applied and Environmental Microbiology, 50(2):532-534.

12. Dao, M. L. and J. J. Ferretti. 1985. *Streptococcus-Escherichia coli* Shuttle Vector pSA3 and Its Use in the Cloning of Streptococcal Genes. Applied and Environmental Microbiology, 49(1):115-119.

13. Shields, H. E. 2006. *Pediococcus pentosaceus* and Plasmids lab book. In possession of Dr. Jeffery R. Broadbent, Utah State University.

14. Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss. 1996. Efficient Insertional Mutagenesis in *Lactococci* and Other Gram-positive Bacteria. Journal of Bacteriology, 178:931-935

15. Low, D., J. A. Ahlgren, D. Horne, D. J. McMahon, C. J. Oberg, and J. R. Broadbent. 1998. Role of *Streptococcus thermophilus* MR-1C Capsular Exopolysaccharide in Cheese Moisture Retention. Applied and Environmental Microbiology, 64(6):2147-2151.