"Lactic Acid Bacteria Genome Consortium" (LABGC). The mission of LABGC is to forward functional genomic studies on the LAB. Each LAB strain is linked to a designated investigator who will be primarily responsible for gap closure and publishing of the genome sequence. During 2002, JGI generated draft sequence of the 11 genomes. This was accomplished by sequencing shotgun small-insert libraries (2-3 kb) of each microbe's DNA to achieve 10X coverage. Where possible, this coverage was supplemented with 5X coverage from large-insert cosmid libraries (40 kb). The sequence data were incorporated into an assembly and then ordered into scaffolds. The draft sequence was then computationally annotated by Oak Ridge National Laboratories. This annotation can be viewed from the Joint Genome Institute web site (http://www.jgi.doe.gov/). This web site also allows researchers to scan through the draft annotation by metabolic pathway or functional gene categories. Moreover, specific DNA or protein searches are possible through a Basic Local Alignment Search Tool (BLAST) tool within the site.

The LABGC group is currently working with Fidelity Systems, a private company, to close all 11 genomes, with completion expected by 2004. Completion and publication of the genome sequences in the LABGC project will be one of the most significant milestones in LAB research since the initial isolation and use of lactic starter cultures nearly 100 years ago. Since these microorganisms are critically involved in the generation of $20 to $30 billion worth of food products in the U.S.A., this milestone will strongly impact the food and beverage fermentation industries. Moreover, the public availability of accumulated sequence data will foster an expansion of genomics research on LAB in the U.S.A. and abroad.

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A Genomic Study of *Leuconostoc mesenteroides* and the Molecular Ecology of Sauerkraut Fermentations

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ABSTRACT: Most vegetable fermentations are carried out without the use of starter cultures, using a technology that has remained virtually unchanged for centuries. As the scale of industrial vegetable fermentations increases worldwide, the disposal of salt (chloride) waste, which is generated during processing of these products, has become a major problem. The development of new technology to reduce the amount of salt used in vegetable fermentations may require a greater understanding of the microbial ecology of these fermentations, and may also require the use of starter cultures.

Introduction

We have investigated the microbial ecology of commercial sauerkraut fermentations in the United States using molecular fingerprinting and DNA sequencing techniques. Our objectives were to: determine if the classical picture of fermentation microbiology was complete; determine if bacteriophages (which have not previously been isolated from vegetable fermentations) were present; determine the impact of bacteriophages on the natural succession of lactic acid bacteria; and determine the impact of bacteriophage on potential starter cultures. We carried out a 2-y study of commercial sauerkraut fermentations, and we found a complex and previously unknown ecology of bacteriophages and host cells. Novel species of lactic acid bacteria were identified and bacteriophages active against potential starter cultures were detected. A PCR

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fingerprinting method was used to follow the progress of unmarked Leuconostoc mesenteroides starter cultures in sauerkraut fermentations. We found that selected starter cultures can predominate in the initial stages of sauerkraut fermentations. Our results indicate that bacteriophages may play an important role in the natural succession of lactic acid bacteria occurring in sauerkraut and other vegetable fermentations. We conclude that the impact of bacteriophages on potential vegetable fermentation starter cultures needs to be carefully evaluated as low-salt, controlled fermentation technology is developed.

Sauerkraut fermentation microflora

The classical picture of the microbial ecology of cabbage fermentation includes 4 predominant species of lactic acid bacteria (LAB): *Leuconostoc mesenteroides, Lactobacillus brevis, Pediococcus pentosaceus*, and *Lactobacillus plantarum* (Pederson and Albury 1969). *Leuconostoc mesenteroides* is the major species in the early, heterofermentative, stage of fermentation, and *L. plantarum* is the major species involved in the late, homofermentative, stage of fermentation. However, many other species of LAB have also been isolated from sauerkraut or related products (Harris and others 1991; Murcia-Martinez and Collins 1991). Recent evidence from our laboratory has indicated an unsuspected diversity of LAB may be present in sauerkraut fermentation (Breidt, Johanningsmeier and Fleming, unpublished). The development of molecular techniques to study microbial ecology has advanced greatly since they were first demonstrated in the mid-1980s. These techniques offer new opportunities for the analysis of the structure and species composition of microbial communities. There is strong evidence that in many environments the genetic diversity, as assessed by molecular techniques, far exceeds the microbial diversity determined by culture and biochemical methods (Ward and others 1990). The objectives of research reported here were to study and understand microbial diversity in commercial sauerkraut fermentations and to sequence and characterize the genome of *L. mesenteroides*, which initiates most vegetable fermentations.

In a 2-y study of the microbial ecology of commercial sauerkraut fermentations, over 1,000 isolates of LAB were obtained from 4 fermentation tanks, as well as over 170 bacteriophage isolates. These fermentations were each carried out with approximately 90 tons of cabbage. The results from microbial and chemical analyses indicated that the fermentation in the 4 commercial sauerkraut fermentation tanks were normal and consistent with those described (Fleming and others 1984). Chemical analysis of brine samples and biochemical characterization of each isolate had confirmed that the fermentation can be categorized into 2 stages, an initial heterofermentative stage, followed by a homofermentative stage, as described by Pederson and Albury (1969). LAB became the predominant species after the fermentation started, as reflected by plate counts on selective and differential agar media.

Molecular ecology

We used an rRNA operon intergenic transcribed spacer (ITS-PCR) database for LAB to identify major bacterial species present in commercial sauerkraut fermentations. *Leuconostoc mesenteroides* is not the only major species found associated with sauerkraut fermentation during the early heterofermentative phase. *Weissella* spp. were recovered together with *L. mesenteroides*. These organisms were the predominant LAB in the early heterofermentative stage (1 to 7 days after the start of fermentation). Previously, *Weissella* spp. were characterized as members of *Leuconostoc* genera (Aguirre and Collins 1993). The newly identified LAB species, *Leuconostoc fallax*, was also present at the early stages of fermentation. Many LAB species isolated during the first 14 d of fermentation had not been previously recovered from sauerkraut fermentations, including: *Weissella* spp., *Lactobacillus argentinum, Lactobacillus coryniformis, Leuconostoc citreum, Lactobacillus paraplantarum,* and *Lactobacillus paracasei*. Surprisingly, we only recovered small numbers of *L. brevis* and *P. pentosaceus*. This contradicted the previous reports that both of these LABs were considered to be major bacterial species involved in sauerkraut fermentation (Pederson and Albury 1969). The ecology of these commercial fermentations has been shown to be more complex than previously reported. The discovery of a variety of LAB species in the sauerkraut fermentation encourages further investigation of the roles of this species in the fermentation of cabbage and perhaps other vegetables.

Bacteriophages

In addition to the ecology of bacteria, we investigated the ecology of bacteriophages (phages) in commercial sauerkraut fermentations to explore the possible role phages may play in microbial succession during the fermentations and to characterize the predominant phage. A total of 171 independent phage isolates, including at least 26 distinct phages, which were further characterized. Host range and the temporal sequence of occurrence of these phages were determined. Bacterial hosts included *Leuconostoc*, *Lactobacillus*, and *Weissella* species, which were identified by ITS-PCR and 16S rDNA sequence analyses. It was found that there were 2 phage-host systems in the fermentations, with the dividing line occurring around day 7 after the start of the fermentations, corresponding to the population shift from heterofermentative to homofermentative LAB. The data suggest that phages may play a role in the microbial ecology and the succession of LAB species in vegetable fermentations. Eight phage isolates that were independently obtained 2 or more times were further characterized. They belonged to the *Myoviridae* or *Siphoviridae* family and showed distinct host ranges and DNA fingerprints. More research is needed to fully evaluate the impact of phages on vegetable fermentations.

Leuconostoc mesenteroides

The predominant microorganism in the early stages of cabbage fermentations is *L. mesenteroides*. This organism has a major impact on the flavor and quality of fermented cabbage products, including sauerkraut and kimchi (Fleming and others 1995). *Leuconostoc* species are epiphytic bacteria that are widespread in the natural environment and play an important role in several industrial and food fermentations. *Leuconostoc mesenteroides* is a facultative anaerobe requiring complex growth factors and amino acids (Reiter and Oram 1982; Garvie 1986). Under microaerophilic conditions, a heterolactic fermentation is carried out. Glucose and other hexose sugars are converted to equimolar amounts of D-lactate, ethanol, and CO_2 via a combination of the hexose monophosphate and pentose phosphate pathways (Demoss and others 1951; Garvie 1986; Gottschalk 1986). Other metabolic pathways include conversion of citrate to diacetyl and acetoin (Cogan and others 1981) and production of dextrans and levan from sucrose (Broker 1977; Alsop 1983). Viscous polysaccharides produced by *L. mesenteroides* are widely recognized as causing product losses and processing problems in the production of sucrose from sugar cane and sugar beets (Tallgren and others 1999). The first observation of the production of polysaccharide "slime" from sugar dates to the earliest days of the science of microbiology. Pasteur attributed this activity to small cocci, presumably *Leuconostoc* species (Pasteur 1861). Commercial production of dextrans and levans by *L. mesenteroides* for use in the biochemical and pharmaceutical industry has been carried out for more than 50 years (Alsop 1983; Sutherland 1996).

Genome sequencing

To further characterize this organism, the whole genome sequence of *L. mesenteroides* ATCC 8293 (the ATCC "type" strain) originally isolated from olive fermentations has been determined. Sequencing was carried out at the U.S. Department of Energy Joint Genome Institute (Walnut Creek, CA). This was part of a larger project to sequence the genomes of 11 LAB by researchers from 7 universities across the U.S., who formed the Lactic Acid Bacteria Genome Consortium or LABGC. Bacteria sequenced included: *Lactobacillus gasseri*, *L. casei*, *L. bulgaricus*, and *L. brevis*, as well as *L. mesenteroides*, *Oenococcus oeni*, *Lactococcus cremoris*, *P. pentosaceus*, *Streptococcus thermophilus*, *Brevibacterium linens,* and *Bifidobacterium longum*.

A shotgun cloning method was used to determine draft sequences to greater than 10X coverage, which resulted in approximately 100 contiguous fragments for *L. mesenteroides*. Gap closing and finishing was carried out by Fidelity Systems, Inc. (Gaithersburg, MD), using a novel "whole genome" sequencing method that does not involve cloning. The finished chromosome of *L. mesenteroides* was found to have 2038395 bp and contains a predicted 1924 protein-encoding genes. Putative biological functions could be assigned to 54% of the predicted proteins. Consistent with the classification of *L. mesenteroides* as heterofermentative LAB, the genome encoded all enzymes required for the 6-phosphogluconate phosphoketolase pathway. Furthermore, *L. mesenteroides* encoded a number of pyruvate dissipating enzymes that are predicted to catalyze the production of many metabolites leading to various end products of fermentation. A large proportion of genes encoded for carbohydrate transport and utilization. The chromosome also contained genes belonging to phage remnants and mobile genetic elements. Currently, annotation and comparative genomics studies of all 11 LAB sequenced as part of this project are underway, and functional genomics studies are also being carried out.

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