ABSTRACT

SOUTHER, NICOLE. Development of a Multiplex PCR to Identify *Lactobacillus acidophilus* NCFM, a Probiotic Organism. (Under the direction of Dr. Todd R. Klaenhammer.)

Lactobacillus acidophilus NCFM is a probiotic organism used in food products and dietary supplements since 1972. A recent study recovered strains of *L. acidophilus* from various food- and probiotic products currently being used in the food industry demonstrating a genetic fingerprint comparable to that of *L. acidophilus* NCFM. Reliable strain identification in commercial products is of paramount in regards to food safety and consistent probiotic features. A multiplex PCR system to identify *Lb. acidophilus* NCFM was established and used to distinguish this strain from other *Lactobacillus* species and *Lactobacillus acidophilus* strains. The genome of this organism revealed the presence of seven distinct transposase elements. Three of these genes were found in multiple, highly conserved copies ranging from two to six replicas, indicating relatively recent multiplication events. A total of four different primer sets were designed around four selected transposase genes to generate semi-conserved PCR products. Various strains of *L. acidophilus*, including NCFM, were tested with the primer sets. The multiplex reaction identified six of these strains as NCFM, or closely related to *Lb. acidophilus* NCFM. Five of the *L. acidophilus* strains showed distinguishing banding patterns. We were able to demonstrate the use of multiplex PCR to identify *L. acidophilus* NCFM and NCFM-related strains, and distinguish these from other species and closely-related strains of *L. acidophilus.*

Development of a Multiplex PCR to Identify *Lactobacillus acidophilus* NCFM – a Probiotic Organism

by

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Biography

Nicole Souther is the daughter of Ricky Souther and Nanci Slane. She has one sister, Lauren Souther, and plenty of aunts, uncles and cousins. She graduated in 2001, from North Carolina State University with a Bachelor of Science degree in Food Science, and a Bachelor of Science degree in Biological Sciences. During her undergraduate studies, she met Alan House, and they have been together ever since. She has worked in the Plant Pathology and Entomology Departments at North Carolina State University, participated in an ecological study abroad program in Brazil and worked at Syngenta Biotechnology, Inc. before returning to North Carolina State University to pursue a Master of Science degree in Food Science, with a minor in Biotechnology. During her graduate studies, she worked under the direction of Dr. Todd Klaenhammer. She is currently working for Wyeth Vaccines.

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Occasionally gratitude for work and effort individuals perform everyday is a nice thing to receive and I have a long list of people I would like to acknowledge. For starter's, I had the privilege to work in a lab filled with talented, ambitious, and genuinely nice people. I was constantly impressed with how dedicated they were to their projects and careers. Also, they kept work days lively and interesting.

I began working in the lab closely with Eric Altermann, and he was the unfortunate soul with the responsibility of training me on bioinformatic software. I have many inadequacies and using computers and anything related to computers is at the top of the list. Eric persevered and his patience and friendship mean a lot to me.

Our lab technician Evelyn Durmaz is an unbelievable wealth of knowledge that is constantly taxed. She knows details ranging from laboratory budgets to science journal citations over the past 20 years. Thanks for always being there to answer questions and to give help.

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though I knew very little about genomics, less about bioinformatics, and despite the fact he knew I didn't understand what he was talking about when I interviewed with him. He took me anyway, and he never once made me feel stupid and to a beginning graduate student, that respect helps you through your mistakes and self doubt. To his credit I learned quite a bit about those subjects so that when I left, we were able to speak freely about my project.

Work is one thing, home is another. I have to mention my roommates, Amanda Pokrzywa and Maria (you might know her as Revonda) Pokrzywa. I lived with both these ladies for four years, and am still living with Amanda. To quote a magnet I purchased recently "A friend is someone who knows all about you and likes you anyway"— this is certainly relevant here. Whenever I was stressed, ill, or difficult, they were still my friends, and ALWAYS supportive. It's a great thing to have someone to go to and complain or vent and who will take your side even if it is ridiculous, I have two such individuals, I am quite lucky. I consider these ladies family, and need to thank them for being such good friends.

Lauren, my sister, is one who leads an extraordinary lifestyle and has done so many cool things even though she is quite young, and has no money. I admire how she is determined to stick with an archaeology program even though she suffers constant ridicule for her decision. Our family has had some rough patches over the past couple of years and her being there has made these times easier for me. She can always make me laugh, and I love having her for a sister.

My parents, Ricky and Nanci, are two of the hardest working people you'll ever meet. You want to see work ethic, look at them. As I get older, I understand more and

more about their sacrifices. The fact I had the luxury to go to college, not to mention graduate school, is largely due to them. I don't have words to express my gratitude to them.

And finally, I have a few words to say about my 'other half', Alan. We have been together for a few years now, and I really love this guy. We talk on the phone and see each other daily and I still get excited when he calls and when I know I am going to see him. I am amazed at his creativity and how he is always delving into various projects. He's a huge part of my life and his advice, support and love keeps me going with a lighter step. My life is richer by having him in it.

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Introduction

Lactic acid bacteria are a diverse group of microorganisms important in food fermentations and as natural inhabitants of the human gastrointestinal tract (Klaenhammer, 1998). Some species belonging to this group of microorganisms are considered probiotic bacteria. Probiotics are living microorganisms which exert health benefits beyond inherent nutrition when ingested in certain numbers (Guarner& Schaafsma, 1998). In order for a bacterial strain to be classified as a probiotic, the microorganism must meet certain criteria. This includes but is not limited to the following: the strain belongs to a genus of human origin; exhibits stability against bile, acid, enzymes and oxygen; has the ability to adhere to intestinal mucosa; is able to colonize or survive passage through the gastrointestinal tract; demonstrates antimicrobial activity; and is safe for human consumption (Kailasapathy & Chin, 2000).

An example of a probiotic microorganism is *Lactobacillus acidophilus* NCFM which was isolated from a human source and is a well characterized probiotic strain (Gilliland et al. 1975). NCFM exhibits probiotic health benefits including alleviation and prevention of diarrhea, alleviation of symptoms of lactose intolerance, immunostimulation, antimicrobial activity, and the reduction of fecal mutagenic enzymes (Percival, 1997; Rao et al., 1999; Sanders & Klaenhammer, 2001; Simenhoff et al., 1996). NCFM has been used commercially since the 1970's and has a history of safe use in consumer products, primarily in yogurts and acidophilus milks (Sanders & Klaenhammer, 2001).

Strain identification in probiotic research and commercial use is essential. The health benefits exhibited by these microorganisms are thought to be strain related

(Lucchini, et al. 1998). Tracking an industrial strain is necessary to ensure commercial product quality and to protect proprietary cultures. This presents a difficult challenge for researchers. Numerous approaches have been taken to develop methods to readily classify and identify probiotic microorganisms. These include both phenotypic and molecular techniques.

Phenotypic tests examine morphological, biochemical, and physiological properties of the microorganisms in question. These tests are often not definitive for species identification because different species within a genus can demonstrate the same fermentation patterns and growth requirements (Andrighetto et. al., 1998). Also, different culture conditions and the diversity of strains can create difficulty in reproducing the results of phenotypic tests (Tannock, 1999).

The use of molecular techniques is a more desirable approach for both species and strain identification and classification of lactic acid bacteria (Schleifer, et. al., 1995). Sequence analysis and comparison of the 16S ribosomal ribonucleic acid (16S rRNA) has been the most extensively used molecular technique to establish phylogenetic relatedness of bacteria (Schleifer, et. al., 1995, Tannock, 2001). Among lactobacilli, variable regions V1, V2, and V3 of the 16S rDNA has species-specific information (Tannock, 1999). Both universal and species specific primers can be used to amplify variable regions of the 16S rRNA via the polymerase chain reaction (PCR). Once amplified, the product can be sequenced to identify the genus and species of the bacterium (Kullen, et al., 2000; Massi, et. al, 2004; Tannock 2001). There are closely-related species with the *L. acidophilus* complex that are difficult to distinguish, even by sequencing the rRNA regions. Notably,

L. johnsonii and *L. gasseri* are very similar over these sequences and can be difficult to distinguish (Altermann, Durmaz, and Klaenhammer, unpublished).

The amplified 16S rRNA fragments can also be digested with restriction enzymes to generate a profile for the microorganism. This technique is called ARDRA, amplified ribosomal DNA with restriction analysis (Andrighetto, et. al., 1998; Grifoni, et. al., 1995; Vaneechoutte, et. al., 1992). This method has been successful for species discrimination of lactobacilli, but not successful in strain differentiation (Drake, et. al., 1996; Kullen, et. al., 2000).

Species of lactic acid bacteria can be differentiated by the ~500 base pair spacer region between the 16S and 23S rRNA genes (Tannock, 2001). Prokaryotes contain the 16S, 23S, and 5S genes in their rRNA genetic loci. These genes are separated by spacer regions which can vary in length and sequence at the genus and species level (Jensen, et. al., 1993). The spacer region between the 16S and 23S rRNA genes can be amplified by PCR and the polymorphisms in the resulting product can be visualized by three approaches. The PCR product can be digested with restriction enzymes and the resulting fragments can yield a profile for a particular species (Barrangou, et. al., 2002; Breidt and Fleming, 1996; Clark, et. al., 2001; Jensen, et. al., 1993). The PCR product can also be used to generate a species specific probe (Barry, et. al., 1990; Barry, et. al. 1991). Another technique is to design specific primers and use stringent PCR conditions to target the intergenic space for the microorganism of interest. This has proved useful in grouping species into individual genera (Berthier & Ehrlich, 1998; Jensen, et. al., 1993; Moschetti, et. al., 2001).

Random amplification of polymorphic DNA (RAPD) is another molecular method to fingerprint bacteria. This method uses random primers and low stringency conditions in the PCR allowing the primers to anneal to the DNA template. The number and location of priming sites will vary among closely related bacteria so that distinguishing fingerprints can be visualized electrophoretically (Clark, et. al., 2001; Tannock, 2001). This method has been successful in differentiating strains of bacteria (Barrangou, et. al., 2002; Drake, et. al., 1996; Fitzsimons, et. al., 1999; Johansson, et. al., 1995). Even though this technique can discriminate bacterial strains, it certainly has a major drawback—it is highly variable. Any changes to the reaction conditions can alter the results of RAPD. Also, because non-specific primers are used, this procedure is very vulnerable to contamination. These characteristics often inhibit the reproducibility of results for a particular fingerprint generated by this method (Clark, et. al., 2001; Fraga, et. al., 2002; Power, 1996)

Restriction endonucleases can also be used to 'cut' chromosomal DNA to generate a fingerprint for a microorganism. A rare-cutting enzyme can be used to generate large fragments of chromosomal DNA which can be separated by pulse field gel electrophoresis (PFGE). This procedure has been successful in generating unique banding patterns for particular strains of bacteria (Clark, et. al., 2001; Gautier, et. al., 1996; Moschetti, et. al., 2001; Schleifer, et. al., 1995). Although this procedure has high discriminatory power, it can generate large quantities of bands which can make interpreting specific patterns difficult (Schleifer, et. al., 1995). It is also a technically challenging and labor intensive procedure (Clark et. al., 2001).

The use of a specific probe on digested DNA can generate a distinctive pattern for a particular bacterial strain (Grifoni, et. al., 1995; Schleifer, et. al., 1995). Probes targeting the rRNA gene sequence are commonly used on digested chromosomal DNA to generate bacterial fingerprints. This is called ribotyping (Tannock, 2001). The 16S and 23S gene sequences have been used to generate a species specific probe to identify lactic acid bacteria, including *Lactobacillus* species (Andrighetto, et. al., 1998; Chagnaud, et. al., 2001; and McCartney, et. al., 1996). However, closely related species often have very similar rRNA sequences and can not be differentiated by ribotyping (Berthier & Ehrlich, 1998).

Other unique DNA sequences in a microorganism can be used as species and/or strain specific markers. Insertion sequence elements have been used as probe targets to generate specific DNA patterns through southern hybridization. This has been done successfully for many species of bacteria, including *Mycobacterium tuberculosis*, *Vibrio cholerae*, and *Bordetella pertussis* and *Salmonella typhimurium* (Bik, et. al., 1996; Mazurek, et. al., 1991; Stanley, et. al., 1993; and van Loo & Mooi, 2002).

Insertion sequence (IS) elements are small, pieces of DNA, < 2.5 kb, with a simple organization and are capable of inserting into multiple sites of a target molecule (Mahillon & Chandler, 1998). These elements contain no genetic information except that required for transposition (Archer, et. al., 1996). Transposition of these elements can result in rearrangements in bacterial genomes (Salvatore, et. al., 2001). Structural changes caused by transposition can cause insertions, deletions, inversions, and translocations (Bartosik, et. al., 2003). The presence of insertion sequences can affect gene expression and virulence in bacteria and can be associated with pathogenicity

islands and drug resistance (Kong et al., 2003; Mahillon & Chandler, 1998; Paulsen, et. al., 2003).

As of 2001, approximately 800 insertion sequences from 196 bacterial species have been identified (Mahillon & Chandler, 2002). As mentioned earlier, these elements have been used as unique molecular markers to identify many bacteria. This approach has been extensively applied to *Mycobacterium tuberculosis*. The mobile element IS*6110* in *Mycobacterium tuberculosis* has been the target used for strain specific identification and to generate a database of restriction fragment length polymorphism, RFLP, patterns for various strains of *M. tuberculosis* (Suffys, et. al., 2000). It has been found that moderate insertional activity is exhibited by IS*6110*. This activity is not excessive but transposition occurs enough to differentiate closely related strains of *M. tuberculosis* (Mazurek, et. al., 1991; Niemann, et. al., 2000).

The *Mycobacterium tuberculosis* complex demonstrates 99.9% similarity at the nucleotide level for the 16SrRNA sequence. Consequently, this area can not be used for strain identification in this complex (Haddad, et. al., 2003). Many studies have found genotyping with IS*6110* probes has high discriminatory power to differentiate strains of *Mycobacterium tuberculosis* (Bifani, et. al., 2000; Bifani, et. al., 2001; Kurepina, et. al., 1998).

Insertion sequence elements have also been identified in probiotic bacteria. *Lactobacillus acidophilus, Lactobacillus plantarum, Lactococcus lactis, Lactobacillus gasseri,* and *Lactobacillus johsonii* all have IS elements present in their genomes (Bolotin, et. al, 2001; Clark, et. al., 2001; Klaenhammer, et. al., 2002; Kleerebezem, et al., 2003; Pridmore et. al., 2003). The wide distribution of IS elements in these bacteria

and the success of using IS elements for identifying other microorganisms provides an opportunity to use mobile elements to differentiate probiotic microorganisms.

A Closer Look at Insertion Sequences in Lactic Acid Bacteria

As mentioned earlier, the nucleotide sequence for the entire genome has been completed for *Lactobacillus acidophilus, Lactobacillus plantarum, Lactococcus lactis, Lactobacillus gasseri,* and *Lactobacillus johsonii* (Bolotin, et. al, 2001; Klaenhammer, et. al., 2002; Kleerebezem, et al., 2003; Pridmore et. al., 2003). Predicted transposase genes belonging to IS families were found within the genome of each of these strains. Many of these putative proteins were found in multiple copies, some were present only once in the genome, and some demonstrated amino acid sequence homology to existing transposase genes of known IS families.

The transposase genes predicted were examined for *Lb. acidophilus*, *Lb. plantarum*, *L. lactis*, and *Lb. johnsonii*. The amino acid sequence for each putative transposase of each of the aforementioned bacteria was used to create a multiple sequence alignment using CLUSTALX (Thompson, et. al., 1997). The resulting dendrogram file was edited using Tree View (version 4.3 © 1997, R. D. M. Page). The tranposase genes selected for analysis were equal to or greater than 150 amino acids in length and demonstrated amino acid sequence identity greater than or equal to 30 % to transposase amino acid sequences in the non-redundant database (http://www.ncbi.nlm.nih.gov/entrez/) and/or the IS database (http://www-IS.biotoul.fr). By using these criteria, many transposase fragments, truncated transposase open reading frames (ORF), unknown proteins, and predicted ORFs identified as ORF A for a

particular transposase were eliminated from the analysis. The predicted IS elements used in this study are all listed in Table 1.

The goal of this analysis was to generate an overview of the distribution of potential IS elements in the genomes of lactic acid bacteria that have been sequenced. This analysis was not intended to demonstrate the phylogenetic relationship among these bacteria and/or IS elements. Nor was this analysis intended to fully characterize the predicted IS elements identified in each of the microorganisms. Rather by searching for the number and identity of potential IS elements in the genomes of these bacteria, the possible use of these mobile elements as molecular markers and tools for identification for probiotic lactic acid bacteria can be considered.

Table 1. IS Elements of Lactic Acid Bacteria Used in Analysis

Microorganism	IS element	Locus Tag	Accession Number	IS Family*
Lb. plantarum	ISP*1*-1	lp_1064	NP_784752	ISL*3*
Lb. plantarum	ISP*1*-2	lp_1463	NP_785074	ISL*3*
Lb. plantarum	ISP*1*-3	lp_1537	NP_785141	ISL*3*
Lb. plantarum	ISP*1*-4	lp_2164	NP_785667	ISL*3*
Lb. plantarum	ISP*1*-5	lp_2617	NP_786032	ISL*3*
Lb. plantarum	ISP*1*-6	lp_3036	NP_786355	ISL*3*
Lb. plantarum	ISP*1*-7	lp_3308	NP_786560	ISL*3*
Lb. plantarum	ISP*2*-1	lp_0216	NP_784041	IS*1182*
Lb. plantarum	ISP*2-2*	lp_0911	NP_784633	IS*1182*
Lb. plantarum	ISP*2*-3	lp_2006	NP_785535	IS*1182*
Lb. plantarum	ISP*2*-4	lp_3496	NP_786704	IS*1182*
Lb. plantarum	ISP*2*-N-terminal fragment	lp_3331	NP_786574	IS*1182*
Lb. plantarum	Unknown Transposase	lp_1212	NP_784877	ISPsy*8*
Lb. plantarum	Unknown Transposase	lp_1288	NP_784937	ISL*3*
Lb. plantarum	Transposase Fragment	lp_2023	NP_785550	ISL*3*
Lb. plantarum	Transposase Fragment	lp_2074	NP_785592	IS*1253*
Lb. johnsonii	ISLjo*1*-1	LJ_0033	NP_964048	IS*30*
Lb. johnsonii	ISLjo*1*-2	LJ_0275	NP_964282	IS*30*
Lb. johnsonii	ISLjo*1*-3	LJ_0372	NP_964396	IS*30*
Lb. johnsonii	ISLjo*1*-4	LJ_0622	NP_965633	IS*30*
Lb. johnsonii	ISLjo*1*-5	LJ_0642	NP_965613	IS*30*
Lb. johnsonii	ISLjo*1*-6	LJ_0772	NP_964626	IS*30*
Lb. johnsonii	ISLjo*1*-7	LJ_1726	NP_965531	IS*30*
Lb. johnsonii	ISLjo*2*-1	LJ_0064	NP_964080	ISL*3*
Lb. johnsonii	ISLjo*2*-2	LJ_0186	NP_964202	ISL*3*
Lb. johnsonii	ISLjo*2*-3	LJ_0813	NP_964668	ISL*3*
Lb. johnsonii	ISLjo*2*-4	LJ_1306	NP_965161	ISL*3*
Lb. johnsonii	ISLjo*2*-5	LJ_1599	NP_965403	ISL*3*
Lb. johnsonii	ISLjo*2*-6 Truncated Fragment	LJ_1040	NP_964896	ISL*3*

Table 1. continued...

Lb. johnsonii	ISLjo*4*	LJ_0765	NP_964618	IS*200-605*
Lb. johnsonii	ISLjo*5*-1	LJ_0692	NP_964544	IS*200-605*
Lb. johnsonii	ISLjo*5*-2	LJ_0693	NP_964545	IS*200-605*
Lb. gasseri	hyp. prot. lactobacillus_1156	Lgas_0017	ZP_00045855	ISL*3*
Lb. gasseri	tnpa-is1253-like streptococcus_1522	Las_0544	ZP_00046377	IS*1253*
Lb. gasseri	cons. hypo_1524	Lgas_0542	ZP_00046375	ISL*3*
Lb. lactis	IS*1077*A	L0466	NP_266208	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*1077*B	L0467	NP_266295	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*1077*C	L0468	NP_266528	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*1077*D	L0469	NP_266795	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*1077*E	L0470	NP_266980	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*1077*F	L0471	NP_268232	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*1077*G	L0472	NP_268304	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*A	L0457	NP_266209	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*B	L0458	NP_266293	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904C*	L0459	NP_266296	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*D	L0460	NP_266526	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*E	L0461	NP_266793	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*F	L0462	NP_266978	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*G	L0463	NP_266983	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*H	L0464	NP_268230	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*904*I	L0465	NP_268302	IS*3*/IS*150*/IS*904*
Lb. lactis	IS*981*A	L0432	NP_266243	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*B***	L200007	AE006247	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*C	L0434	NP_266813	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*D	L0433	NP_266878	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*E	L0436	NP_267351	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981F*	**	NC_002662	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*G	L0438	NP_267404	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*H	L0435	NP_267700	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*I	L0439	NP_267847	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*981*J	L0440	NP_268183	IS*2*, IS*3*, IS*150*, IS*600*, IS*629*, IS*861*, IS*904* and ISL*1*
Lb. lactis	IS*983*A	L0443	NP_266837	IS*30*
Lb. lactis	IS*983*B	L0444	NP_266859	IS*30*
Lb. lactis	IS*983*C	L0445	NP_267088	IS*30*
Lb. lactis	IS*983*D	L0446	NP_267459	IS*30*
Lb. lactis	IS*983*E	L0447	NP_267515	IS*30*
Lb. lactis	IS*983*F	L0448	NP_267671	IS*30*
Lb. lactis	IS*983*G	L0449	NP_267854	IS*30*

Table 1. continued..

Lb. lactis	IS*983*H	L0450	NP_268042	IS*30*
Lb. lactis	IS*983*I	L0451	NP_268065	IS*30*
Lb. lactis	IS*983*J	L0452	NP_268088	IS*30*
Lb. lactis	IS*983*K	L0453	NP_268094	IS*30*
Lb. lactis	IS*983*L	L0454	NP_268161	IS*30*
Lb. lactis	IS*983*M	L0455	NP_268219	IS*30*
Lb. lactis	IS*983*N	L0456	NP_268292	IS*30*
Lb. lactis	IS*983*O	L00457	NP_268340	IS*30*
Lb. lactis	IS*905*	L24515	NP_267360	IS*1191*
Lb. acidophilus	IS*605*-1	La377	n/a	IS*605*
Lb. acidophilus	IS*605*-2	La1464	n/a	IS*605*
Lb. acidophilus	IS*605*-3	La1487	n/a	IS*605*
Lb. acidophilus	IS*605*-4	La1533	n/a	IS*605*
Lb. acidophilus	IS*605*-5	La1569	n/a	IS*605*
Lb. acidophilus	IS*150*-1	La127	n/a	IS*150*
Lb. acidophilus	IS*150*-2	La614	n/a	IS*150*
Lb. acidophilus	IS*150*-3	La1299	n/a	IS*150*
Lb. acidophilus	IS*150*-4	La1475	n/a	IS*150*
Lb. acidophilus	IS*150*-5	La1868	n/a	IS*150*
Lb. acidophilus	IS*150*-6	La1954	n/a	IS*150*
Lb. acidophilus	IS*1272*-1	La77	n/a	IS*1272*
Lb. acidophilus	IS*1272*-2	La1907	n/a	IS*1272*
Lb. acidophilus	IS*1420*	La1420	n/a	ISHp*609*
Lb. acidophilus	IS*1722*	La1722	n/a	ISL*6*
Lb. acidophilus	IS*1723*	La1723	n/a	ISDL*4*
Lb. acidophilus	IS*1881*	La1881	n/a	IS*605*

*IS elements were classified under a particular family if a family was designated to the element under its GenBank submission. If no family was assigned to a particular element, then the amino acid sequence of the IS element was used to search the IS protein database (http://www-IS.biotoul.fr) and the non-redundant protein database (http://www.ncbi.nlm.nih.gov/entrez/) for significant matches

**No locus tag assigned. This element mentioned in the *L. lactis* genome paper (Bolotin, et. al., 2001), but the complete nucleotide sequence and amino acid sequence could not be found in GenBank.

***No amino acid sequennce for IS*981*B was deposited in GenBank. Because the nucleotide sequence for this element was exactly the same as the nucleotide sequence for the other copies of IS*981*, it was assumed the amino acid sequence was the same as well.

IS Elements in *Lactobacillus plantarum* WCFS1

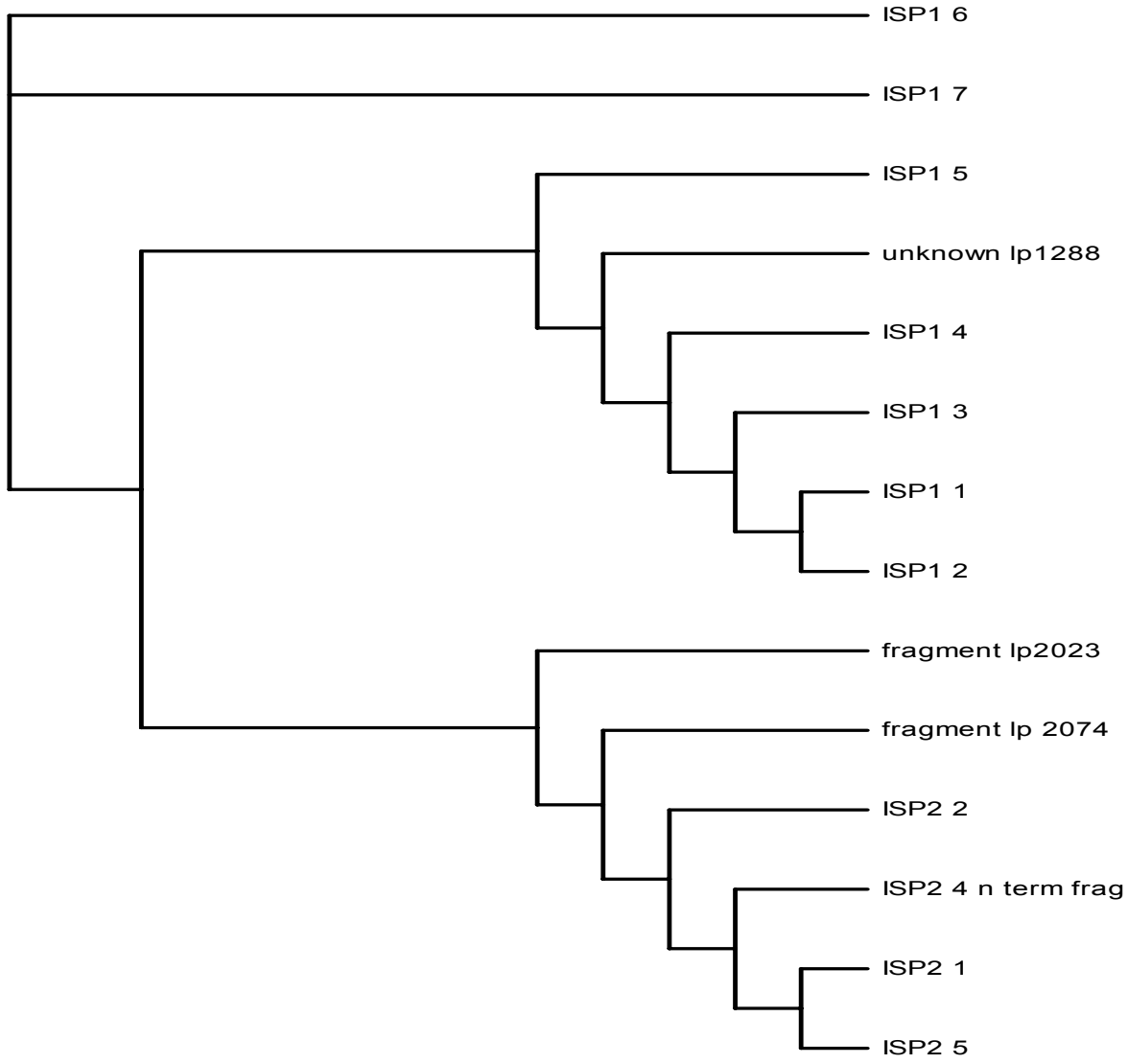
Four possible transposase genes were identified in *Lb. plantarum* WCFS1 (Figure 1). Two of these genes, ISP*1* and ISP*2*, were found in multiple copies. ISP*1* was present in 9 copies. Seven of these elements were identified as ISP*1* in GenBank. The eighth copy at locus 1288 was an identified transposase with 94 % amino acid sequence identity to the ISP*1* transposases. The ninth copy was an unidentified transposase fragement at locus 2023 which also demonstrated 94 % amino acid sequence identity to ISP*1* elements. ISP*1* transposases demonstrated approximately 36 % identity to ISL*3*, accession number CAA55730. ISL*3* was first identified in *Lactobacillus delbruekii*

subsp. *bulgaricus* (Germond, et. al., 1995). Members of the ISL*3* family have been found in many other bacteria including *Bacillus halodurans*, *Enterococcus faecalis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* (Mahillon & Chandler, 2002).

The other possible transposase found in multiple copies was ISP*2*. This predicted gene was present in four complete copies along with the fragment of another copy of this element. This element demonstrated 32 % identity to the amino acid sequence for a transposase belonging to the IS*1182* family isolated from *Staphylococcus intermedius*, accession number AAG42229. IS*1182* is associated with the transposon Tn5405 which carries resistance genes to aminoglycosides in *Staphylococcus aureus* (Derbise, et. al., 1996; Derbise et. al., 1997). In *Lb. plantarum* WCFS1, neither Tn5405 or aminoglycoside resistance genes were identified.

An unknown putative transposase identified at locus 1212 demonstrated 34 % amino acid sequence identity to ISPsy*8*, accession number NP_794798. A transposase fragment at locus 2074 demonstrated 33 % amino acid sequence identity to a transposase of IS*1253*, accession number AAN63779.

Figure 1. Putative Transposase Genes in *Lactobacillus plantarum* WCFS1



IS Elements in *Lactobacillus johnsonii* NCC 533

Lactobacillus johnsonii NCC 533 had several putative transposase genes identified throughout its genome (Figure 2). One element identified as ISjo*1* was present in 7 copies. This element demonstrated approximately 41 % amino acid sequence identity to a transposase gene belonging to the IS*30* family, accession number NP_622395. IS*30* transposase genes tend to have a single ORF composed of 293-383 codons (Mahillon & Chandler, 2002). ISjo*1* was indeed a single ORF composed of 341 codons.

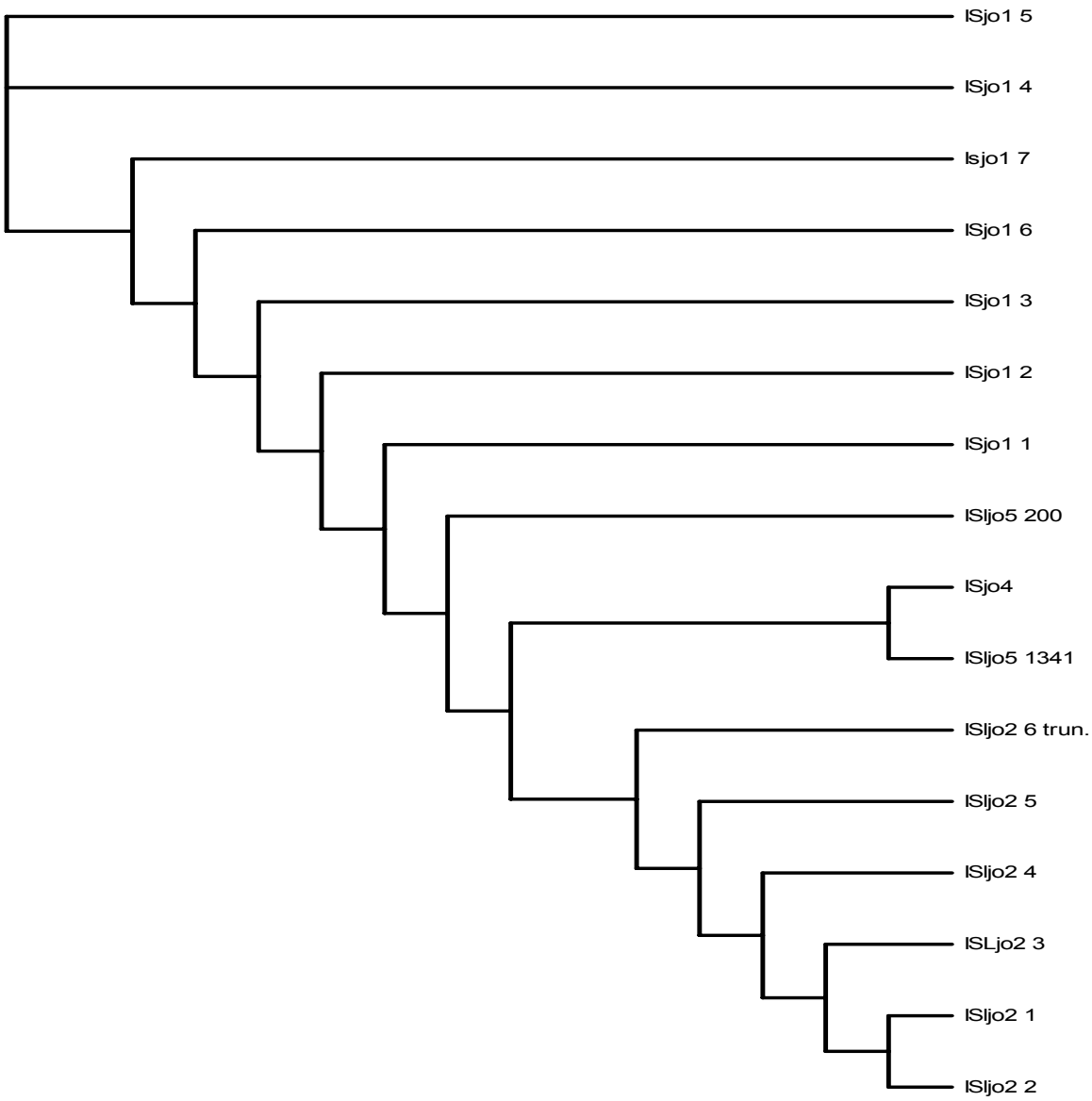
Another putative transposase found in multiple copies was ISjo*2*. This element was found in 5 complete copies and a truncated sixth copy. This element was classified as a transposase belonging to the ISL*3* family, and it demonstrated a 51 % amino acid sequence identity to a ISL*3* element isolated from *Lactobacillus delbrueckii*, accession number CAA55730.

Three other IS elements identified in NCC 533 were found to belong to the IS*200-605* family. In general, IS200-*605* contains transposase genes with two separate reading frames transcribed divergently. The smaller reading frame consists of a transposase of the IS*200* family and the larger reading frame encodes a transposase of the IS*1341* family (Mahillon & Chandler, 2002). IS*605* elements have been widely characterized in *Helicobacter pylori* (Kersulyte, et. al., 1998; Tomb et. al., 1997). For ISjo*4*, the IS database, http://www-is.biotoul.fr, revealed the putative protein had 28 % identity to the protein sequence of an IS*1341* element. This element also had 51 % amino acid sequence identity to an ISL*3* element, accession number CAA55730. No IS*200* element was found for ISjo*4*. ISjo*4* was designated in GenBank as belonging to IS*200-605* family, however

because no IS*200* was found for this transposase and because it demonstrated a higher identity to ISL*3*, this particular transposase may belong to the ISL*3* family.

ISjo*5*-1 and ISjo*5*-2 were found to have amino acid sequence homology to IS*1341* and IS*200* elements, respectively. ISjo*5*-1 demonstrated 30 % amino acid identity to an IS*1341* element in the IS database and ISjo*5*-2 demonstrated 35 % amino acid identity to an IS*200* element present in *Helicobacter pylori* strain 26695, accession number NP_207212. Because ISjo*5*-1 and ISjo*5*-2 were adjacent to each other in the genome and in opposite orientations, it is reasonable to assume that these predicted ORFs compose a whole IS*200-605* element in *Lb. johnsonni* NCC 533.

Figure 2. Putative Transposase Genes in *Lactobacillus johnsonii* NCC 533



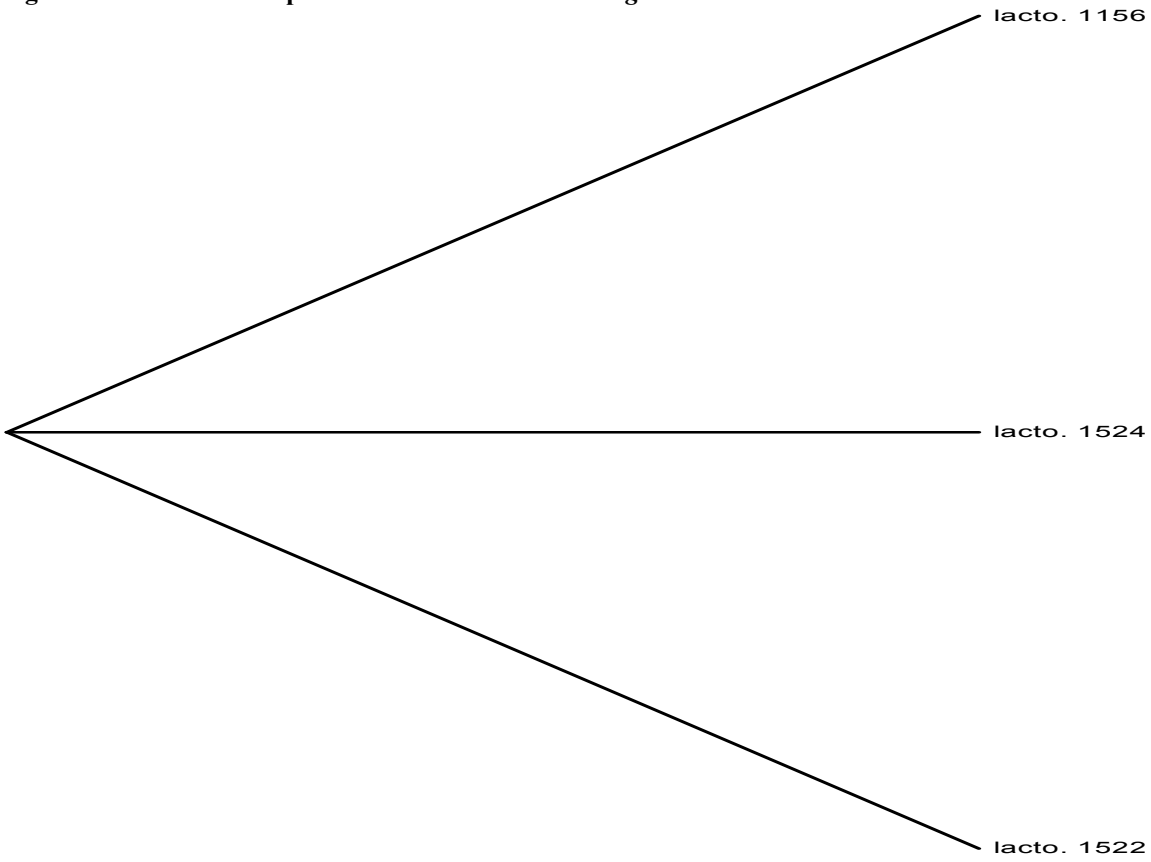
IS Elements in *Lactobacillus gasseri* ATCC 33323

Out of all the lactic acid bacteria examined, *Lactobacillus gasseri* ATCC 33323 had the fewest putative transposases identified in its genome (Figure 3). Only three predicted IS elements met the criteria for this analysis, the ORF must be greater than or equal to 150 amino acids and the predicted ORF must demonstrate at least 30 % homology to a transposase amino acid sequences in either the non-redundant and/or the IS database.

A predicted transposase with the locus tag Lgas_0017 showed 50 % amino acid sequence identity to an ISL*3* element isolated from *L. delbrueckii*, accession number CAA55730. Another putative transposase demonstrated 34 % homology to a tnpA-IS*1253*-like element in *Streptococcus thermophilus*, accession number AAN63779. The third possible transposase, with the locus tag Lgas_0542, also demonstrated homology to an ISL*3* element, CAA55730.

It was interesting that *L. gasseri* harbored so few IS-elements (3) in comparison to the many found in *L. johnsonii* (16), considering how closely related these two species are in genomic content and structure (Pridmore, et. al., 2004). The predicted transposase gene with the locus tag Lgas_0017 did show approximately 70 % amino acid homology to the ISLjo*2* elements in *L. johnsonii.*

Figure 3. Putative Transposase Genes in *Lactobacillus gasseri* ATCC 33323



IS Elements in *Lactococcus lactis* subsp. *lactis* IL1403

Lactococcus lactis subsp. *lactis* IL1403 genome contained the greatest amount of IS elements among all of the other five lactic acid bacteria presented in this paper (Figure 4). Five different transposase genes of IS families were predicted, and four of these were present in multiple copies.

One element, labeled IS*1077* was present in 7 copies. This element showed 47 % amino acid sequence identity to an IS*150*-like transposase in *Enterococcus faecium*, accession number AAM29175 and 45 % identity to an IS*3* transposase in *Bacillus cereus* ATCC 10987, accession number NP_977451. The IS*3* family is a large group of insertion sequences. The main feature of this family is two overlapping reading frames, *orf*A and *orf*B (Sekine, et al., 1994; Walker & Klaenhammer, 1994). During transposition, programmed translational frameshifting generates a fusion protein from both open reading frames, ORFAB (Mahillon & Chander, 1998). IS*150* is a member of the larger IS*3* family and has been found to have a frameshifting frequency of approximately 50% (Mahillon & Chandler, 1998; Vögele, et al., 1991). The IS*1077* transposase ORFs demonstrated amino acid sequence to *orf*A of the IS*150* like element in *Enterococcus faecium*, and also to a transposase identified as *orf*B of the IS*3* element in *Bacillus cereus*.

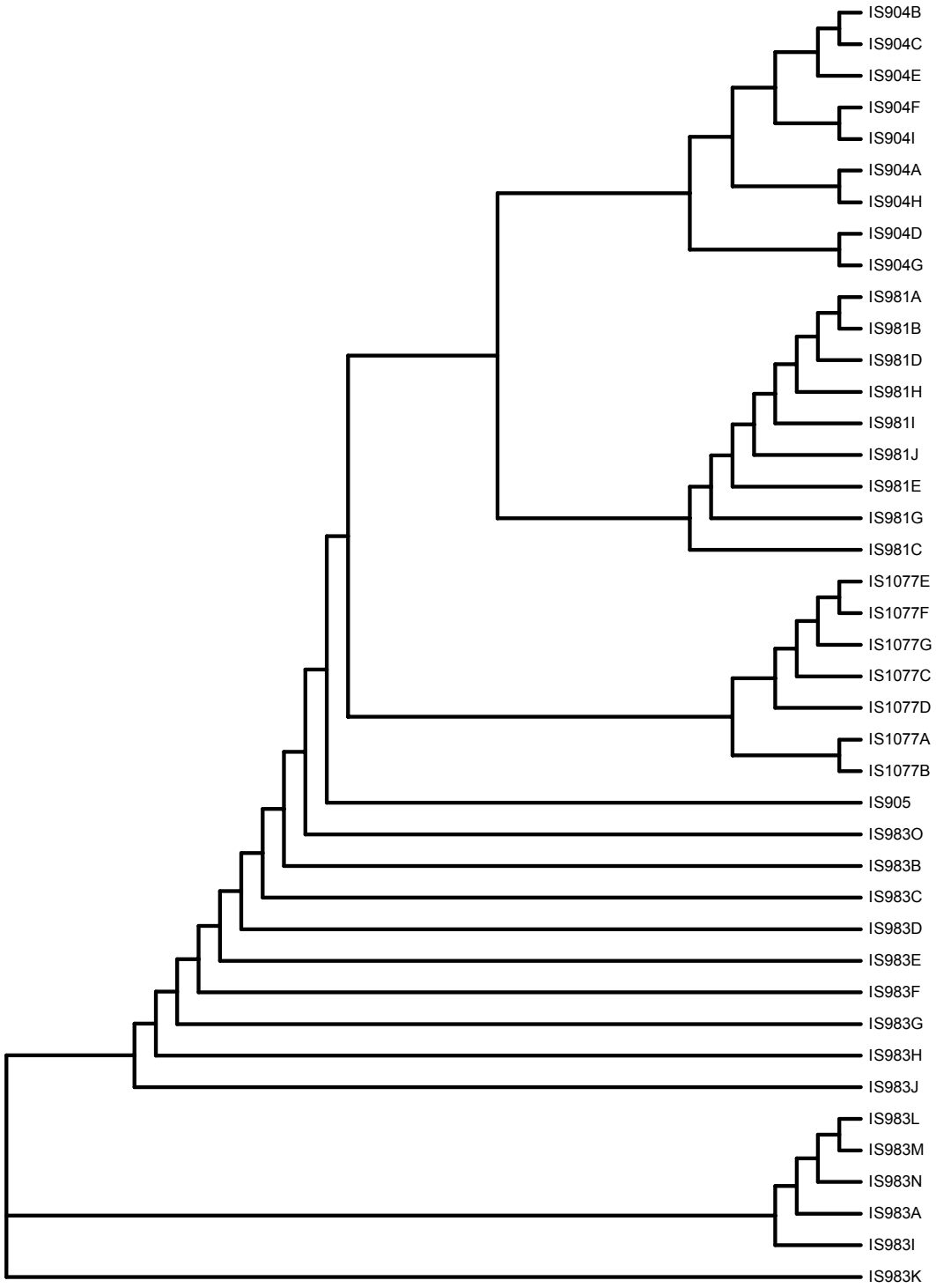
Predicted transposase genes labeled IS*904* were present in 9 copies throughout the genome. The amino acid sequence for this mobile element was also classified as an IS*3*-like tranposase, demonstrating approximately 41 % amino acid sequence identity to an IS*3* transposase in *Brucella melitensis*, accession number AAL59363.

Putative transposase, IS*981*, was found in 10 copies in IL1403. This protein showed homology to members of the IS*3* elements, including 34 % amino acid identity to an IS*600* transposase in *Shigella flexneii*, accession number NP_839677, and approximately 33 % protein identity to another transposase of the IS*600* family in *Escherichia coli* O157: H7, accession number NP_286669. Interestingly, IS*600* elements have been found flanking the shiga toxin operon in *Shigella dysenteriae*1 and its insertional activity is believed to have resulted in loss of sequence in the shiga toxin-encoding lambdoid prophage, making it defective (McDonough & Butternton, 1999).

Another transposase found in multiple copies was IS*983*, which was present in 15 copies. This element had 50 % amino acid identity to IS*1239* transposase in *Streptococcus pyogenes*, accession number NP_606772. IS*1239* has been found in multiple copies in *Steptococcus pyogenes* and is a member of the IS*30* family of insertion sequences (Kapur, et al., 1994).

The putative transposase, IS*905*, was present in a single copy in IL1403. This putative protein demonstrated approximately 94 % amino acid identity to an IS*1191* transposase in *Streptococcus thermophilus*, tnp-IS*1191*, accession number AAN63693.

Figure 4. Putative Transposase Genes in *Lactococcus lactis* subsp. *lactis* IL1403



IS Elements in *Lactobacillus acidophilus* NCFM

Seven different putative transposase genes were identified in NCFM, with three of them present in multiple copies (Figure 5). These three different predicted proteins demonstrated homology to transposase genes belonging to the IS*150*, IS*605*, and IS*1272* families.

An IS*1272*-like putative transposase was found in two copies. This possible transposase demonstrated 39 % amino acid sequence identity to an IS*1272* transposase in *Staphylococcus aureus*, accession number YP_040780. IS*1272* elements are thought to be important in the horizontal transfer of methicillin resistance genes in *Staphylococcus* species (Kobayashi, et al., 1999).

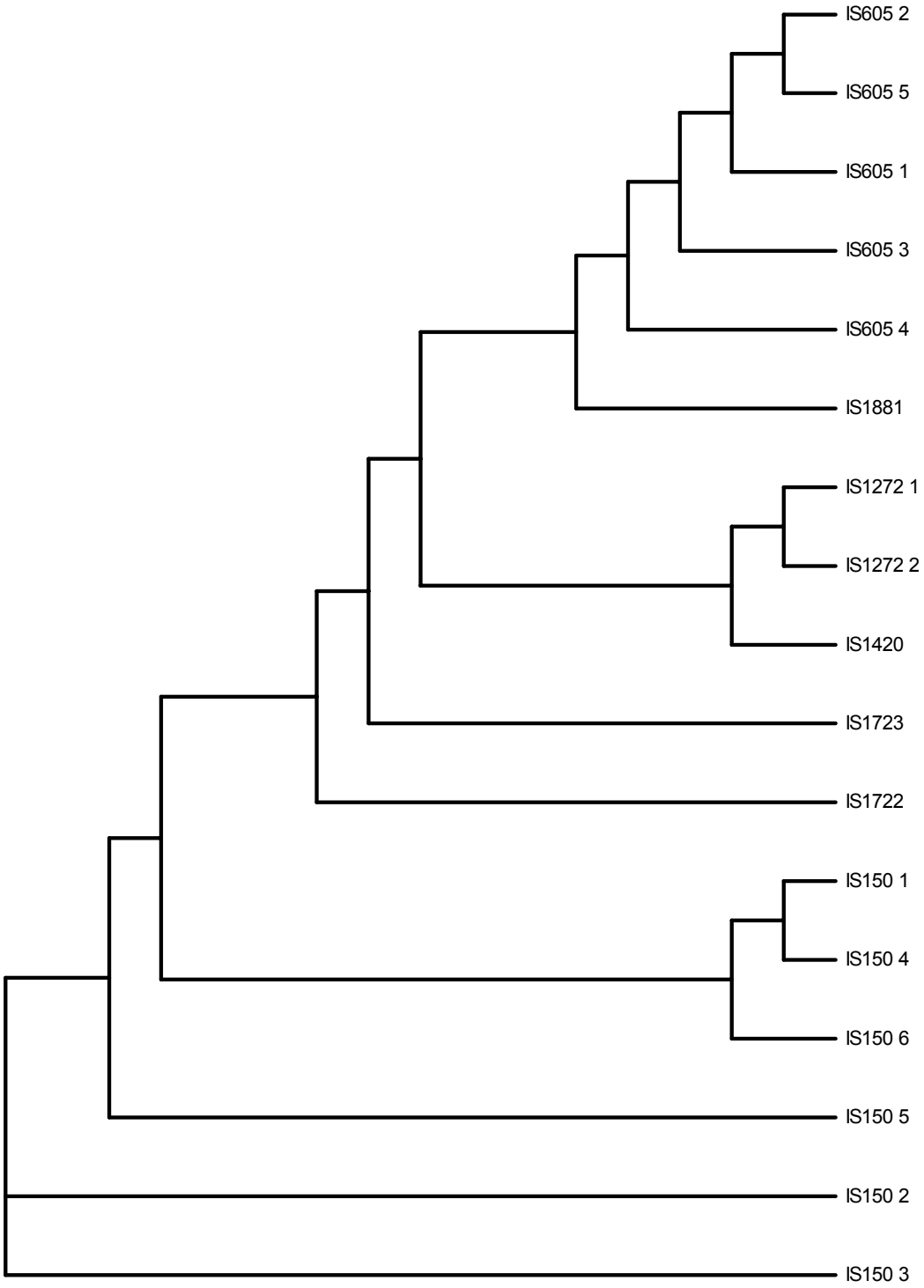
A transposase found in six copies throughout NCFM had greater than 40 % amino acid identity to the Orf B of IS*150* elements in *Escherichia coli*, accession numbers AAB18535 and NP_065294. This transposase also demonstrated homology to a transposase of ISL*6* in *Lactobacillus delbrueckii* subsp. *lactis*, accession number AAK8366, and to a transposase of the transposon th3692 in *Lactobacillus crispatus*, accession number AAQ01501.

The third transposase present in multiple copies demonstrated homology to transposase genes belonging to the IS*605* family. This element was present in five copies throughout the genome and interestingly demonstrated approximately 88 % protein identity to the transposase of ISjo*4* in *L. johnsonii* NCC 533, accession number NP_964618 and 63 % identity to an unknown transposase in *L. delbrueckii* subsp. *lactis*, accession number AAQ060905.

Four other putative transposase genes were present in single copies in NCFM. One predicted transposase, IS*1420* demonstrated greater than 30 % protein identity to the transposase of ISHp*609* in *Helicobacter pylori*, accession number AAR83266. IS*1722* had 83 % protein identity to a transposase composing the transposon 3692 in *L.actobacillus crispatus*, accession number AAQ01501. In *L. crispatus* CHCC3692, transposon 3692 contains an erythromycin resistance gene (Stroman, et. al., 2003).

The predicted transposase IS*1723* demonstrated 50 % amino acid sequence identity to the transposase of ISDL*4* in *Lactobacillus delbruekii* subsp. *lactis*, accession number CAC79140 and approximately 49 % amino acid sequence identity to ISL*4* transposase in *Lactobacillus delbrueckii* subsp. *bulgaricus*, accession number AAK83659. The four predicted transposase in a single copy was IS*1881*. This element had approximately 40 % identity to IS*605*-like elements in *Streptocmyces avermitilis*, accession numbers NP_821235 and BAC67756.

Figure 5. Putative Transposase Genes in *Lactobacillus acidophilus* NCFM



Concluding Remarks

The lactic acid bacteria examined in this paper all had multiple transposase genes representing many IS families, albeit the number in *L. gasseri* was significantly lower, comparatively. Among the probiotic lactobacilli, only one major transposase family, ISL3, was represented across the different species of *L. gasseri, L. johnsonii, and L. plantarum*. In this regard, it was interesting that *L. acidophilus* did not harbor an ISL3-like element, even though 17 IS elements were found in the genome. Moreover, over the 4 probiotic lactobacilli, 7 different transposase families were represented and none of these was shared between any two species.

A significant proportion of individual transposases were also found in multiple copies. The distribution and copy number of these mobile elements provide potential loci for genomic shuffling. IS elements are also known to influence gene expression and serve as vehicles for lateral gene transfer. The variation in the numbers and types of putative transposase genes identified among these lactic acid bacteria could provide targets for molecular identification that would allow for discrimination among closely related species and strains of bacteria.

DEVELOPMENT OF A MULTIPLEX PCR TO IDENTIFY *LACTOBACILLUS ACIDOPHILUS* NCFM

Introduction

Probiotics are living microorganisms which exert health benefits beyond inherent nutrition when ingested in certain numbers (Guarner & Schaafsma, 1998). Recently this definition has been updated by the International Scientific Association of Probiotics and Prebiotics to Probiotics are defined as "live microorganisms which, when administered in adequate amounts confer a health benefit on the host." (Reid, et. al., 2003). Some of these proposed health benefits include antimicrobial activity, alleviation of symptoms of lactose intolerance, diarrhea prevention and alleviation, and immunostimulation (Scheinback, 1998; Ouwehand, et. al., 2002). *Lactobacillus acidophilus* is a probiotic species implicated in the aforementioned benefits. It is also believed that consumption of *L. acidophilus* helps establish the natural microflora after antibiotic treatment, prevents the occurrence of vaginitis and provides anticarcinogenic activity (Klaenhammer, 1998; Sanders & Klaenhammer, 2001; Wagner et al. 1997). *L. acidophilus* NCFM, isolated from a human source, is a well characterized probiotic strain (Gilliland et al. 1975). This strain has been shown to survive passage through the gastrointestinal tract, to adhere to the intestinal tissue, and to reduce fecal mutagenic enzyme activity (Sanders & Klaenhammer, 2001; Rao et al. 1999, Sui, et. al., 2002). It has been used commercially since the 1970's and has a history of safe use in consumer products, primarily yogurts and acidophilus milks. These qualities led to the selection of *L. acidophilus* NCFM for complete nucleotide sequencing of its genome (Klaenhammer et al. 2002).

In silico analyses of the sequenced genome has revealed volumes of information about NCFM, including potential gene targets that are likely to be important to its probiotic properties. With respect to many possible probiotic cultures, it is realized widely that various health benefits can be strain related (Lucchini, et al. 1998). Manufacturers of commercial probiotic products must ensure the proper culture identity of the product. *L. acidophilus* NCFM is a commercial strain, which continues to be studied in various human clinical trials (Sanders & Klaenhammer, 2001; Rao et. al. 1999; Scheinbach, 1998). A quick and accurate method for identification of this microorganism recovered from clinical samples is also desirable.

Attempts to identify *L. acidophilus* NCFM have included both traditional and molecular approaches. Molecular techniques utilized have included pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), and 16S rRNA sequencing (Clark et al, 2001; Kullen et al 2000). These methods have been met with some success, but they are generally time consuming, labor intensive, and/or technically difficult. A PCR based approach would enable the selection of specific targets and provide a quick and reliable method of identification.

In this study we describe a multiplex PCR approach to quickly identify *L. acidophilus* NCFM. The sequenced genome revealed the presence of several transposase genes scattered throughout. Because these mobile genetic elements can exist in variable numbers and locations among highly related microorganisms, they were chosen as potential candidates for unique molecular targets of a multiplex PCR.

Materials & Methods

Computational Analysis of Transposase Genes in NCFM

Genomic sequencing and open reading frame predictions were performed using GAMOLA as described by Altermann and Klaenhammer, 2003. The nucleotide sequences and the amino acid sequences of predicted transposase and integrase genes were aligned using CLUSTALX (Thompson, et al., 1997) and a subsequent neighbor joining phylogenetic tree was produced using the amino acid sequence multiple alignment. The multiple sequence alignment (msa) output file produced by CLUSTALX was imported into GeneDoc (Nicholas and Nicholas, 1997) for editing. Msa files were imported into MEGA2.1 and there the phylogenetic tree was calculated and visualized (Kumar, et al., 2001).

Bacterial Strains

The bacterial strains used in this study are listed in Table 2. The species identities of all *L. acidophilus* strains were confirmed by 16S rRNA sequencing as described by Kullen et al., 2000. *L. acidophilus* strains were propagated at 37C in MRS broth (Difco Laboratories, Detroit, Michigan).

Table 2. *Lactobacillus acidophilus* Strains Used in Study

Strain	Origin	Date Obtained
NCK 56/NCFM	NCFM/N2	1988
NCK 1263	University of Minnesota	1999
NCK 336	ATCC 4356	1984
NCK 830	Snowbrand Milk Products Co., Ltd.	1996
NCK 1005	Marshall Products	1997
NCK 821	Snowbrand Milk Products Co., Ltd	1996
NCK 826	Snowbrand Milk Products Co., Ltd	1996
NCK 935	Marshall Products	1997
NCK 825	Snowbrand Milk Products Co., Ltd	1996
NCK 828	Snowbrand Milk Products Co., Ltd	1996

Table 2. continued..

NCK 822	Snowbrand Milk Products Co., Ltd	1996
NCK 829	Snowbrand Milk Products Co., Ltd	1996

DNA Extraction

The protocol for DNA extraction was modified from procedure described by Clark et al. 2001. Cells from 3mL of an overnight culture were harvested by centrifugation at 14,000 rpm for 5 minutes. Pellets were washed with distilled H_2O and then resuspended in 0.5 ml of a solution containing 10 mg/ml lysozyme, 20% sucrose, 10 mM Tris pH 8, 10 mM EDTA pH 8, 50 mM NaCl, and 25 µl of 10% SDS (Sigma, St. Louis, MO, USA). This cell suspension was then incubated at 55 C for 30 minutes and/or until lysis solutions became clear. Then 20 µl of proteinase K solution (20 mg/ml) was added followed by incubation at 60 C for 1 hour. Three phenol-chloroform extractions were performed using 500 µl of phenol:chloroform-isoamyl-alcohol (25:24:1), including a centrifugation step at 14,000 rpm for 5 minutes, followed by the transfer of the aqueous phase to a clean tube. DNA precipitation was accomplished by the addition of 50 µl of 3 M sodium acetate and 1 ml of cold 95% ethanol and centrifugation at 14,000 rpm for 15 minutes. The supernatant was removed and the resulting DNA pellet was dissolved in 50µl TE and 2 µl RNase (500 U/ml). As an alternative, a DNA extraction kit from Mo Bio Labs, Carlsbad, CA could be used.

Primers and PCR Reactions

Clone Manager software (Version 6.0, SciEd Central) was used to design primer sets targeting putative transposase genes belonging to IS families IS*150*, IS*605*, and IS*1272*. PCR reaction mixtures contained 5 ul (20 ng/ul) genomic DNA, 2.5 µL 10X

buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 4 µl dNTP mixture (1 mM each), 0.25 µl Taq polymerase (1 U/µl), 5 µl of each primer (20 µM), and double distilled water, d_2H_2O, for a final volume of 25 µl. The cycling parameters for these reactions included a 2 minute initial melting step at 94 C followed by thirty one cycles consisting of a 30 second melting step at 94 C, a 30 second annealing step at 55 C, and a 4 minute extension step at 72 C, and a final extension step at 72 C for seven minutes.

Multiplex PCR

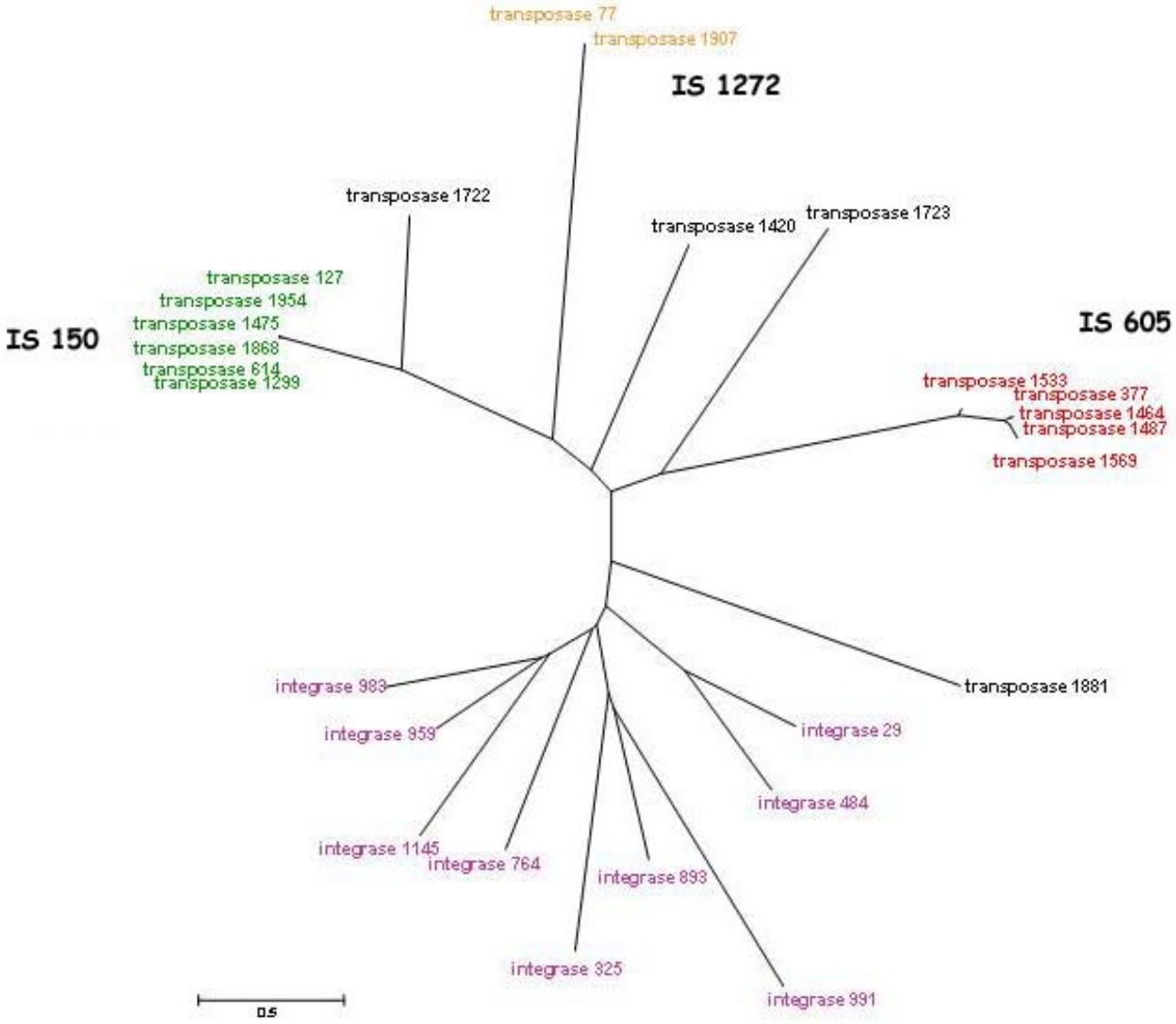
The reaction mixture and cycling parameters were optimized for a multiplex reaction that yielded amplicons from NCFM genomic DNA. The final recipe for this reaction contained 100 ng DNA, 2.5µl 10X buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 4µl dNTP mixture (1 mM each), 1.25µl IS*1272* (20 µM), 1.25µl p_77 (20 µM), 2.5 µl IS*150* (20 µM), 1.25 µl p_1954 (20 µM), 1.25 µl IS*605* (2 µM), 1.25 µl p_614 (2 µM), 1.25 µl p_1569 (2 µM), 1µl Taq polymerase (1U/µl), and d_2H_2O for a final volume of 25 µl. The cycling parameter had a 10 minute initial melting step at 94 C. After this step, Taq polymerase was added to the reaction mixture. This was followed by 35 cycles of a 30 s melting step at 94 C, a 30 s annealing step at 55 C, and a 4 min extension step at 72 C. A final extension at 72 C for 7 minutes completed the amplification. This multiplex reaction was used to screen twelve strains of *Lb. acidophilus*, including NCFM (Table 2).

Results

Computational Analysis of Transposase Genes

Seventeen putative transposase genes and nine putative phage-related integrase genes were identified throughout the genome of NCFM. Msa files were made using the amino acid sequences for each transposase and integrase identified in NCFM. These msa files were used as input for CLUSTALX so a phylogenetic tree could be produced (Figure 6). Seven distinct transposase genes were revealed and three of these were present in multiple copies ranging from two to six replicas. These three transposase genes could be grouped into three separate IS families, IS*150* (six copies), IS*1272* (two copies), and IS*605* (five copies).

Figure 6. Putative Transposase Genes in NCFM



For the three transposase genes present in multiple copies, msa files using the nucleotide sequences were created and compared to reveal highly conserved nucleotide sequences among the copies of IS*150*, IS*1272*, and IS*605* (Figure 7). Many single point

mutations were observed among the copies of IS*605*, a majority of these were found in the transposase at the open reading frame 1533, tr_1533 (Figure 7 C).

Figure 7. Partial Multiple Sequence Alignment for Transposase Genes in IS Families

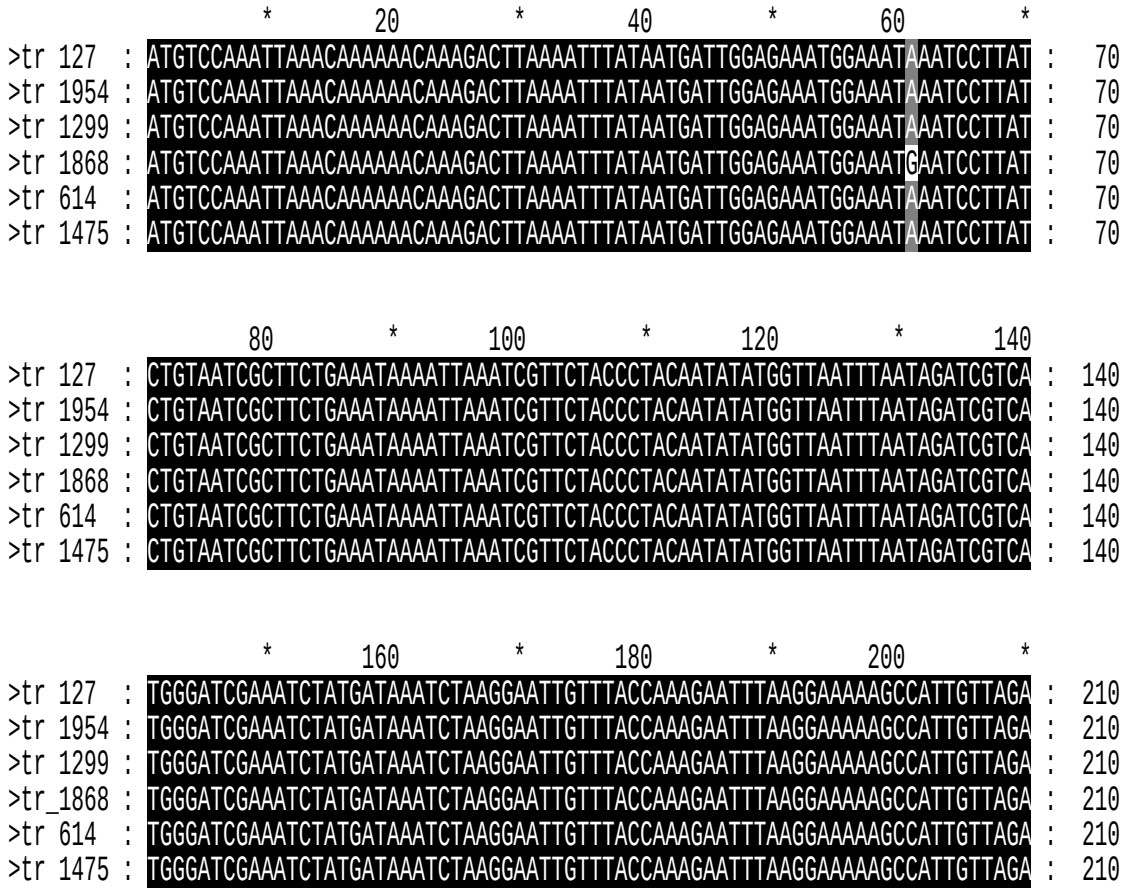


Figure 7A. Partial Multiple Sequence Alignment for Transposase Genes in IS*150*

Figure 7B. Partial Multiple Sequence Alignment for Transposase Genes in IS*1272*.

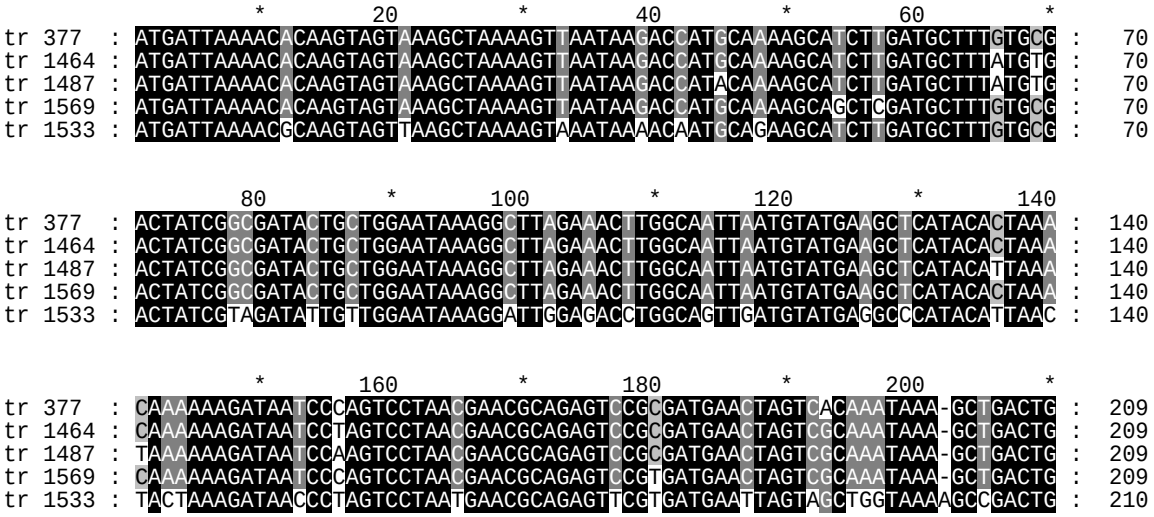
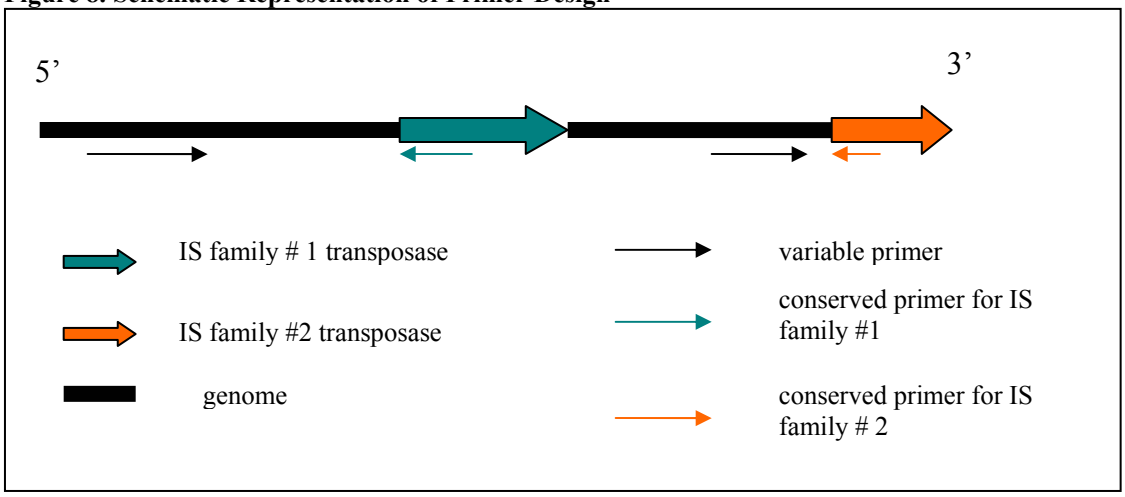


Figure7C. Partial Multiple Sequence Alignment for Transposase Genes in IS*605*

Design and Screening of Individual Primer Sets

The three distinct transposase genes present in multiple copies were used as targets for PCR primers that might yield strain specific amplicons. The copies of IS*150*, IS*605*, and IS*1272* were so highly conserved, it was possible to design one primer for each of the three IS families at the 5' end of the transposase gene and a variable primer in an adjacent region of the genome (Figure 8). Thereby, both the individual transposase and the locus of the transposase would be targets of the PCR reaction.

Figure 8. Schematic Representation of Primer Design



Clone Manager software was used to design 3 conserved primers, one for IS*150*, IS*1272*, and IS*605* (Table 3). This software was also used to design 13 variable primers adjacent to the 5' end of each individual transposase gene (primers not shown). These primer sets were tested on NCFM and on two other strains of *L. acidophilus,* NCK 1263 and ATCC 4356. These two strains had been differentiated from NCFM previously by pulse field gel electrophoresis and restriction fragment length polymorphisms (Clark et al., 2001).

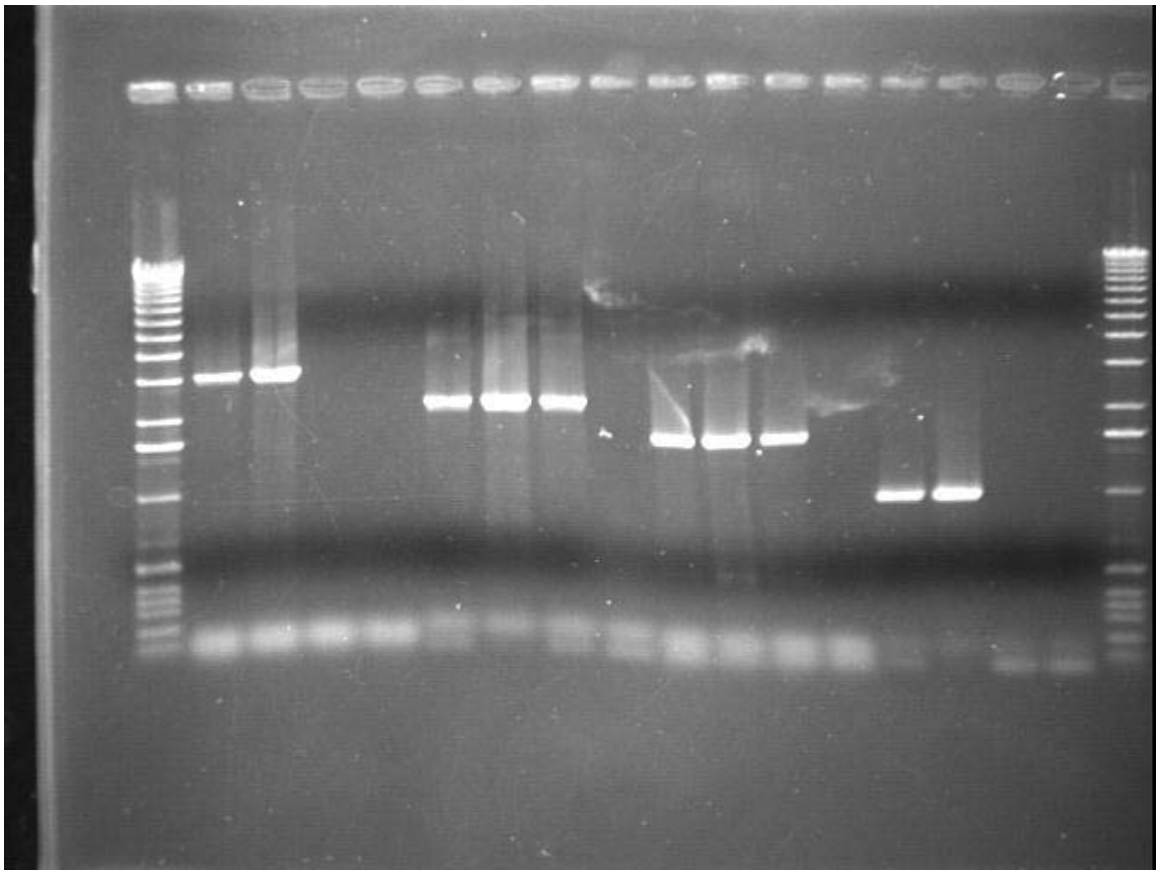
Table 3. Primer Sets in Multiplex Reaction

Conserved Primer	Conserved Primer Sequence	Variable Primer	Variable Primer Sequence	Expected Product Size
IS*1272*	5'-GAT AAG CAG GGC GGC CTG TTT TAG CAG TTT-3'	p_77	5'-GAT TCT GAT TTT GCG TTA ATC TAA CAG TGA-3'	~3100bp
IS*150*	5'-AAT TAT ACA AGA TAC CGT TGC TTC TTA GTC C-3'	p_614	5'-CTG AAA ATG ACT TAT CTC GTG CCA TTA TTT C-3'	~960bp
IS*150*	5'-AAT TAT ACA AGA TAC CGT TGC TTC TTA GTC C-3'	p_1954	5'-AAG CTG AAC CAT TAT TCT GTT CTT CAA CAT A-3'	~2200bp
IS*605*	5'-GCC TTT ATT CCA GCA GTA TCG CCG ATA GTC-3'	p_1569	5'-ACT GAC GAG ATG GCT TAT GAA GCC GGT AGT-3'	~1700bp

The primer set adjacent to the transposase at ORF 77 (p_77) and the conserved primer for IS*1272* family formed a 3100 bp product with NCFM and NCK 1263, but not for ATCC 4356 (Figure 9, Lanes 2, 3, 4). The individual primer set using a conserved primer in the IS*150* family and a primer adjacent to the transposase at ORF 614 (p_614) formed a 960 bp product for NCFM and NCK 1263, but not with ATCC 4356 (Figure 9, Lanes 14, 15, and 16). These two primer sets were chosen for the multiplex PCR. Two more primer sets were selected to complete the multiplex PCR so that a distinctive ladder for NCFM could be observed. One of these primer sets was composed of the conserved primer for the IS*150* family and a variable primer adjacent to the transposase at ORF 1954 and produced a 2200 bp product for NCFM, NCK1263 and ATCC 4356 (Figure 9, Lanes 6, 7, and 8). The other primer set consisted of a conserved primer for the IS*605* family and a variable primer adjacent to the transposase at ORF 1569 and formed a 1700 bp product for NCFM, NCK 1263, and ATCC 4356 (Figure 9, Lanes 10, 11, and 12).

Figure 9. Gel photo of PCR products for individual primer sets used in the Multiplex PCR.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



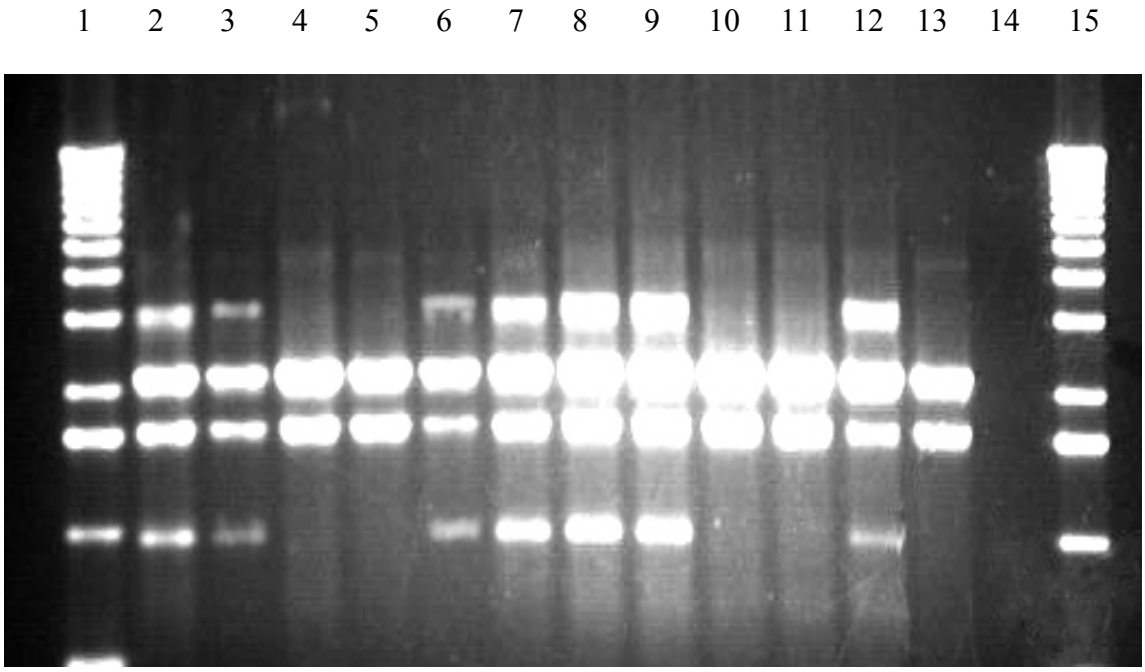
Lane 1, 1 kb ladder; Lane 2, NCFM-primers IS*1272* and p_77; Lane 3, NCK 1263-primers IS*1272* and p_77; Lane 4, ATCC 4356 – primers IS*1272* and p_77; Lane 5, d_2H_2O – primers IS*1272* and p_77; Lane 6, NCFM – primers IS*150* and p_1954; Lane 7, NCK 1263 – primers IS*150* and p_1954; Lane 8, ATCC 4356 – primers IS*150* and p_1954; Lane 9, d_2H_2O – primers IS*150* and p_1954; Lane 10, NCFM – primers IS*605* and p_1569; Lane 11, NCK 1263 – primers IS*605* and p_1569; Lane 12, ATCC 4356 – primers IS*605* and p_1569; Lane 13, d_2H_2O – primers IS*605* and p_1569; Lane 14, NCFM – primers IS*150* and p_614, Lane 15, NCK 1263 – primers IS*150* and p_614; Lane 16, ATCC 4356 – primers IS*150* and p_614; Lane 17, d_2H_2O – primers IS*150* and p_614; Lane 18, 1 kb ladder.

Multiplex PCR Screening of *Lb. acidophilus* Strains

The four primer sets listed in Table 3 were chosen for the multiplex PCR. The reaction mixture and the cycling parameters were optimized and subsequently tested on twelve industrial strains of *L. acidophilus*, including NCFM. Five of the strains screened showed a different banding pattern than that of NCFM. These strains did not form

products with the primer set IS*1272* and p_77 and the primer set IS*150* and p_614. The other six strains screened, formed the same products from the multiplex PCR as NCFM. The results from these reactions are shown in Figure 10 and summarized in Table 4.

Figure 10. Gel Photo of Products of Multiplex PCR for industrial *L. acidophilus* strains.



Lane 1, 1 kb ladder; Lane 2, NCFM; Lane 3, NCK 1263; Lane 4, ATCC 4356; Lane 5, NCK 830; Lane 6, NCK 1005; Lane 7, NCK 821; Lane 8, NCK 826; Lane 9, NCK 935; Lane 10, NCK 825; Lane 11, NCK 828; Lane 12, NCK 822; Lane 13, NCK829; Lane 14, d_2H_2O; Lane 15, 1 kb ladder.

Table 4: Results from Multiplex PCR Screening of *Lb. acidophilus* Strains

Lane	Sample	#Products Formed	P_77 & IS*1272* (~3100bps)	P_614 & IS*150* (~960bps)	P_1569 & IS*605* (~1700bps)	P_1954 & IS*150* (~2200bps)
2	NCFM	4	+	+	+	+
3	NCK1263	4	+	+	+	+
4	NCK 336	2	-	-	+	+
5	NCK 830	2	-	-	+	+
6	NCK 1005	4	+	+	+	+
7	NCK 821	4	+	+	+	+
8	NCK 826	4	+	+	+	+
9	NCK 935	4	+	+	+	+
10	NCK 825	2	-	-	+	+
11	NCK 828	2	-	-	+	+
12	NCK 822	4	+	+	+	+
13	NCK 829	2	-	-	+	+

Discussion

A multiplex PCR was developed to identify and differentiate NCFM from closely related strains of *L. acidophilus*. Predicted mobile elements were chosen as potential candidates for this reaction because of the variability in location, copy number, and the presence or absence of these elements that can exist among closely related strains. In NCFM, seventeen putative transposase genes and nine putative phage-related integrase genes were identified. Seven distinct transposase genes were present in NCFM's genome and three of these were found in multiple copies (Figure 6).

IS elements are small segments of DNA that are capable of inserting at multiple sites in a target molecule. They are generally less than 2 kb in length and contain no known genes unrelated to insertion function (Campbell et al., 1977). More than 700 IS

elements have been identified in both eukaryotic and prokaryotic organisms (Mahillon & Chandler, 2002). These genetic elements are capable of participating in chromosomal rearrangements and can thereby contribute to the diversity of a species within a given genus and to the diversity of strains within a given species (Mahillon & Chandler, 1998; Lawrence et al., 1992). They have been found in many bacterial genomes including *Lactobacillus* spp. and *Lactococcus* spp. (Bolotin, et. al, 2001; Klaenhammer, et. al., 2002; Kleerebezem, et al., 2003; Pridmore et. al., 2003). The diversity which can exist among highly related microorganisms with respect to these mobile elements led to the selection of three distinct putative transposase genes found in multiple copies in NCFM for the targets of a multiplex PCR.

The high nucleotide sequence conservation of these elements allowed the primer sets tested to use a conserved primer for each IS family and a variable primer for each individual transposase. The individual primer sets were tested on NCFM against two other strains of *L. acidophilus*, NCK 1263 and ATCC 4356. ATCC 4356 has been distinguished from NCFM previously by pulse-field gel electrophoresis (Walker, et. al., 1996) and both ATCC 4356 and NCK 1263 have been distinguished from NCFM by RFLP using a distinct transposase probe (Clark et. al, 2001).

Two of the individual primer sets demonstrated a differential banding pattern for ATCC 4356 and were chosen for the multiplex PCR. Two more individual primer sets were selected so a distinctive banding pattern for NCFM could be produced. Strains previously identified as *L. acidophilus* by 16S rRNA sequencing as described by Kullen et. al., 2001 were used to test the multiplex reaction. The multiplex PCR identified six of the strains tested, including NCK 1263, as NCFM or closely related to NCFM. Two of

these strains, NCK 1005 and NCK 935, had previously demonstrated the same RFLP pattern as NCFM (Clark, et al., 2001). They also generated the same amplicon fingerprint as NCFM from the multiplex PCR, providing additional evidence these strains are NCFM. Five of the strains tested, including ATCC 4356, yielded distinguishing banding patterns from the multiplex PCR when compared to NCFM. This paper defines the primers and conditions for a multiplex PCR which can identify *L. acidophilus* NCFM and differentiate NCFM from other closely related strains.

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