5-1987

Plasmid-Linked Maltose Utilization in Lactobacillus spp. Isolated from Meat

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PLASMID-LINKED MALTOSE UTILIZATION IN *LACTOBACILLUS* SPP. ISOLATED FROM MEAT

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1987

ACKNOWLEDGMENTS

I would like to express my appreciation to Dr. J. K. Kondo, my major advisor, for his guidance and encouragement in the writing of this thesis as well as throughout the experimentation.

My sincere thanks goes to Dr. D. T. Bartholomew for providing cultures, and I would like to thank Dr. F. J. Post and Dr. D. P. Cornforth for serving as members of my committee and reviewing this thesis.

I especially thank Dr. M. A. Salih and my laboratory fellows, particularly D. J. Freedman and S. A. Woskow, whose friendship, companionship, and discussions always keep me up.

Finally, this is dedicated to my parents and brother for their love, support, and encouragement, and to my fiance, Chia-Yiu, for his love and understanding.

Mei-Ling Liu

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ABSTRACT

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by

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Utah State University, 1987

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Five strains of *Lactobacillus plantarum* and four strains of *Lactobacillus* species isolated from fresh meat were examined for the presence of plasmid DNA. All strains examined contained between one and five plasmids ranging in molecular mass from 1.3 to 51.6 (Mdal). Plasmid-curing experiments suggest that maltose utilization is associated with a 51 Mdal plasmid in *Lactobacillus* sp. DB29 and 42 Mdal plasmids in *Lactobacillus* spp. DB27, DB28, DB31. Southern blot DNA-DNA hybridization showed homology between the maltose plasmid from *Lactobacillus* sp. DB29 and several plasmids from the other *Lactobacillus* spp.

(50 pages)

CHAPTER I

INTRODUCTION

Fermented meat products have been an important part of the human diet since ancient times. Fermented sausage is the result of a complex biological, chemical, and physical process. A successful sausage fermentation is dependent on the proper development of fermentative microorganisms in raw, comminuted meat. It has been shown that lactic acid bacteria, mainly Lactobacillus spp., are the major group of organisms found in sausage manufacturing. The rapid conversion of added sugar to lactic acid by these bacteria is largely responsible for the characteristic flavor and shelf-life associated with sausage.

In traditional meat fermentations, lactic acid bacteria are selected by applying a system of physical, chemical, biochemical, and environmental constraints to prevent the growth of undesirable microorganisms. However, the quality of traditionally fermented sausage has been variable and difficult to control. Following the successful use of starter cultures in dairy fermentations, Lactobacillus starter cultures were also introduced to the meat industry as an acidulant to achieve greater uniformity and to shorten processing schedules.

Despite the benefits in using starter cultures, most sausage manufacturers still rely on traditional processing methods. This is mainly due to the inability of commercial starter strains to produce the desired characteristics unique to their product.

Recently corn syrups which contain maltose and dextrose were suggested to be a carbohydrate source for growth of lactic acid bacteria

in sausage manufacture. Use of corn syrup and other inexpensive carbohydrate sources in sausage fermentations would be economically advantageous.

The development of efficient starter strains which are able to perform predictably and efficiently under the varying conditions of meat fermentations would be highly desirable. Microbiological, biochemical, and genetic analysis of meat starter strains is needed to define strains of commercial value. Also, with recent advances in genetic engineering it may be possible to genetically improve preexisting meat starter strains.

Therefore, the purpose of this study was to use microbiological, biochemical, and genetic analysis to characterize Lactobacillus spp. which have been associated with meat products. This thesis is divided into four chapters. Chapter I is an introduction to the topic and format of the thesis. Chapter II describes a historical review of sausage manufacture and biotechnology of lactobacilli. Chapter III contains the results from this study, which will be submitted for publication in the Journal of Applied and Environmental Microbiology. The summary and conclusions are found in Chapter IV.

CHAPTER II

HISTORICAL REVIEW

Sausage is one of the oldest forms of fermented foods in the world. Sausage making developed gradually from the simple process of salting and drying meats to preserve that which could not be consumed at once. Historically it was observed that addition of salt and sugar to ground meat, followed by an incubation period, was conducive to preservation and resulted in a palatable product. Throughout history several distinctive sausage types were developed in various geographical regions. These sausage types were distinctive in flavor, texture, size, and shape. The successful manufacture of fermented sausage is dependent on the controlled conversion of sugar to lactic acid by lactic acid bacteria (13). Lactic acid bacteria may be present naturally or introduced to the meat mixture during handling or processing. Members of the genus Lactobacillus, the predominant flora in fermented sausage, have received much attention for their role in sausage manufacture. Recently development of starter cultures consisting of lactobacilli has been successful. Methods to improve meat starter strains by genetic engineering, exemplified by dairy starter cultures, are presently under development.

Manufacturing Process

The manufacturing process of fermented sausages consists of grinding the meats; blending them with salt, nitrate or nitrite, spices and sugar; and then stuffing the mixture into casings. The sausages

are then transferred to a "green" room where fermentation takes place. Temperature, relative humidity, and time of incubation in the "green" room are carefully controlled. These factors differ depending on the type of sausage desired. If a smoked variety is desired, the smoking usually takes place at the same time.

Manufacture of fermented sausages is dependent on the types of microorganisms in the raw, comminuted meat. Initially the microbial flora of fresh, refrigerated, or frozen meat is mainly Gram-negative, catalase-positive, aerobic bacteria. In a natural fermentation the manufacture process promotes a shift towards a Gram-positive, catalase-negative, microaerophilic flora (19). Selective growth of this group is favored due to the anaerobic nature of the meat mixture and the presence of salt (19, 52, 57). Lactic acid bacteria, mainly Lactobacillus spp., have been found to grow, eventually dominating the bacterial flora of naturally fermented products (18, 19, 52). L. plantarum and L. casei are usually found as the representative species. Atypical lactobacilli have also been shown to be a major group found on a variety of meat products (18, 54). The activity of certain lactobacilli is largely responsible for the characteristics associated with fermented sausage. The major role of these lactobacilli is to rapidly and reliably produce lactic acid from added sugars. Production of lactic acid imparts the tangy flavor and characteristic texture associated with fermented sausage. Lactic acid also decreases pH, which aids in the formation of cured meat color and inhibits the growth of foodborne pathogens and spoilage bacteria.

Use of Starter Cultures

Traditionally, fermented sausages have been made by cultivating natural microflora, often resulting in products with variable qualities. Failures may occur if nonfermentative microorganisms or heterofermentative lactic acid bacteria predominate. Insufficient lactic acid production results in products defective in flavor, aroma, texture, and appearance. Also, a potential health hazard results when the proper pH is not achieved. Some manufacturers obtained better consistency by "back-slopping," a method in which meat from a previous successful fermentation is added to the new sausage mix. This method provides a massive inoculum of desired bacteria but can also result in the growth of undesired bacteria.

With the successful use of starter cultures in milk fermentations during the last 30 years, starter cultures have been developed for the meat industry (14). The development of starter cultures for meat fermentations involved the isolation and identification of the microorganisms responsible for the desired effect, followed by their addition to the meat at the appropriate stage. Smith and Palumbo (61, p. 997) defined meat starter cultures as "viable microorganisms added directly to meat to improve the keeping quality, improve the safety, and/or enhance consumer acceptability of the meat product."

Research in fermented meat technology has not kept pace with other areas of fermented food processing such as dairy fermentations. Meat is more complicated to study than milk products because of its nonuniformity and nonfluid nature. In addition, research with the effects of bacterial-pure cultures is very difficult since fermented meats are not sterile or pasteurized. The effects of undesired

contaminant bacteria cannot be eliminated (50).

In preliminary meat starter trials it was found that most of the strains which were used in dairy fermentations were unable to grow in the meat mixtures due to their salt and/or nitrite intolerance (29). Subsequent attempts utilized lactobacilli, which were the predominant flora in meat mixtures, but these strains did not survive lyophilization, which was the chief method of distributing starters at that time. In the 1950s Pediococcus acidilactici (previously designated P. cerevisiae) was successfully used as a meat starter culture (14, 15, 51). With the development of frozen culture concentrates and the improvement of freeze-drying techniques, lactobacilli were successfully marketed to the meat industry.

Micrococcus spp. are also used in meat starter cultures because they possess catalase activity as well as the ability to reduce nitrate to nitrite (11). Today Micrococcus (49, 52), Lactobacillus (20, 52), and Pediococcus (14) are successfully utilized as meat starter cultures. The addition of pure cultures of starter bacteria in meat mixtures speeds up the fermentation process, lowers the pH rapidly, and takes control of the development of proper microflora. Thus, the finished product is more uniform, losses are minimized, and flavor is improved.

The major role of starter lactobacilli and pediococci in meat fermentations is to produce lactic acid from carbohydrates rapidly. The availability of fermentable carbohydrates in raw meat is the growth-limiting factor. Suppliers of starter cultures for sausage fermentations suggest that at least 0.75% dextrose must be added to meat mixtures for proper culture growth and acid production (3, 4).

Utilization of Fermentable Carbohydrate in Fermented Sausage

Glucose (dextrose) is the main carbohydrate that is added to the sausage mixture. Other carbohydrate sources, primarily sucrose and corn syrups, are alternatives to dextrose in some fermented sausages (34, 37). The rate of fermentation of various carbohydrates, including commercially available corn syrups, has been determined during a 24 h summer sausage fermentation at 38°C using a frozen concentrate starter culture of Pediococcus acidilactici (1). It was found that maltose yielded 78% as much acid compared to dextrose, while lactose and dextrin showed little acid development. Corn syrup, a hydrolysis product from corn starch, contains varying quantities of the simpler carbohydrates (maltose and dextrose) as well as higher molecular weight fractions. The availability of simpler carbohydrates was the limiting factor in the fermentation. These findings indicate that it is possible to use maltose or corn syrup instead of dextrose, or a combination of maltose or corn syrup with dextrose, to yield a satisfactory fermentation. From an industrial viewpoint, use of corn syrup in sausage formations would be economically advantageous.

The ability of lactic acid bacteria to utilize maltose varies depending on the species and strains used. In testing the fermentative ability of 129 bacterial isolates from dry sausage, Urbaniak and Pezacki (66) reported that all isolates fermented dextrose, several fermented sucrose, and a few fermented maltose.

Therefore, strains used for sausage manufacture should be carefully selected, especially if different carbohydrates are added to the meat formulation. Use of genetic engineering techniques is an important strategy to develop new and improved starter cultures for use in dairy

fermentations (12, 36, 46). Recent genetic studies in lactobacilli show that there is also potential to develop improved strains for use in meat fermentations.

Plasmids in Lactobacilli

Genetic studies in lactobacilli have shown that most strains contain plasmid DNA, thus providing essential tools for using gene cloning techniques for genetic studies and for strain improvement. In 1976 Chassy et al. (7) provided the first evidence for plasmids in lactobacilli. Since then several reports document the prevalence of plasmid DNA in lactobacilli (Table 1). Most of these plasmids are cryptic, having no defined function. However, some of them encode a number of biological properties and provide an extraordinary tool for genetic engineering (Table 2). Phenotypic properties which have been associated with plasmids include lactose metabolism in L. casei subsp. casei 64H (8), N-acetylglucosamine utilization and reduced lactic acid production in L. helveticus subsp. jugurti S36-2 (60), tetracycline and erythromycin resistance in L. fermentum LF601 (27), antibiotic resistance in L. reuteri and L. acidophilus (68), and bacteriocin production in L. acidophilus 11759 (33).

The stability of plasmids in commercial L. plantarum meat starter strain DSM-No 1959 was studied by von Husby and Nes (71). Over a period of 10 years two of the six plasmids appeared to form a larger cointegrate plasmid. The other plasmids were stable and remained unchanged.

TABLE 1. Distribution of plasmids in lactobacilli

Strain	Plasmid size	Reference
L. casei subsp. *rhamnosus* OC19	19 (Mdal)	(7)
L. casei subsp. *casei* 64H	23 (Mdal)	(7, 8)
L. casei subsp. *casei*	NR[1]	(67)
L. helveticus subsp. *jugurti* S13-8	16.45, 13.03, 11.83 (kb)	(60)
L. helveticus subsp. *jugurti* S36-2	13.17 (kb)	(60)
L. helveticus	NR[1]	(67)
L. fermentum LF601	37.5, 16.7, 10.1, 8.5 (Mdal)	(27)
L. acidophilus PA3	13.7, 6.3 (Mdal)	(32)
L. acidophilus 11759	26 (Mdal)	(33)
L. acidophilus	NR[1]	(67, 68)
L. reuteri	NR[1]	(67, 68)
L. heterohiochi	NR[1]	(67)
L. bulgaricus	NR[1]	(67)
L. plantarum MD and NF	12.3, 10.3, 7.4, 5.5, 3.1, 2.65 (Mdal)	(48)
L. plantarum S and CH-1	12.2, 9.2, 6.5 (Mdal)	(48)
L. plantarum N	15.4, 7.4 (Mdal)	(48)
L. plantarum ATCC 8014	14.9, 14.2, 5.6, 1.35 (Mdal)	(48)
L. plantarum ATCC 14917	4.6 (Mdal)	(48)
L. plantarum DSM-No 1959	6.5, 7.6, 13.4, 18.1, 25.2, 30.0 (kb)	(71)
L. plantarum 340	8 (kb)	(72)
L. plantarum 343	9, 2.75 (kb)	(72)
L. plantarum 352	15, 10, 8.5, 7, 4, 2.8 (kb)	(72)
L. plantarum 704	15, 2.5 (kb)	(72)
L. plantarum 1193	11.5 (kb)	(72)
L. plantarum 1752	7 (kb)	(72)
Lactobacillus sp.	NR[1]	(42)
Lactobacillus sp.	2.2 (Mdal)	(45)
Lactobacillus sp. DRI	30 (Mdal)	(55)

[1]NR: the sizes of plasmids were not reported.

TABLE 2. Functional properties of plasmids in lactobacilli

Strain	Plasmid size	Plasmid phenotype	Reference
L. casei subsp. *casei* 64H	23 Mdal	Lactose metabolism	(8)
L. helveticus subsp. *jugurti* S36-2	13.17 kb	N-acetylglucosamine utilization and reduced lactic acid production	(60)
L. fermentum LF601	37.5 Mdal	Tetracycline resistance	(27)
	10.1 Mdal	Erythromycin resistance	(27)
L. acidophilus 11759	26 Mdal	Bacteriocin production	(33)

Gene Transfer in Lactobacilli

Plasmids which code for certain biological properties may be transferred to target organisms by several different gene transfer systems. In other lactic acid bacteria, such as the group N streptococci, transduction, conjugation, protoplast transformation, and protoplast fusion are well documented (22, 36). These gene transfer techniques are important for genetic analysis and for the improvement of starter cultures using genetic engineering principles. However, transferring plasmids in lactobacilli has been very difficult, and further research is needed. Genetic studies have recently been reviewed by Batt and Sinskey (6).

Transduction

Bacteriophage-mediated transduction is a classical method of transferring genes between strains (41). In lactobacilli there have been numerous reports of phages in various species (10, 16, 63, 74). However, only one successful transduction between *Lactobacillus* spp. has been reported. Tohyama et al. (64) demonstrated generalized transduction of *L. salivarius* auxotrophic (lysine, proline, serine) mutants to prototrophy at a frequency of 10^{-7} to 10^{-8} using a temperate phage, PLS-1. They were also able to transduce lactose-fermenting ability at a similar frequency.

Conjugation

Conjugation is a gene transfer process in which a donor cell comes into physical contact with a recipient cell and transfers a replica of genetic information to the recipient cell (21). An erythromycin resistance encoding plasmid, pAMβ1, originally isolated from

Streptococcus faecalis, has been transferred into and between several Lactobacillus species (23, 59, 69, 72). A S. faecalis erythromycin and chloramphenicol resistance plasmid, pIP501, has been transferred to L. plantarum but could not be transferred between strains of L. plantarum (72). In 1981 Chassy and Rokaw (9) described the transfer of plasmid-mediated lactose metabolism by a "conjugation-like" process between two strains of L. casei.

Transformation

The uptake of naked or free DNA by a cell occurs by a process termed transformation. A number or organisms have been successfully transformed by polyethylene glycol (PEG)-induced DNA uptake by protoplasts (24, 26, 30, 35, 53, 56). In lactobacilli the major barrier in developing recombinant DNA technology is the lack of transformation systems (26). One problem in the development of transformation systems in lactobacilli has been the presence of potent DNases produced by many strains (58). However, the formation of L. casei protoplasts has been reported by Tomochika et al. (65) and Lee-Wickner and Chassy (39), the formation of L. reuteri protoplasts has been reported by Vescovo et al. (70), and the transformation of both L. bulgaricus and L. helveticus using a chimeric vector has been reported by Batt (5). Shimizu-Kadota and Kudo (58) successfully transfected L. casei protoplasts with phage DNA encapsulated in liposomes formed from lecithin.

Protoplast Fusion

Two protoplasts are induced to fuse, and recombination of genetic information may occur by a process termed protoplast fusion. Protoplast fusion between two strains of L. fermentum has been reported by Iwata et

al. (28).

Gene Cloning

The development of cloning systems in lactobacilli has been hampered by the lack of efficient transformation systems. However, Lactobacillus genes have been cloned into other genera where transformation and vector DNAs are readily available. The gene coding for β-D-phosphogalactoside galactohydrolase in L. casei was successfully cloned into pBR322, an Escherichia coli vector (38). A cryptic 3.0 kb plasmid from L. helveticus and the erythromycin resistance marker originally from pAMβ1 were cloned into the E. coli vector pBR325 to form a chimeric vector, pLBC104EM. This chimeric vector was successfully constructed in L. helveticus and L. bulgaricus (6). Cryptic plasmids isolated from L. casei strains have been cloned into both E. coli and Streptococcus sanguis by using shuttle vectors (40).

Summary

Lactobacilli have played an essential role in the production of fermented meat products by imparting many of the desirable characteristics associated with these foods.

In natural meat fermentations, salt and fermentable carbohydrates are added to provide a favorable selective environment where the lactobacilli can rapidly proliferate. Following the successful use of starter cultures in dairy fermentations, lactobacilli were introduced into the sausage mixture as starter cultures because of their lactic acid production ability and salt tolerance. Because of the complex nature of the substrate, the selection of efficient and reliable strains

is essential. Genetic engineering techniques have the potential to improve strains used in meat fermentations.

CHAPTER III

PLASMID-LINKED MALTOSE UTILIZATION IN *LACTOBACILLUS* SPP. ISOLATED FROM MEAT

Introduction

The lactobacilli are widely used as starter cultures in dairy, sausage, alcoholic beverage, pickling, and silage fermentations for their ability to produce lactic acid from various carbohydrates. It has been shown that plasmids are widely distributed among members of the genus *Lactobacillus* (7, 8, 25, 27, 32, 33, 42, 45, 47, 48, 55, 60, 67, 68, 72). Although most plasmids are cryptic, some of the functions of these plasmids have been reported. The metabolism of lactose in some strains of *L. casei* is associated with a 23 megadalton (Mdal) plasmid (8). The loss of N-acetylglucosamine fermentation and reduced lactic acid production were accompanied by the loss of a 13.2 kilobase (8.2 Mdal) plasmid in a strain of *L. helveticus* subsp. *jugurti* (60). Tetracycline and erythromycin resistance in a strain of *L. fermentum* was linked to 37.5 and 10.1 Mdal plasmids respectively (27). In *L. acidophilus* and *L. reuteri*, some antibiotic-resistant characteristics are plasmid linked (68). The determinant for bacteriocin production was coded by a 26 Mdal plasmid in a strain of *L. acidophilus* (33). However, the lack of genetic markers has made the study of plasmids of lactobacilli difficult.

In this study it was found that maltose utilization by atypical *Lactobacillus* spp. isolated from fresh meat was an unstable trait and that the loss of maltose utilization in these strains correlates with

the loss of specific plasmids.

Materials and Methods

Bacterial Strains and Media

All strains used in this study are listed in Table 3. Cultures were maintained by biweekly transfer in lactobacilli MRS broth (Difco Laboratories, Detroit, Mich.) (17). Cultures were characterized by using the API 50L *Lactobacillus* identification system (API System, S. A. La Balme les Grottes, Montalieu Vercieu, France) as recommended by the manufacturer.

Isolation and Purification of Plasmid DNA

Plasmid DNA was isolated by the method of Anderson and McKay (2), with modification. Cultures were grown for 16-18 h in MRS broth and transferred as a 2% inoculum into fresh MRS broth. After 2 h incubation at 32°C, cells were harvested by centrifugation and lysed by lysozyme-sodium dodecyl sulfate treatment.

Plasmid DNA was further purified by cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium density gradient centrifugation as previously described (31). Gradient fractions containing plasmid DNA were pooled and extracted with equal volumes of CsCl-saturated n-propanol to remove EtBr. CsCl was removed, and plasmid DNA was concentrated in TE (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) buffer by ultrafiltration using Amicon Centricon-30 microconcentrators (Amicon Corporation, Danvers, Mass.) according to the instructions of manufacturer. Ultrafiltration was performed at least three times.

TABLE 3. *Lactobacillus* strains and plasmids

Strain	Plasmid sizes (Mdal)	Relevant phenotype	Comment and source
L. plantarum ATCC 4008	18.5, 4.8, 1.3	Mal^+	ATCC 4008
L. plantarum DB16	39.8, 20.9, 7.1	Mal^+	Obtained from Trumark
L. plantarum DB21	43.3, 21.2, 12.7, 8.3, 7.2	Mal^+	Obtained from Hansen's rediset LT2
L. plantarum DB24	30.7, 10.3	Mal^+	Obtained from ABC custom culture 18
L. plantarum DB25	20.4, 16.0, 13.3, 9.8, 2.9	Mal^+	Obtained from B Heller
Lactobacillus sp. DB27	41.6, 28.3, 19.9	Mal^+	Obtained from USU culture collection, previously designated as mutton LBS 10-4[a]
Lactobacillus sp. DB28	42.4, 28.7, 20.1	Mal^+	Obtained from USU culture collection, previously designated as mutton MRS 5-5[a]
Lactobacillus sp. DB29	51.6	Mal^+	Obtained from USU culture collection, previously designated as beef MRS 3-3[a]
Lactobacillus sp. DB31	42.4, 30.2, 21.6	Mal^+	Obtained from USU culture collection, previously designated as beef LBS 3-2[a]
Lactobacillus sp. ML291	None	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB29
Lactobacillus sp. ML292	24.7	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB29
Lactobacillus sp. ML271	28.3, 19.9	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB27
Lactobacillus sp. ML272	28.3	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB27
Lactobacillus sp. ML281	28.7, 20.1	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB28
Lactobacillus sp. ML282	28.7	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB28
Lactobacillus sp. ML311	30.2, 21.6	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB31
Lactobacillus sp. ML312	30.2	Mal^-	Plasmid-cured derivative of *Lactobacillus* sp. DB31

[a]These isolates from fresh beef and mutton were identified as strains of atypical lactobacilli by API test and other biochemical test (73).

Agarose Gel Electrophoresis

The examination of plasmid DNA was performed on 0.6% horizontal agarose slab gels in Tris-borate buffer (0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA). Electrophoresis was carried out at 100 volts for 4 h at 4°C. The gel was stained in ethidium bromide (1 µg/ml) for 30 min and photographed under UV illumination. E. coli V517 (43) plasmids were used as standards for molecular mass determinations.

Plasmid Curing

Cultures grown overnight in MRS broth supplemented with 2% maltose instead of glucose (MRS-M) were transferred to fresh MRS broth containing 100 µg/ml acriflavin and incubated for 24 h at 32°C. MRS broth and MRS-M broth were also inoculated and incubated to serve as controls. After serial dilution, cultures were plated on MRS-indicating agar (MRS-M agar containing 0.001% bromocresol purple) and anaerobically incubated at 32°C for 48 h using a BBL GasPak Anaerobic System (BBL Microbiology Systems, Cockeysville, Md.). Individual maltose-negative (Mal^-) colonies were scored, isolated, and subcultured in MRS broth for examination of plasmid profiles.

Restriction Endonuclease Analysis

Plasmid DNA was digested with restriction enzymes listed in Table 4. Restriction digestions were performed according to the instructions of the manufacturer (International Biotechnologies, Inc., New Haven, Conn.). Analysis of fragments was conducted by using horizontal gel electrophoresis under the same conditions as previously described for plasmid analysis using lambda-HindIII fragments as reference mobility fragments.

TABLE 4. Restriction enzyme analysis of pML291 and pML292

Restriction enzyme	Plasmid	Number of fragments	Calculated size of fragments (kb)	Total size (kb)
PvuII	pML291	12	26.5, 11.5, 9.3, 7.7, 7.7, 6.6, 3.5, 2.3, 2.0, 1.4, 1.2, 0.7	80.4
	pML292	5	26.5, 3.5, 2.3, 1.2, 0.7	34.2
SmaI	pML291	5	28.5, 23.2, 20.2, 2.4, 2.3	76.6
	pML292	4	23.2, 20.2, 2.4, 2.3	48.1

DNA-DNA Hybridization

Southern blot DNA-DNA hybridization was performed with a commercially available DNA-DNA blot hybridization system (BluGene, Bethesda Research Laboratories, New York, N.Y.). Plasmid DNA was electrophoresed through an agarose gel under the same conditions as previously described for plasmid analysis and then transferred to nitrocellulose paper by the Southern method (62), as modified by Maniatis et al. (44). Plasmid, pML291, was digested with BclI and nick translated using Biotinyl-11-dUTP as a label. Preparation of the nick-translated biotinylated probe and hybridization were performed according to the instructions of the manufacturer, except that hybridization was carried out at 68°C.

Results

Characterization of Lactobacilli

Results of the API 50L *Lactobacillus* identification test are listed in Table 5. None of the strains isolated from meat could be identified to a species level. They were considered to be atypical *Lactobacillus* species, which is consistent with previous studies (73).

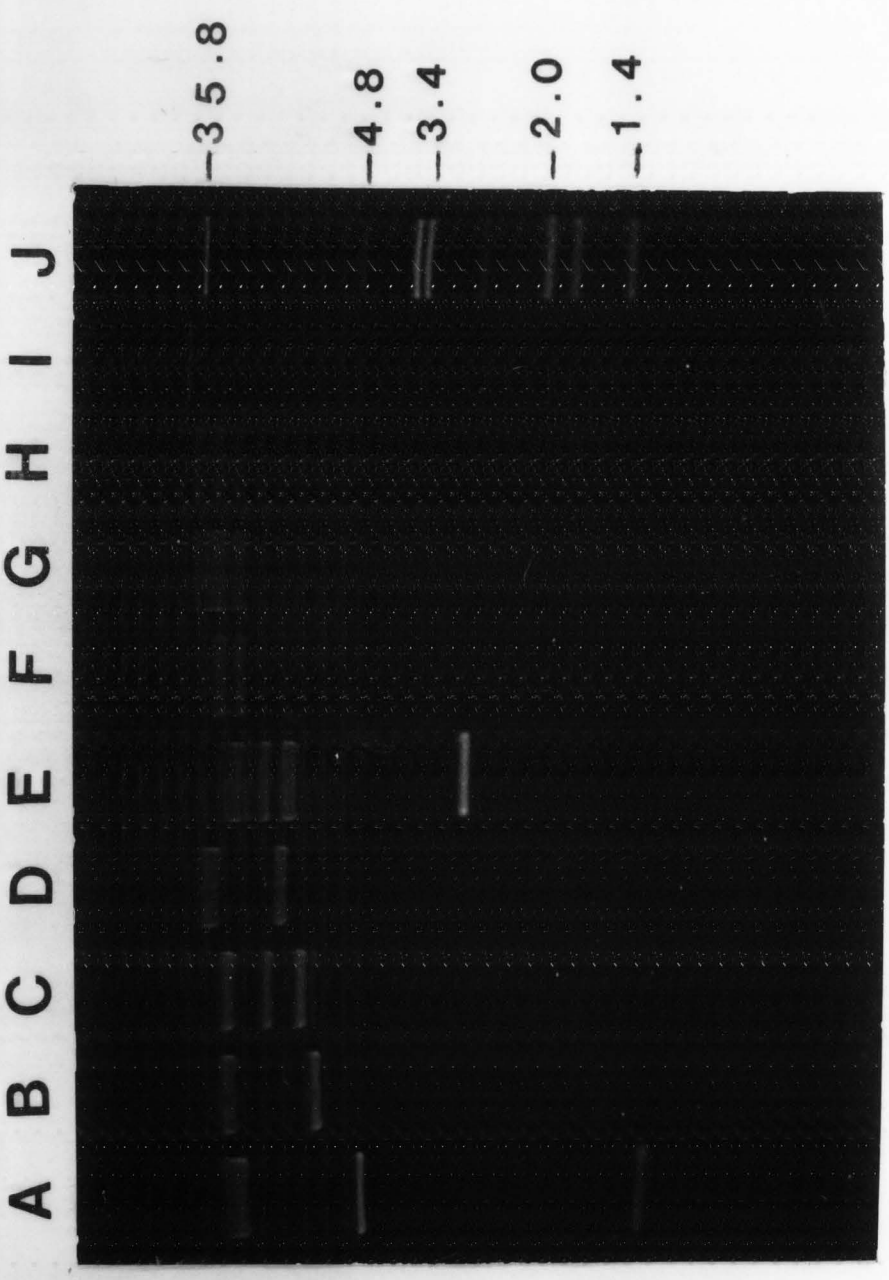
Distribution of Plasmids in Lactobacilli

Agarose gels of CsCl-EtBr gradient purified plasmids isolated from strains used in this study are shown in Fig. 1. These strains harbor between one and five distinct plasmids ranging in mass from 1.3 to 51.6 Mdal as shown in Table 3.

TABLE 5. Utilization of substrates by lactobacilli (API test)

Strain	ATCC 4008	DB 16	DB 21	DB 24	DB 25	DB 27	DB 28	DB 31	DB 29	ML 291	ML 292
0 Control	-	-	-	-	-	-	-	-	-	-	-
1 Glycerol	-	+	+	-	-	-	-	-	-	-	-
2 Erythritol	-	-	-	-	-	-	-	-	-	-	-
3 D-Arabinose	-	-	-	-	-	-	-	-	-	-	-
4 L-Arabinose	-	+	+	+	-	+	+	+	+	+	+
5 Ribose	-	+	+	+	+	+	+	+	+	+	+
6 D-Xylose	-	+	+	-	-	-	-	-	-	-	-
7 L-Xylose	-	-	-	-	-	-	-	-	-	-	-
8 Adonitol	-	-	-	-	-	-	-	-	-	-	-
9 B-Methyl-Xyloside	-	-	-	-	-	-	-	-	-	-	-
10 Galactose	+	+	+	+	+	+	+	+	+	+	+
11 D-Glucose	+	+	+	+	+	+	+	+	+	+	+
12 D-Fructose	+	+	+	+	+	+	+	+	+	+	+
13 D-Mannose	+	+	+	+	+	+	+	+	+	+	+
14 L-Sorbose	-	-	-	-	-	-	-	-	-	-	-
15 Rhamnose	-	+	+	-	+	-	-	-	-	-	-
16 Dulcitol	-	-	-	-	-	-	-	-	-	-	-
17 Inositol	-	-	-	-	-	-	-	-	-	-	-
18 Mannitol	+	+	+	-	+	-	-	-	-	-	-
19 Sorbitol	+	+	+	-	+	-	-	-	-	-	-
20 a-Methyl D-mannoside	+	-	-	-	+	-	-	-	-	-	-
21 a-Methyl D-glucosid	-	+	+	-	-	-	-	-	-	-	-
22 N-Acetyl-Glucosamine	+	+	+	+	+	+	+	+	+	+	+
23 Amygdalin	+	+	+	+	+	-	-	-	-	-	-
24 Arbutin	+	+	+	-	+	-	-	-	-	-	-
25 Esculin	+	+	+	+	+	+	+	+	-	-	-
26 Salicin	+	+	+	+	+	+	+	+	-	-	-
27 Cellobiose	+	+	+	+	+	+	+	+	-	-	-
28 Maltose	+	+	+	+	+	+	+	+	+	-	-
29 Lactose	+	+	+	+	+	+	+	-	-	-	-
30 Melibiose	+	+	+	+	+	+	+	+	-	-	-
31 Saccharose	+	+	+	+	+	+	+	+	+	+	+
32 Trehalose	+	+	+	+	+	+	+	+	+	+	+
33 Inuline	-	-	-	-	-	-	-	-	-	-	-
34 Melezitose	+	-	-	-	+	-	-	-	-	-	-
35 D-Raffinose	+	+	+	-	-	-	-	-	-	-	-
36 Starch	-	-	-	-	-	-	-	-	-	-	-
37 Glycogen	-	-	+	-	-	-	-	-	-	-	-
38 Xylitol	-	-	-	-	-	-	-	-	-	-	-
39 B-Gentiobiose	+	+	+	+	+	-	-	-	-	-	-
40 D-Turanose	-	+	-	-	+	-	-	-	-	-	-
41 D-Lyxose	-	-	-	-	-	-	-	-	-	-	-
42 Tagatose	-	-	-	-	-	-	-	-	-	-	-
43 D-Fucose	-	-	-	-	-	-	-	-	-	-	-
44 L-Fucose	-	+	+	-	-	-	-	-	-	-	-
45 D-Arabitol	-	+	+	-	+	-	-	-	-	-	-
46 L-Arabitol	-	-	-	-	-	-	-	-	-	-	-
47 Gluconate	-	-	-	+	-	+	+	+	+	+	+
48 2-Keto-Gluconate	-	-	-	-	-	-	-	-	-	-	-
49 5-Keto-Gluconate	-	-	-	-	-	-	-	-	-	-	-

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from strains used in this study. Plasmids were isolated from L. plantarum ATCC 4008 (lane A), L. plantarum DB16 (lane B), L. plantarum DB21 (lane C), L. plantarum DB24 (lane D), L. plantarum DB25 (lane E), Lactobacillus sp. DB27 (lane F), Lactobacillus sp. DB28 (lane G), Lactobacillus sp. DB31 (lane H), Lactobacillus sp. DB29 (lane I), and E. coli V517 (lane J). Numbers represent the E. coli reference plasmid sizes in Mdal.


A
B
C
D
E
F
G
H
I
J
35.8
4.8
3.4
2.0
1.4

Production of Mal$^-$ Variants with Acriflavin

After acriflavin treatment the strains isolated from meat, DB27, DB28, DB29, and DB31, yielded Mal$^-$ variants which were isolated and identified on MRS-indicating agar as white colonies. The colonies of parental Mal$^+$ strains were yellow due to rapid acid production from maltose metabolism. The frequencies of plasmid curing are shown in Table 6. After three consecutive transfers in the presence of acriflavin, the production of Mal$^-$ variants ranged from 94.7 to 99.4%. To determine if the observed loss of maltose metabolism was accompanied by the loss of any plasmids in these strains, 20 Mal$^-$ isolates from each strain were examined for the presence of plasmid DNA. The majority of the Mal$^-$ isolates from DB29 lost a 51.6 Mdal plasmid. These mutants are exemplified by loss of pML291 by strain ML291. However, a Mal$^-$ isolate designated as ML292 contained a smaller plasmid (pML292) of 24.7 Mdal, as shown in Fig. 2. In strain DB27 some of the Mal$^-$ variants, such as ML271, lost the 41.6 Mdal plasmid; while others, such as ML272, lost 41.6 and 19.9 Mdal plasmids. Mal$^-$ variants of DB28, exemplified by ML281, lost the 42.4 Mdal plasmid; and the other Mal$^-$ isolates, such as ML282, lost both 42.4 and 20.1 Mdal plasmids. Similarly, some of the Mal$^-$ variants isolated from DB31, such as ML311, lost a 42.4 Mdal plasmid; and the others, such as ML312, lost both 42.4 and 21.6 Mdal plasmids, as shown in Fig. 3. Comparison of DB29 with Mal$^-$ ML291 and ML292 using the API 50L *Lactobacillus* identification system indicated that only maltose utilization was different, as shown in Table 5. Maltose utilization appeared to be very stable in the *L. plantarum* strains as no Mal$^-$ mutants could be isolated under the same conditions.

TABLE 6. Frequency of isolating Mal$^-$ variants from atypical Lactobacillus spp. during plasmid-curing experiments

Strain	Percentage of Mal$^-$ variants generated		
	1st transfer	2nd transfer	3rd transfer
DB27[a]	57.6%	96.4%	99.1%
DB27[b]	0.0%	0.0%	0.0%
DB27[c]	0.0%	0.0%	0.0%
DB28[a]	61.4%	99.2%	99.4%
DB28[b]	0.0%	0.0%	0.0%
DB28[c]	0.0%	0.0%	0.0%
DB29[a]	43.5%	64.2%	94.7%
DB29[b]	0.0%	0.0%	0.0%
DB29[c]	0.0%	0.0%	0.0%
DB31[a]	73.6%	91.0%	97.4%
DB31[b]	0.0%	0.0%	0.0%
DB31[c]	0.0%	0.0%	0.0%

[a]Cultures were grown in lactobacilli MRS medium containing 100 µg/ml of acriflavin.

[b]Cultures were grown in lactobacilli MRS medium.

[c]Cultures were grown in MRS-M broth.

FIG. 2. Agarose gel electrophoretic patterns of plasmid DNA isolated from Mal$^-$ mutants of Lactobacillus sp. DB29. ML291 (lane A), ML292 (lane B), DB29 (lane C), and E. coli V517 reference mobility plasmids (lane D). Numbers represent the reference plasmic sizes in Mdal. Chr designates location of the chromosomal DNA.

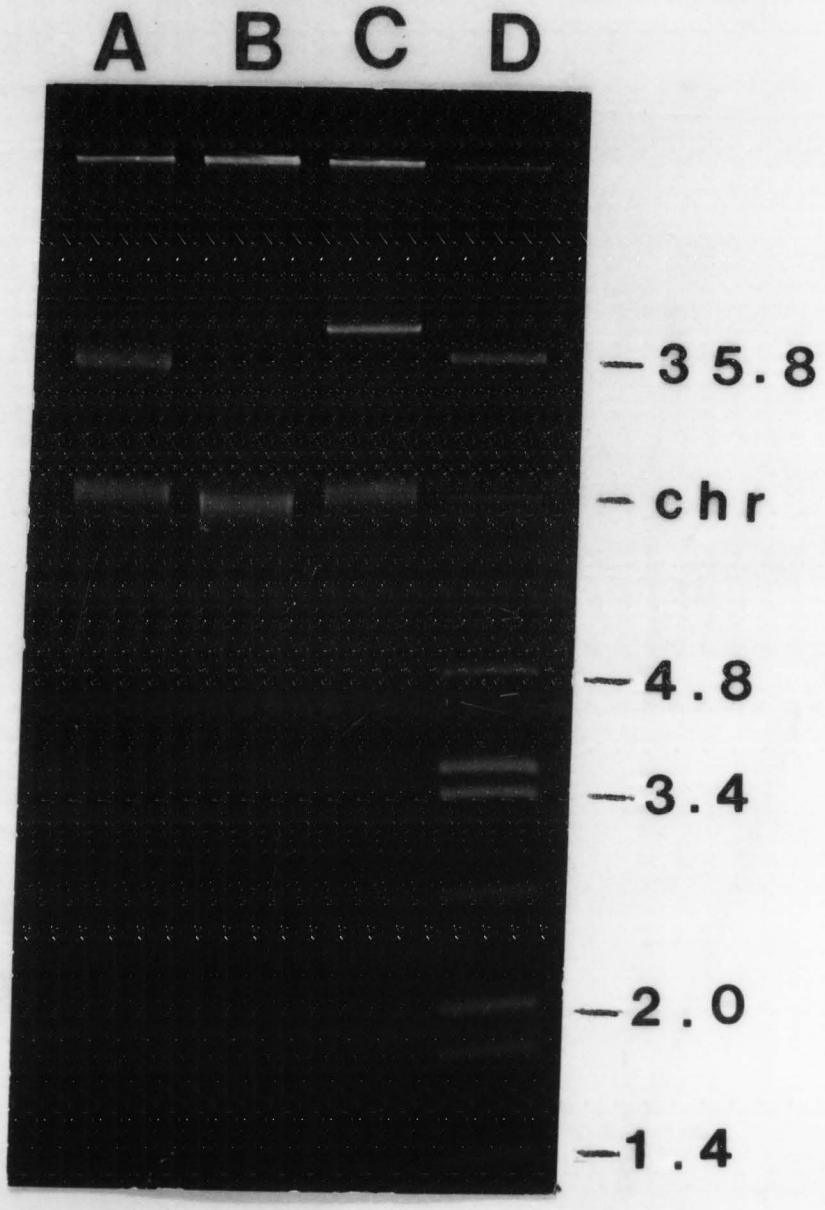
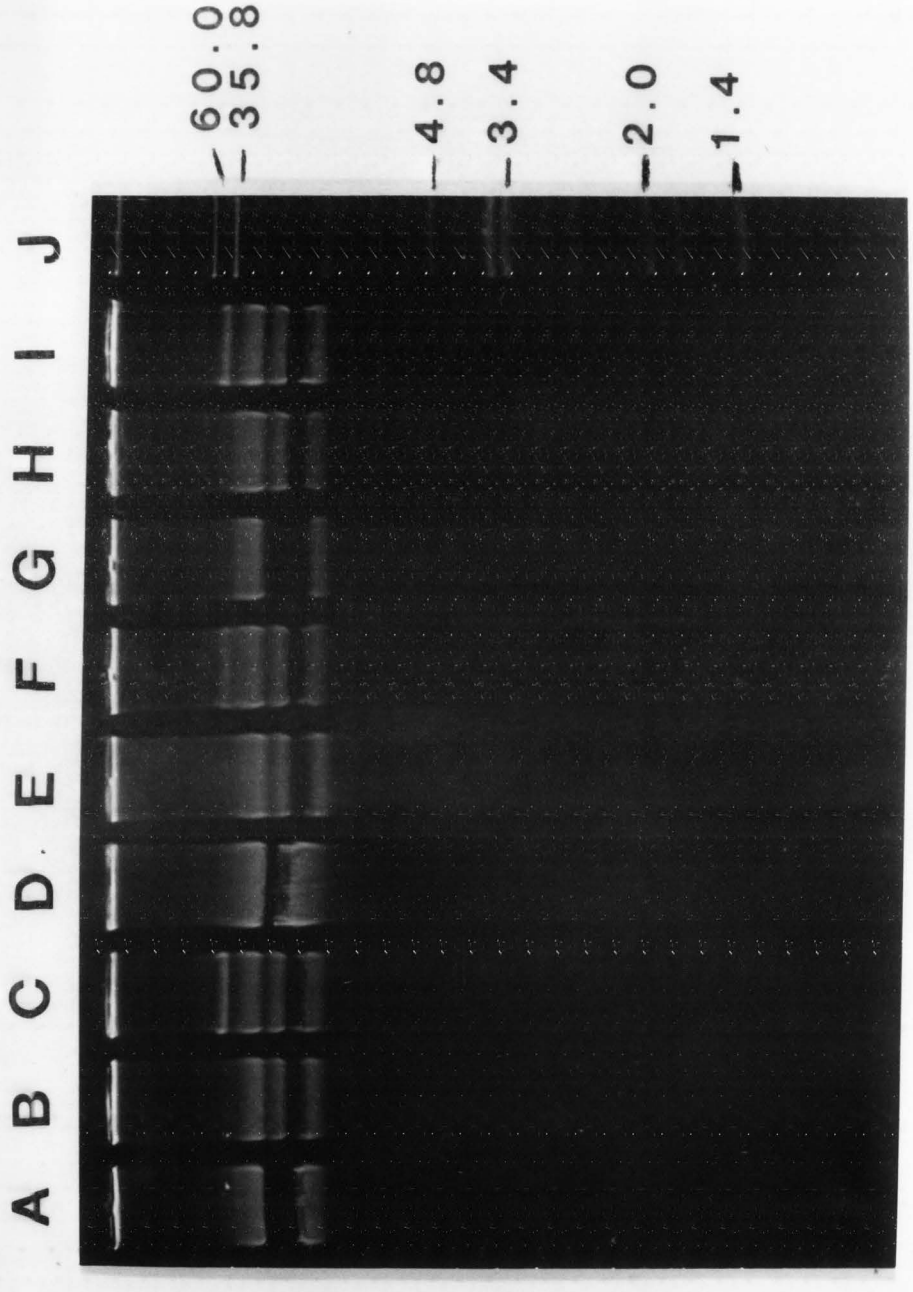

A
B
C
D
35.8
chr
4.8
3.4
2.0
1.4

FIG. 3. Agarose gel electrophoretic patterns of plasmid DNA isolated from Mal$^-$ mutants of Lactobacillus spp. DB27, DB28, and DB31. ML272 (lane A), ML271 (lane B), DB27 (lane C), ML282 (lane D), ML281 (lane E), DB28 (lane F), ML312 (lane G), ML311 (lane H), DB31 (lane I), and Salmonella typhimurium LT2 and E. coli V517 (lane J). Numbers represent the S. typhimurium and E. coli reference plasmid sizes in Mdal.


A B C D E F G H I J
60.0
35.8
4.8
3.4
2.0
1.4

Restriction Endonuclease Analysis

Evidence for the relatedness of pML291 and pML292 was obtained by restriction enzyme analysis, as shown in Table 4 and in Fig. 4 and 5. Restriction digestion with PvuII and SmaI generated many identical fragments, suggesting that pML292 is a deletion derivative of pML291.

DNA-DNA Homology Among pML291 and Plasmids Isolated from Strains DB27, DB28, and DB31

The possible relatedness of the maltose plasmid, pML291, from strain DB29 and plasmids isolated from strains DB27, DB28, and DB31 was examined. Results of the hybridization in Fig. 6 showed homology between pML291 and several plasmids from strains DB27, DB28, and DB31. Strong hybridization between pML291 and the 42 Mdal plasmids from DB27, DB28, and DB31 suggests that the 42 Mdal plasmids may also code for maltose utilization.

Discussion

The major role of starter lactobacilli in the production of fermented sausage is to produce lactic acid rapidly and reliably from added sugars. Glucose (dextrose) is the major carbohydrate that is added to sausage mixtures, but cheaper carbohydrate sources such as corn syrup, which contains dextrose and maltose, are preferable (1). Therefore, strains for use in sausage manufacture should be carefully selected based on their carbohydrate-fermentation capabilities. In this study the carbohydrate-fermentation capabilities and the stability of these characteristics were studied in strains of lactobacilli.

Five strains of L. plantarum and four atypical Lactobacillus spp. isolated from fresh meat were biochemically characterized (Table 5).

FIG. 4. Agarose gel electrophoretic patterns of PvuII restriction fragments of pML291 (lane A) and pML292 (lane B).

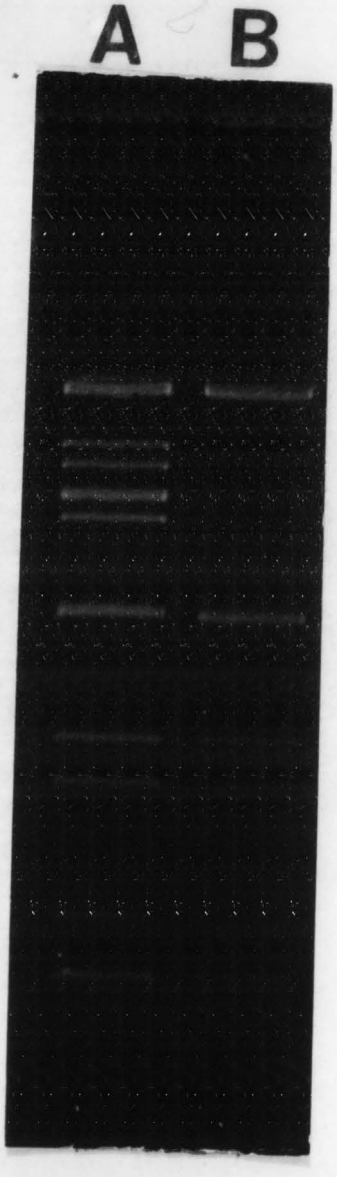

A
B

FIG. 5. Agarose gel electrophoretic patterns of SmaI restriction fragments of pML291 (lane A), pML292 (lane B), and lambda-HindIII restriction mobility reference fragments markers. The numbers represent sizes (kb pairs) and position of marker bands.

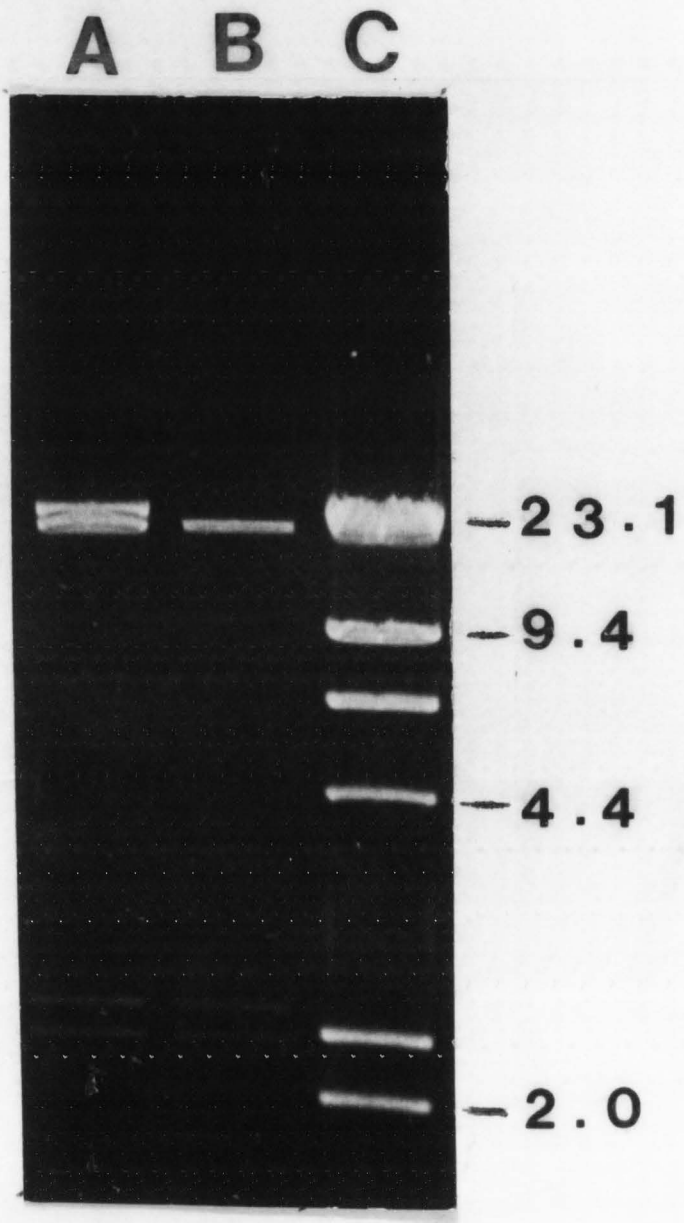
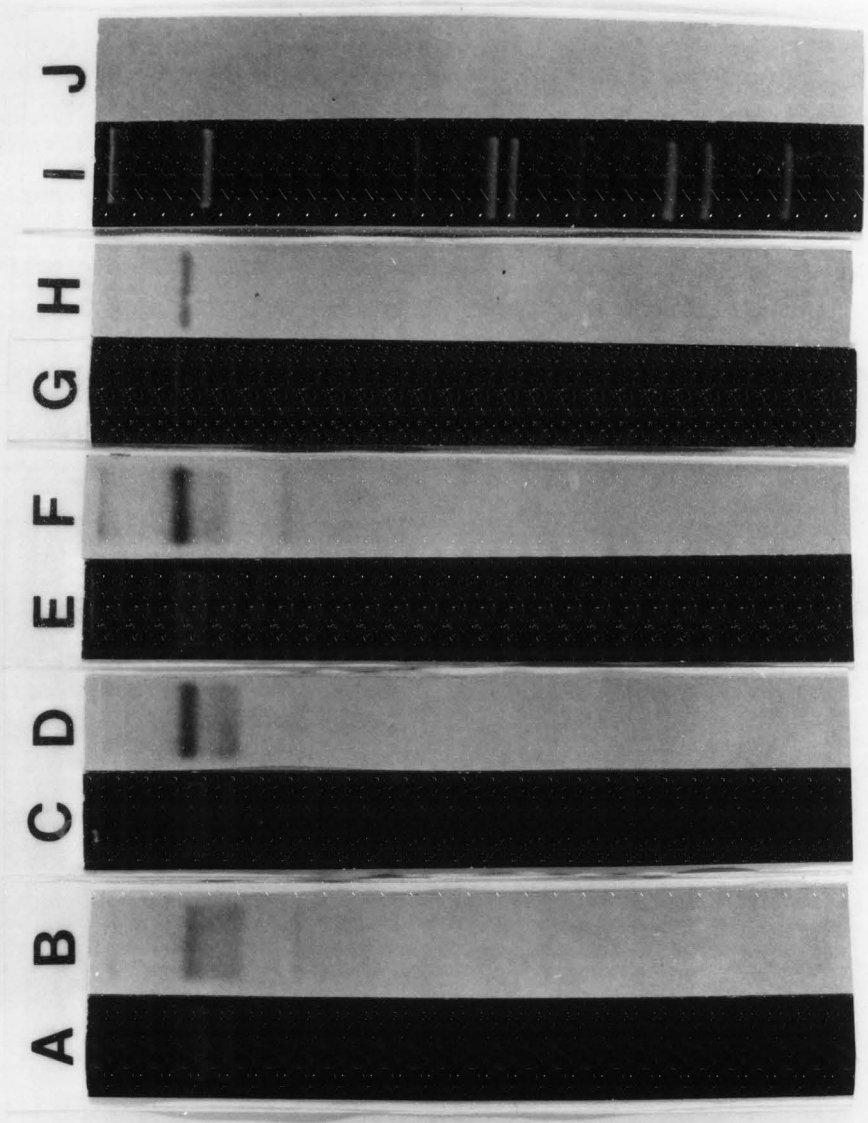

A
B
C
23.1
9.4
4.4
2.0

FIG. 6. DNA-DNA blot hybridization of biotinylated pML291-BclI fragments. Agarose gel electrophoretic patterns of plasmid DNA from DB27 (lane A), DB28 (lane C), DB31 (lane E), DB29 (lane G), and E. coli V517. After Southern transfer of DNA from a 0.6% agarose gel to nitrocellulose, biotinylated pML291-BclI fragments hybridized to: 28.3 and 19.9 Mdal plasmids in DB27 (lane B), 28.7 and 20.1 Mdal plasmids in DB28 (lane D), 30.2 and 21.6 Mdal plasmids in DB31 (lane F), and 51.6 Mdal plasmid in DB29 (lane H).


A
B
C
D
E
F
G
H
I
J

Plasmid analysis showed that these strains contained between one and five distinct plasmids ranging in molecular mass from 1.3 to 51.6 Mdal (Table 3). Growth of these strains in the presence of acriflavin showed that maltose utilization was an unstable trait in atypical Lactobacillus sp. strains DB27, DB28, DB29, and DB31. Between 94.7 and 99.4% Mal^- variants were isolated after three consecutive transfers (Table 6). In contrast, maltose utilization by L. plantarum appeared to be very stable under the same conditions.

In Lactobacillus DB29 loss of maltose metabolism was also accompanied by the loss of pML291 or by loss of a 26.9 Mdal segment of pML291 (Fig. 2). Restriction analysis suggests that pML292 is a deletion derivative of pML291 (Table 4, Fig. 4 and 5). In strains DB27, DB28, and DB31, loss of maltose metabolism was correlated to the loss of a plasmid of approximately 42 Mdal. Southern blot DNA-DNA hybridization using pML291 as a probe showed strong hybridization to the 42 Mdal plasmids. These results support curing data and suggest that the 42 Mdal plasmids also code for maltose utilization. Attempts to transform ML291 with pML291 were unsuccessful.

The evidence presented suggests that maltose metabolism is a plasmid-associated trait in the atypical Lactobacillus spp. isolated from fresh meat. In the development of genetic engineering techniques, these plasmids could be used as genetic markers to develop gene transfer systems and cloning vectors for lactobacilli. From an industrial viewpoint, if gene transfer systems for these plasmids are developed, it will be very useful to transfer these genes to Mal^- starter cultures. Also, cloning the maltose utilization genes of these plasmids into high copy number stable plasmids will increase gene dosage and possibly

influence the rate of acid production. Therefore, corn syrups which contain maltose and dextrose could be used as efficiently as dextrose alone in meat fermentations. This would be economically advantageous for the meat processing industry.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Successful sausage fermentation is dependent on the conversion of fermentable carbohydrates to lactic acid rapidly and reliably by fermentative microorganisms. However, the fermentable carbohydrates source in raw meat is limited. Dextrose is the principle carbohydrate added in sausage manufacture. Recently corn syrups containing dextrose and maltose have been suggested to be used instead of dextrose for economic reasons. However, the ability of meat starter strains to utilize maltose is variable as many strains are unable to ferment maltose.

In this study five strains of L. plantarum and four strains of atypical Lactobacillus species isolated from fresh meat were examined for the presence of plasmid DNA. The plasmid profiles of all strains examined showed that these strains harbored between one and five distinct plasmids ranging in molecular mass from 1.3 to 51.6 Mdal. After treatment with acriflavin, strains DB27, DB28, DB29, and DB31 yielded a high percentage of Mal^- variants. Plasmid analysis showed that Mal^- variants from these strains were plasmid-cured derivatives. However, no Mal^- variants from L. plantarum strains could be isolated.

The curing experiments suggested that the 51.6 Mdal plasmid (pML291) in DB29 and the 42 Mdal plasmids in DB28, DB29, and DB31 code for maltose metabolism. Southern blot DNA-DNA hybridization using pML291-BclI fragments as a probe showed strong hybridization to the 42 Mdal plasmids. The results suggest that the 51.6 and 42 Mdal plasmids

may contain similar DNA sequence for maltose metabolism.

The results of this study suggest that maltose metabolism is a plasmid-mediated trait in the atypical Lactobacillus strains isolated from meat. This will provide the groundwork for further studies to improve meat starter cultures by gene transfer systems and will aid in developing genetic engineering techniques in lactobacilli.

LITERATURE CITED

1. Acton, J. C., R. L. Dick, and E. L. Norris. 1977. Utilization of various carbohydrates in fermented sausage. J. Food Sci. 42:174-178.
2. Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. 46:549-552.
3. Anonymous. 1969. Description and use of Lactacel. Merck Technical Service Bull. IS-8010. Merck Chemical Div., Merck & Co., Rahway, N. J.
4. Anonymous. 1972. Hansen's Redi-Set cultures for meat products. Bull. Chr. Hansen's Laboratory, Inc., Milwaukee, Wis.
5. Batt, C. A. 1986. Genetic engineering of Lactobacillus. Food Technol. 40(1):95-98.
6. Batt, C. A., and A. J. Sinskey. 1984. Current status of genetic engineering of Lactobacillus. Presented during UNIDO symposium on Importance of Lactic Acid Fermentation in the Food Industry, Mexico City, Nov.
7. Chassy, B. M., E. Gibson, and A. Giuffrida. 1976. Evidence for extrachromosomal elements in Lactobacillus. J. Bacteriol. 127: 1576-1578.
8. Chassy, B. M., E. M. Gibson, and A. Giuffrida. 1978. Evidence for plasmid-associated lactose metabolism in Lactobacillus casei subsp. casei. Curr. Microbiol. 1:141-144.

9. Chassy, B. M., and E. Rokaw. 1981. Conjugal transfer of lactose plasmids in Lactobacillus casei, p. 590. In S. Levy, R. Clowes, and E. Koenig (eds.), Molecular biology, pathogenesis, and ecology of bacterial plasmids. Plenum Press, New York.

10. Coetzee, J. N., and H. C. De Klerk. 1962. Lysogeny in the genus Lactobacillus. Nature 194:505.

11. Coretti, K. 1977. Starterkulteren in der Fleischwirtschaft. Fleischwirtschaft 57:386-394.

12. Davies, F. L., and M. L. Gasson. 1983. Genetics of dairy cultures. Ir. J. Food Sci. Technol. 7:49-60.

13. Deibel, R. H. 1974. Technology of fermented, semi-dried and dried sausages, p. 57-60. In Proc. Meat Ind. Res. Conf., Am. Meat Inst. Found., Washington, D. C.

14. Deibel, R. H., and C. F. Niven, Jr. 1957. Pediococcus cerevisiae: A starter culture for summer sausage. Bacteriol. Proc. 1957:14-15.

15. Deibel, R. H., G. D. Wilson, and C. F. Niven, Jr. 1961. Microbiology of meat curing IV. A lyophilized Pediococcus cerevisiae starter culture for fermented sausage. Appl. Microbiol. 9:239-243.

16. De Klerk, H. C., J. N. Coetzee, and J. J. Theron. 1963. The characterization of a series of Lactobacillus bacteriophages. J. Gen. Microbiol. 32:61-67.

17. DeMan, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130-135.

18. Egan, A. F. 1983. Lactic acid bacteria of meat products. Antonie van Leeuwenhoek 49:327-336.

19. Erichsen, I. 1983. Fermented fish and meat products: The present position and future possibilities, p. 271-286. In T. A. Roberts and F. A. Skinner (eds.) Food microbiology: Advances and prospects. Academic Press, London.

20. Everson, C. W., W. E. Danner, and P. A. Hammes. 1970. Bacterial starter cultures in sausage products. J. Agr. Food Chem. 18:570-571.

21. Freifelder, D. M. 1983. Molecular biology. A comprehensive introduction to prokaryotes and eukaryotes. Sci. Books Int., Inc., Portola Valley, Calif.

22. Gasson, M. J. 1983. Genetic transfer systems in lactic acid bacteria. Antonie van Leeuwenhoek 49:275-282.

23. Gibson, E. M., N. M. Chace, S. B. London, and J. London. 1979. Transfer of plasmid-mediated antibiotic resistance from streptococci to lactobacilli. J. Bacteriol. 137:614-619.

24. Gotz, F., B. Krentz, and K. H. Schleifer. 1983. Protoplast transformation of Staphylococcus carnosus by plasmid DNA. Mol. Gen. Genet. 189:340-342.

25. Hofer, F. 1977. Involvement of plasmids in lactose metabolism in Lactobacillus casei suggested by genetic experiments. FEMS Microbiol. Lett. 1:167-170.

26. Hopwood, D. A. 1981. Genetic studies with bacterial protoplasts. Annu. Rev. Microbiol. 35:237-272.

27. Ishiwa, H., and S. Iwata. 1980. Drug resistance plasmids in Lactobacillus fermentum. J. Gen. Appl. Microbiol. 26:71-74.

28. Iwata, M., M. Mada, and H. Ishiwa. 1986. Protoplast fusion of Lactobacillus fermentum. Appl. Environ. Microbiol. 52:392-393.

29. Jensen, L. B., and L. Paddock. 1940. Sausage treatment. U. S. Pat. 2,225,783.

30. Katsumata, R., A. Ozaki, T. Oka, and A. Furuya. 1984. Protoplast transformation of glutamate-producing bacteria with plasmid DNA. J. Bacteriol. 156:306-311.

31. Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin. 1978. Improved lysis of group N streptococci for isolating and rapid characterization of plasmid deoxyribonucleic acid. Appl. Environ. Microbiol. 35:592-600.

32. Klaenhammer, T. R., and S. M. Sutherland. 1980. Detection of plasmid deoxyribonucleic acid in an isolate of Lactobacillus acidophilus. Appl. Environ. Microbiol. 39:671-674.

33. Kleeman, E. G., and T. R. Klaenhammer. Evidence for plasmid linkage of Lactacin F, a bacteriocin produced by Lactobacillus acidophilus. J. Dairy Sci. 66(Suppl. 1):67.

34. Komarik, S. L., D. K. Tressler, and L. Long. 1974. Food products formulary: Meats, poultry, fish, shellfish. Avi Publishing Co., Westport, Conn.

35. Kondo, J. K., and L. L. McKay. 1982. Transformation of Streptococcus lactic protoplasts by plasmid DNA. Appl. Environ. Microbiol. 43:1213-1215.

36. Kondo, J. K., and L. L. McKay. 1985. Gene transfer systems and molecular cloning in group N streptococci: A review. J. Dairy Sci. 68:2143-2159.

37. Kramlich, W. E., A. M. Pearson, and F. W. Tauber. 1973. Processed Meats. Avi Publishing Co., Westport, Conn.

38. Lee, L.-J., J. B. Hansen, E. K. Jagusztyn-Krynicka, and B. M. Chassy. 1982. Cloning and expression of the β-D-phosphogalactoside galactohydrolase gene of Lactobacillus casei in Escherichia coli K-12. J. Bacteriol. 152:1138-1146.

39. Lee-Wickner, L.-J., and B. M. Chassy. 1984. Production and regeneration of Lactobacillus casei protoplasts. Appl. Environ. Microbiol. 48:994-1000.

40. Lee-Wickner, L.-J., and B. M. Chassy. 1985. Characterization and molecular cloning of cryptic plasmids isolated from Lactobacillus casei. Appl. Environ. Microbiol. 49:1154-1161.

41. Lewin, B. 1977. Gene expression. Vol. 3. Plasmid and phages. John Wiley & Sons, Inc., New York, NY.

42. Lin, J. H.-C., and D. C. Savage. 1985. Cryptic plasmids in Lactobacillus strains isolated from the murine gastrointestinal tract. Appl. Environ. Microbiol. 49:1004-1006.

43. Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen. 1978. A multiple plasmid-containing Escherichia coli strain: Convenient source of size reference plasmid molecules. Plasmid 1:417-420.

44. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

45. McCormick, E. L., and D. C. Savage. 1983. Characterization of Lactobacillus sp. strain 100-37 from the murine gastrointestinal tract: Ecology, plasmid content, and antagonistic activity toward Clostridium ramosum H1. Appl. Environ. Microbiol. 46:1103-1112.

46. McKay, L. L. 1983. Functional properties of plasmids in lactic streptococci. Antonie van Leeuwenhoek 49:259-274.

47. Morelli, L., M. Vescovo, and V. Bottazzi. 1983. Plasmids and antibiotic resistance in Lactobacillus helveticus and Lactobacillus bulgaricus isolated from natural whey culture. Microbiologica 6: 145-154.

48. Nes, I. F. 1984. Plasmid profiles of ten strains of Lactobacillus plantarum. FEMS Microbiol. Lett. 21:359-361.

49. Niinivaara, F. P. 1955. Uber den Einfluss von Bakterienreinkulturen auf die Reifung und Umrotung der Rohwurst. Acta Agralia Fennica 84:95-101.

50. Niinivaara, F. P., M. S. Pohja, and S. E. Komulainen. 1964. Some aspects about using bacterial pure cultures in the manufacture of fermented sausages. Food Technol. 18:25-31.

51. Niven, Jr. C. F., R. H. Diebel, and G. D. Wilson. 1958. The AMIF sausage starter culture. Am. Meat Inst. Found. Circ. 41:5-25.

52. Nurmi, E. 1966. Effect of bacterial inoculations on characteristics and microbial flora of dry sausage. Acta Agralia Fennica 108:1-77.

53. Reid, S. J., E. R. Allcock, D. T. Jones, and D. R. Woods. 1983. Transformation of Clostridium acetobutylicum protoplasts with bacteriophage DNA. Appl. Environ. Microbiol. 45:305-307.

54. Reuter, G. 1975. Classification problems, ecology and some biochemical activities of lactobacilli of meat products, p. 221-229. In J. G. Carr, C. V. Cutting, and G. C. Whiting (eds.), Lactic acid bacteria in beverages and food. Academic Press, London.

55. Romero, D. A., and L. L. McKay. 1985. Isolation and plasmid characterization of a Lactobacillus species involved in the manufacture of fermented sausage. J. Food Prot. 48:1028-1035.

56. Santamaria, R., J. A. Gil, J. M. Mesas, and J. F. Martin. 1984. Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in Brevibacterium lactofermentum. J. Gen. Microbiol. 130:2237-2246.

57. Sharpe, M. E. 1962. Lactobacilli in meat products. Food Manuf. 37:582-589.

58. Shimizu-Kadota, M., and S. Kudo. 1984. Liposome-mediated transfection of Lactobacillus casei spheroplasts. Agric. Biol. Chem. 48:1105-1107.

59. Shrago, A. W., B. M. Chassy, and W. J. Dobrogosz. 1986. Conjugal plasmid transfer (pAMβ1) in Lactobacillus plantarum. Appl. Environ. Microbiol. 52:574-576.

60. Smiley, M. B., and V. Fryder. 1978. Plasmids, lactic acid production, and N-acetyl-D-glucosamine fermentation in Lactobacillus helveticus subsp. jugurti. Appl. Environ. Microbiol. 35:777-781.

61. Smith, J. L., and S. A. Palumbo. 1983. Use of starter cultures in meats. J. Food Prot. 46:997-1006.

62. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

63. Stetter, K. O. 1977. Evidence for frequent lysogeny in lactobacilli: Temperate bacteriophages within the subgenus Streptobacterium. J. Virol. 24:685-689.

64. Tohyama, K., T. Sakurai, and H. Arai. 1971. Transduction by temperate phage PLS-1 in Lactobacillus salivarius. Jpn. J. Bacteriol. 26:482-487.

65. Tomochika, K., M. Funabashi, A. Nagata, T. Fuji, and Y. Kanemasa. 1982. Protoplast formation of Lactobacillus casei induced by endo-N-acetylmuramidase. Jpn. J. Bacteriol. 37:777-785.

66. Urbaniak, L., and W. Pezacki. 1975. The lactic acid forming microflora of dry sausage and the technologically determined changes it undergoes (in German). Fleischwirtschaft 55:229.

67. Vescovo, M., V. Bottazzi, P. G. Sarra, and F. Dellaglio. 1981. Evidence of plasmid deoxyribonucleic acid in Lactobacillus. Microbiologica 4:413-419.

68. Vescovo, M., L. Morelli, and V. Bottazzi. 1982. Drug resistance plasmids in Lactobacillus acidophilus and Lactobacillus reuteri. Appl. Environ. Microbiol. 43:50-56.

69. Vescovo, M., L. Morelli, V. Bottazzi, and M. J. Gasson. 1983. Conjugal transfer of broad-host-range plasmid pAMβ1 into enteric species of lactic acid bacteria. Appl. Environ. Microbiol. 46:753-755.

70. Vescovo, M., L. Morelli, P. S. Cocconcelli, and V. Bottazzi. 1984. Protoplast formation, regeneration and plasmid curing in Lactobacillus reuteri. FEMS Microbiol. Lett. 23:333-334.

71. von Husby, K. O., and I. F. Nes. 1986. Changes in the plasmid profile of Lactobacillus plantarum obtained from commercial meat starter cultures. J. Appl. Bacteriol. 60:413-417.

72. West, C. A., and P. J. Warner. 1985. Plasmid profiles and transfer of plasmid-encoded antibiotic resistance in Lactobacillus plantarum. Appl. Environ. Microbiol. 50:1319-1321.

73. Woodbury, B. L. 1984. Effects of lactic acid bacteria on residual nitrite in a summer style sausage. Thesis, Utah State University.

74. Yokokura, T., S. Kodaira, H. Ishiaw, and T. Sakurai. 1974. Lysogen in lactobacilli. J. Gen. Microbiol. 84:277-284.