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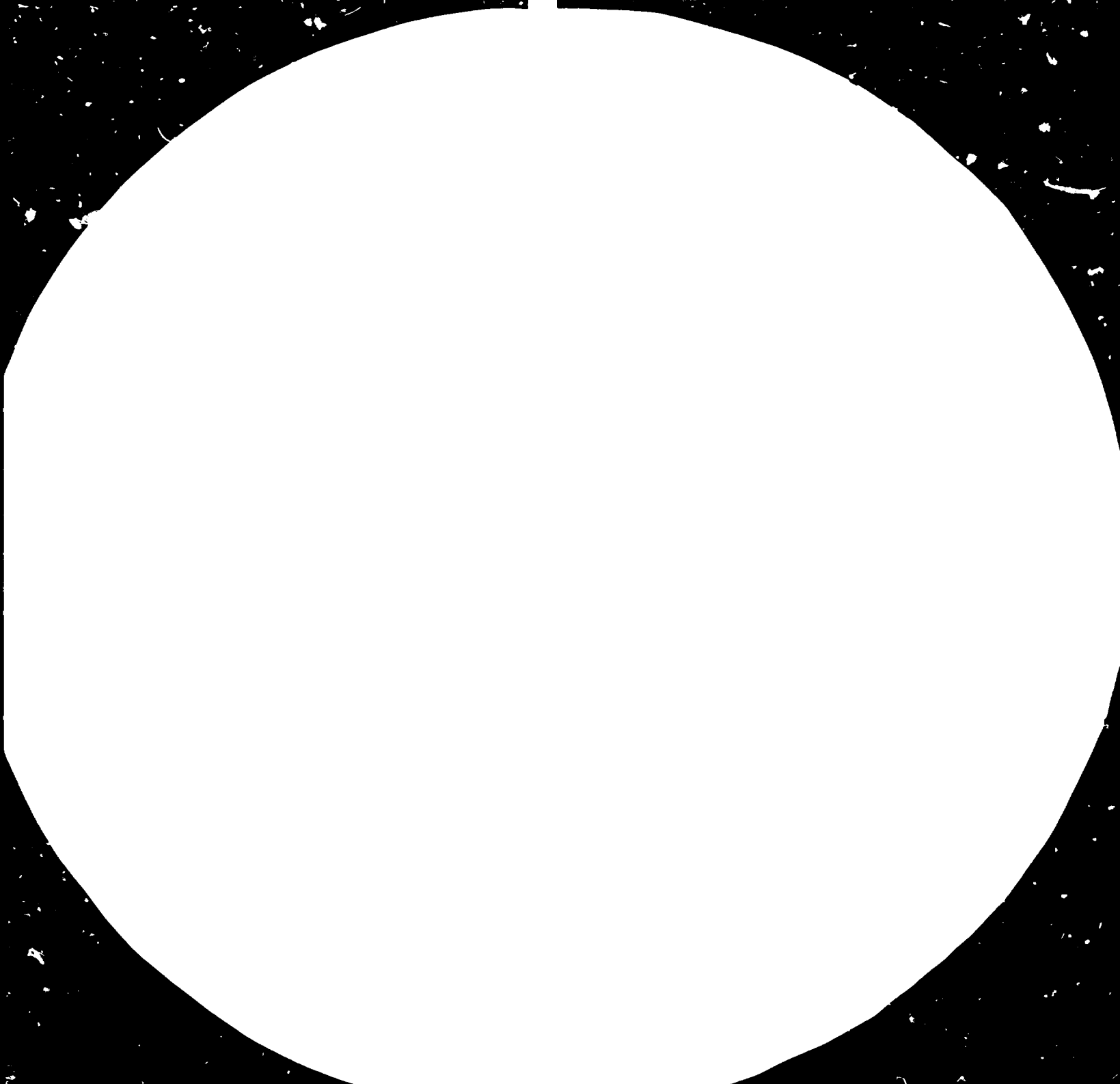

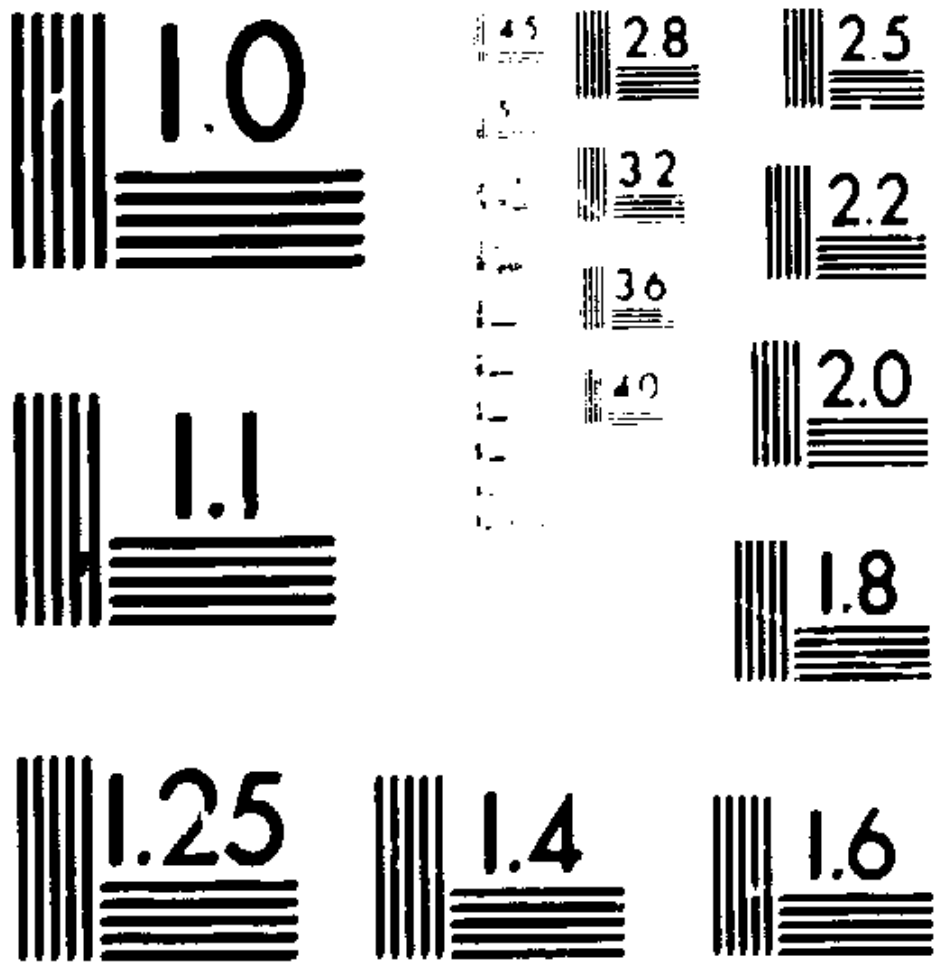

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CURRENT STATUS OF GENETIC ENGINEERING OF LACTOBACILLUS*

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CONTENTS

BACKGROUND

1. Lactobacillus play a central role in various food fermentation processes as well as for the production of lactic acid. Despite their economic importance relatively little effort has been directed toward elucidating their genetics or biochemistry. Recent advances in the field of genetic engineering offer exciti .g prospects for strain improvement and manipulating catalytic activities if this technology can be extended to Lactobacillus. Currently, several groups are developing recombinant DNA technology for Lactobacillus bulgaricus and Lactobacillus casei. These efforts have resulted in the elucidation of plasmid function, construction of chimeric vectors and the establishment of a transformation system for introducing these chimeras into Lactobacillus.

I. INTRODUCTION

2. Recent significant developments in biotechnology have resulted in the following:

- o New techniques are being developed for the directed genetic manipulation of microorganisms, plants and animals, thus generating new types of cells and plants with desirable metabolic properties
- o New knowledge is evolving on the properties of enzymes which catalyze reactions of potential interest. It may be possible, soon, by the use of recombinant DNA procedures to generate new enzymes which catalyze unique chemical transformations to produce novel materials, chemicals, and ingredients.
- o Tissue cultures have proven to be effective for

producing metabolites with biochemical, pharmaceutical and food applications.

- o Plants resistant to environmental stresses such as drought, frost, and herbicides with increased nutritional and functional quality can now be developed.

3. Before considering advances in genetic engineering of Lactobacillus it is critical to answer the following question. What are the benefits that can be gained from a program in genetic engineering of Lactobacillus?

4. Biotechnology directed toward the general area of lactic acid food fermentations can bring significant economic benefits at both the macro- and microeconomic levels. Macroeconomic interests can be served by:

- o more reliable supply of critical foodstuffs,
- o more efficient use of capital employed in food processing,
- o lower energy consumption in food processing,
- o providing added value usage for agricultural commodities currently in surplus.

At the microeconomic level of individual food sectors, benefits will come from:

- o more effective production yields,
- o higher productivity in processing raw materials,
- o improved ability to meet the consumer's demand for natural food stuffs, and
- o less waste, improved processing characteristics, consistent quality, flavor, and greater nutritional value.

5. Also critical for justifying genetic engineering in Lactobacillus, it is important to determine what are the specific needs. A logical way to answer this question of specific needs is to determine what role will Lactobacillus play in the biotechnology paradigm summarized in Figure 1. The program has to have a technical and economic justification based upon improved yields and productivity.

6. The classification of the various stages in a process using Lactobacillus, can be described using the biotechnology paradigm (Figure I). For example, the production of yoghurt can be described as follows:

- o The milk and non-fat dry milk additives would constitute the raw materials.
- o The Lactobacillus and Streptococcus thermophilus carry out the bioconversion of the raw materials.
- o The bioreactor is the vessel or vat in which the bioconversion process is carried out.
- o Product recovery and delivery are the downstream processing and packaging of the final product.

7. Thus, any improvements to a biotechnology process can occur in any of the aforementioned areas. Clearly, any improvement in the rates of acidification by the Lactobacillus starter culture which results in faster set times, would improve this biotechnological process. In the specific role as a biocatalyst improvements in lactic acid fermentation processes can be envisioned through genetic engineering. Improved yields and productivity are likely to be a result of strategies that simply increase gene dosage. Novel bioreactor designs also are likely to result from a genetic engineering approach. Similar strategies for improving any other stages in this biotechnological process can be

designed.

8. Lactic acid bacteria are important microorganisms that affect raw material usage. They add value to material such as silage, pickles, milk, cheese, meat, baked products, as well as other raw materials. Lactic acid is an antimicrobial agent and therefore the fermentation of various products with Lactobacilli can serve to preserve these products. Other benefits attributed to Lactobacilli fermentations are increased digestability of certain nutrients. Specific improvements in product quality in terms of flavor, texture and storage ability can be readily attained by applications of genetic engineering to lactic acid fermentations.

9. In certain cases, Lactobacilli are used for the production of lactic acid, although this fermentation process competes with chemical synthesis. In a region rich in biomass or where a readily available source of fermentable material is available, i.e., from a large cheese operation, fermentation processes for lactic acid are likely to be competitive with chemical synthesis.

10. Lactobacillus sp. produces a wide variety of compounds reported to be active against undesirable microorganisms (Speck, 1981). These antimicrobial compounds are used as additives for the preservation of food in Europe or alternatively, Lactobacilli themselves can be used. For example, Lactobacillus starter cultures could be used to repress growth of pathogenic organisms in raw milk, extend the shelf-life of cottage cheese and other dairy foods, sausages, ground beef and deboned poultry meat.

11. Lactobacillus delbruckii is traditionally used in the production of soy sauce (Wang and Hesseltine, 1981). Lactobacillus are among the pri-

mary genera of bacteria successfully utilized as meat starter cultures (Bacus and Brown, 1981).

12. Lactobacillus brevis and Lactobacillus plantarum are among the lactic acid bacteria responsible for the natural fermentations of vegetables (Fleming and McFeeters, 1981).

II. CLASSIFICATION

13. The Lactobacilli are a group of gram(+) rod-shaped bacterium which do not form spores. They are distinguished from other gram(+) bacilli by their production of lactic acid. The classification of the various Lactobacillus species is based upon their optimum growth temperature, the type of lactic acid produced and the spectrum of carbohydrates fermented (Buchanan and Gibbons, 1974). The homofermentative species can produce either D or L lactic acid or both. Examples of homofermentative Lactobacillus are L. lactis, L. bulgaricus, L. casei, L. acidophilus and L. plantarium. The heterofermentative species produce in addition to lactic acid, varying amounts of other end products including acetate, carbon dioxide, and ethanol. Examples of heterofermentative species are L. fermentum, L. brevis, L. buchneri and L. cellobiosus. Lactobacillus can ferment a wide variety of mono-, di- and trisaccharides carbohydrates.

III. GENETICS

14. The elucidation of the genetics of Lactobacillus have lagged behind many other microorganisms. Needed are more fundamental studies on metabolism and enzymology identity and biosynthesis of materials involved in flavor and texture. The exact reasons for the lack of progress are not

clear but may involve the complex nutritional requirements which prohibit such studies. Another factor is the current use of Lactobacilli for fermentation of dairy products such as yoghurt. The important attributes for most strains used are imprecise and cannot be readily categorized by an objective phenotype. The properties of a desirable yoghurt culture cannot be described in precise terms which lend themselves to biochemical and genetic characterization. Consequently, no significant genetic studies have been reported for Lactobacillus.

15. On the other hand, the importance of Lactobacilli and their fermented dairy products has stimulated new areas which could benefit from a genetic technology for strain improvement. An attractive alternative to classical methods of strain improvement (i.e., mutagenesis and selection) is the development of a recombinant DNA system. A recombinant DNA would facilitate the introduction of new catalytic activities for amplifying existing but rate-limiting metabolic events. In addition, a recombinant DNA system could be integrated into the current technology available for both Escherichia coli and Bacillus subtilis. This would allow the complete characterization of the gene and its product.

16. The needs and definition of a genetic engineering program for Lactobacillus is summarized in Figure II.

IV. DEVELOPMENT OF A RECOMBINANT DNA SYSTEM

17. A recombinant DNA system requires two important components, a vector and a transformation protocol (Figure III). The vector functions to maintain the cloned DNA sequence in the host during replication of its host. A vector can be either an extrachromosomal element, e.g., a plasmid, or an integrative bacteriophage. A vector must have a selectable

marker which serves to distinguish the transformed cell from the rest of the population. There is a need for food-grade selectable markers. The most frequent selectable marker is a gene which confers antibiotic resistance.

18. The second requirement is a transformation protocol. This permits the introduction of a vector into the host. A transformation protocol is critical since any in vitro DNA manipulations necessitate a method for its introduction back into the host. Conjugation or transduction are useful for moving DNA from one cell to another even between different genera. These techniques are limited, however, they can be coupled to circumvent a transformation provided the desired host can be conjugated to a transformable host. This has been exploited in several gram(-) systems. For example Rhizobium can be conjugated to Escherichia coli which can be readily transformed (Tait et al., 1983).

V. VECTORS

19. Plasmids have been reported in a wide variety of Lactobacillus species. Plasmids have been identified, for example, in L. casei (Chassy et al., 1978), and in L. acidophilus (Klaenhammer, 1984). The classic method for assigning plasmid function is to cure the strain of its plasmid and determine if any biochemical function is lost. The function of most Lactobacillus plasmids for the most part is unknown and they remain cryptic. A notable exception is pLZ64, a plasmid found in L. casei (Lee et al., 1982). pLZ64 (Figure IV) is a 35 Kb plasmid. pLZ64 is one of the several plasmids to be found in L. casei, and has been shotgun cloned in E. coli. A gene coding for the enzyme phospho-β-D-galactosidase has been located on this plasmid. The β-D-phospho-

galactoside galactohydrolase gene is expressed in E. coli and a functional enzyme is produced indistinguishable from that isolated from L. casei.

20. As mentioned previously the most desirable selectable markers are antibiotic resistance genes. The Streptococcus and Staphylococcus have numerous antibiotic resistance genes which are carried on a variety of plasmids. No antibiotic resistance plasmids have been positively identified in Lactobacillus, although some circumstantial evidence exists (Vescovo et al., 1982).

21. A significant fact in the identification of a selectable marker for Lactobacillus is the report of its conjugation to Streptococcus faecalis and the transfer and expression of the erythromycin resistance gene of pAMβ1 (Vescovo et al., 1983; Gibson et al., 1979). The conjugal plasmid pAMβ1 can be transferred into L. acidophilus and L. casei.

22. The expression of the erythromycin resistance gene in Lactobacillus provides an important component for construction of a vector. If the erythromycin resistance gene could be coupled to a replicon which functional in Lactobacillus it would constitute a useful vector. Although pAMβ1 was transferred into Lactobacillus, it is unclear from the reported results if the replicon function or if the entire plasmid or a portion (presumably including the erythromycin resistance gene) was integrated.

23. Numerous plasmids have been reported in Lactobacillus and several promising replicon candidates exist (Ishiwa and Iwata, 1980; Morelli et al., 1983). The desirable attributes of a replicon donor are that it is of a small size and it contains several unique, well distributed restriction sites for cloning it in several orientations. L. casei con-

tains several small plasmids ranging in size from 3-35 Kb, and most plasmid containing strains carry a multiple plasmid profile. L. helveticus, L. acidophilus, L. lactis, L. plantarum and L. bulgaricus also contain numerous plasmids of varying sizes (Klaenhammer, 1984). No detailed restriction maps are reported for any of these plasmids with the exception of pLZ64 (Lee et al., 1982).

24. The host range of these Lactobacillus plasmids is not known and the possibility of a replicon from L. casei functioning in, for example, L. bulgaricus cannot be predicted.

25. The interspecies relationships between Lactobacillus sp. has been established on the basis of G-C ratios and rRNA of the DNA (Figure V; Kandler, 1984). The L. helveticus, L. bulgaricus, L. lactis and L. acidophilus are closely related while the mesophilic L. casei, L. brevis and L. plantarum represent distant relatives. Although the ability of any given plasmid to replicate in a heterologous host cannot be predicted empirically a closely related species would be the most desirable choice for a replicon.

26. Our efforts to develop a recombinant DNA system for L. bulgaricus and L. helveticus therefore focused on identifying possible replicons from L. bulgaricus and L. helveticus. Initially, several strains of L. bulgaricus and L. helveticus were screened for plasmids using a modification of existing protocols (Chassy and Guiffrida, 1980; Hansen and Olson, 1978). The results are shown in Table 1. Curicusly, no confirmable plasmids were found in any L. bulgaricus strains tested. Although satellite bands were observed on agarose gels they may in fact be artifacts since they could not be isolated as extrachromosomal elements. Plasmids were observed in both L. helveticus NCSU10 (this

strain is also classified as L. bulgaricus 10, Klaenhammer, 1984) and LBC692. The single plasmid from L. helveticus LBC692, designated pBSNH692B is 3 Kb and was chosen as the potential replicon. Restriction mapping of pBSNH692B revealed several unique restriction sites for inserting a selectable marker (Figure VI). To facilitate the construction of the vector, pBSNH692B was intially cloned into pBR325 via the single HindIII site and in another construction via the single EcoRI site. To these E. coli/L. helveticus chimeras the erythromycin resistance gene was added from pVA838, a Streptococcus/E. coli chimera (Macrina et al., 1982) resulting in two final vectors pLBC104EM and pLBCH692EM (Figures VII and VIII). These vectors presumably contained replicons (Figures IX and X) which could function in Lactobacillus as well as selectable markers. The only method to confirm the efficacy of these vectors was to transform them successfully into Lactobacillus.

VI. TRANSFORMATION

27. Transformation is the uptake of extracellular DNA and requires that they be competent. What constitutes the competence state is not clear, but it allows the DNA to pass through the cell wall. A cell can become competent naturally or it can be induced by cation treatment. Alternatively, the cell wall may be removed and the resulting protoplast transformed (Figure XI). In the absence of any reported natural competence state or cation treatment, the most direct method for transformation is using protoplasted cells. The requirements of a protoplast procedure is that sufficient cell wall is removed to permit DNA uptake but that the cell remains viable and can later regenerate its cell wall and resume normal replication.

28. Lactobacillus can be protoplasted using either lysozyme or mutanolysin or both (Chassy and Guiffrida, 1978; Klaenhammer, 1984). To prevent rupture of the osmotically sensitive protoplasts an osmotic stabilizer must be included. The choice of osmotic stabilizer should take into account its compatibility with the protoplasts and possible deleterious effects on cell regeneration. Several osmotic stabilizers are currently used including soribtol, sucrose and succinate although L. bulgaricus and L. helveticus protoplasts are more stable in raffinose (Batt et al., 1984) and the results are summarized in Table 2.

29. A competent protoplast is exposed to the DNA and the DNA is fused into the cell using polyethylene glycol (PEG). The addition of PEG is essential and has been employed for transforming a wide variety of microorganisms (Klebe et al., 1983). After PEG is added the protoplasts must be regenerated to yield viable cells which can be replicated. The exact requirements for regeneration cannot be determined empirically and the conditions vary for each organism.

30. The regeneration of L. bulgaricus and L. helveticus protoplasts proved to be most problematic. These protoplasts could not regenerate on any standard medium (e.g., MRS or LCM) even upon the addition of supplements such as raffinose. The regeneration of L. bulgaricus and L. helveticus could only be accomplished in LME medium and required for 2-4 days to recover (Batt et al., 1984).

31. Transformation of both L. bulgaricus and L. helveticus was successfully accomplished using the chimeric vectors pLBC104EM and pLBCH692EM. The erythromycin gene was expressed in both L. bulgaricus and L. helveticus with the former resistant to 2-5 g/ml and the latter resistant to >200 g/ml erythromycin (Table 3). The plasmids are apparently main-

tained at a low copy number, and they have suffered some deletions. Current efforts in our laboratory are focusing on characterizing these rearrangements and determining the final form of the vector.

32. Once a successful protoplast regeneration protocol has been established, other genetic transfer procedures are possible, i.e., transfection (Shimizu-Kadota and Kudo, 1984; Shimizu-Kadota and Touchida, 1984;. This approach is also being investigated in our laboratories with food-grade Lactobacillus (Chow et al., 1984). We have isolated Lactobacillus bulgaricus phage DNA from phage ϕ CH_2. This DNA has then been used to transfect Lactobacillus bulgaricus protoplasts. Studies are now underway to characterize the molecular properties of the phage in order to develop additional tools for an effective of cloning system for Lactobacillus. Such research should also further elucidate the mechanism of phage infection and result in more effective ways to prevent loss of starter cultures due to phage infection.

VII. CONCLUSIONS

33. Our efforts have resulted in the development of a rudimentary system for the insertion of recombinant DNA molecules in L. bulgaricus and L. helveticus. The functionality of this system is currently being evaluated. Questions still remain with regard to the stability and expression of foreign DNA sequences in Lactobacillus. It is unclear if the delicately balanced lactic acid biochemistry can support significant genetic manipulation. The introduction of a "new" protein may not be feasible given the low energy (ATP) obtained from the fermentaion of hexose sugars. This can only be evaluated experimentally.

34. At this stage, our recombinant DNA system for engineering Lacto-

bacillus is not suitable for food-grade usage. The DNA sequences in the vector from E. coli and the erythromycin resistance gene must be substituted with more acceptable selectable markers. Our group is currently constricting vectors with food grade markers such as resistance to the antibiotic nisin.

35. The long-term prospects for genetically engineering Lactobacillus are significant. A probable limitation in the development of new strains with unique catalytic activities will be the fundamental knowledge concerning the biochemistry of certain of these catalytic properties. For example, the engineering of a strain which can yield a strawberry flavor will be impeded by limited understanding of the essential components of strawberry flavor as well as how these compounds can be enzymatically generated. Concurrent with the advances in the genetic engineering of Lactobacillus must be the elucidation of the fundamental knowledge concerning flavors, proteolysis, saccharification and other areas pertinent to these types of biotechnological processes.

36. In summary, the basis of a recombinant DNA system for food grade Lactobacillus species has been established. A variety of numerous applications are now possible. Examples include:

- o Proteins
  - o enzymes
  - o proteases lipases
- o Gene dosage
  - o rates of acid production
- o Novel biochemical pathways
  - o flavor
- o Development of novel regulatory mechanisms
- o Product gene products with special physio-chemical properties.

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Table 1

Plasmids Identified in *Lactobacillus* sp.

Strain		Size (Kb)	Number Observed
Lactobacillus bulgaricus	B009	--	ND
	B010	--	ND
	B015	--	ND
	B022	--	ND
	B025	--	ND
	B026	--	ND
	B031	--	ND
	OSU-CH2	--	ND
Lactobacillus helveticus	NCSU 10	2-20	6
Lactobacillus helveticus	LBC692	3	1

ND - Non detected

Table 2

Effect of Washing with Sorbitol or Raffinose on Viability of *Lactobacillus* Protoplasts

Strain		Initial	After Sorbitol	After Raffinose
L. bulgaricus	B031	4.0×10^7	5.4×10^4	1.3×10^6
	B39	1.0×10^8	<10	2.0×10^7
	CH2	1.0×10^8	5.6×10^4	1.2×10^8
	B	3.0×10^7	1.5×10^4	2.0×10^5

Table 3

Stability of *Lactobacillus* Transformants With and Without Erythromycin Pressure

Strain	Erythromycin (ug/ml)	CFU/ml +EM[1]	CFU/ml -EM[2]
L. bulgaricus CH2[3]	0	2.4×10^8	2.1×10^8
	1	3.8×10^8	7.0×10^7
	20	$<1 \times 10^5$	$<1 \times 10^5$
L. helveticus 692[4]	0	1.9×10^6	1.8×10^7
	1	5.9×10^6	1.6×10^7
	20	1.1×10^6	1.4×10^7

1. *Lactobacillus* transformants transferred through two litmus milks with 1 µg/ml.

2. *Lactobacillus* transformant transferred through two litmus milks without erythromycin.

3. *L. bulgaricus* CH2 transformants grew in 1 µg/ml erythromycin.

4. *L. helveticus* 692 transformants grew in 200 µg/ml erythromycin.

Figure I. Biotechnology Paradigm.

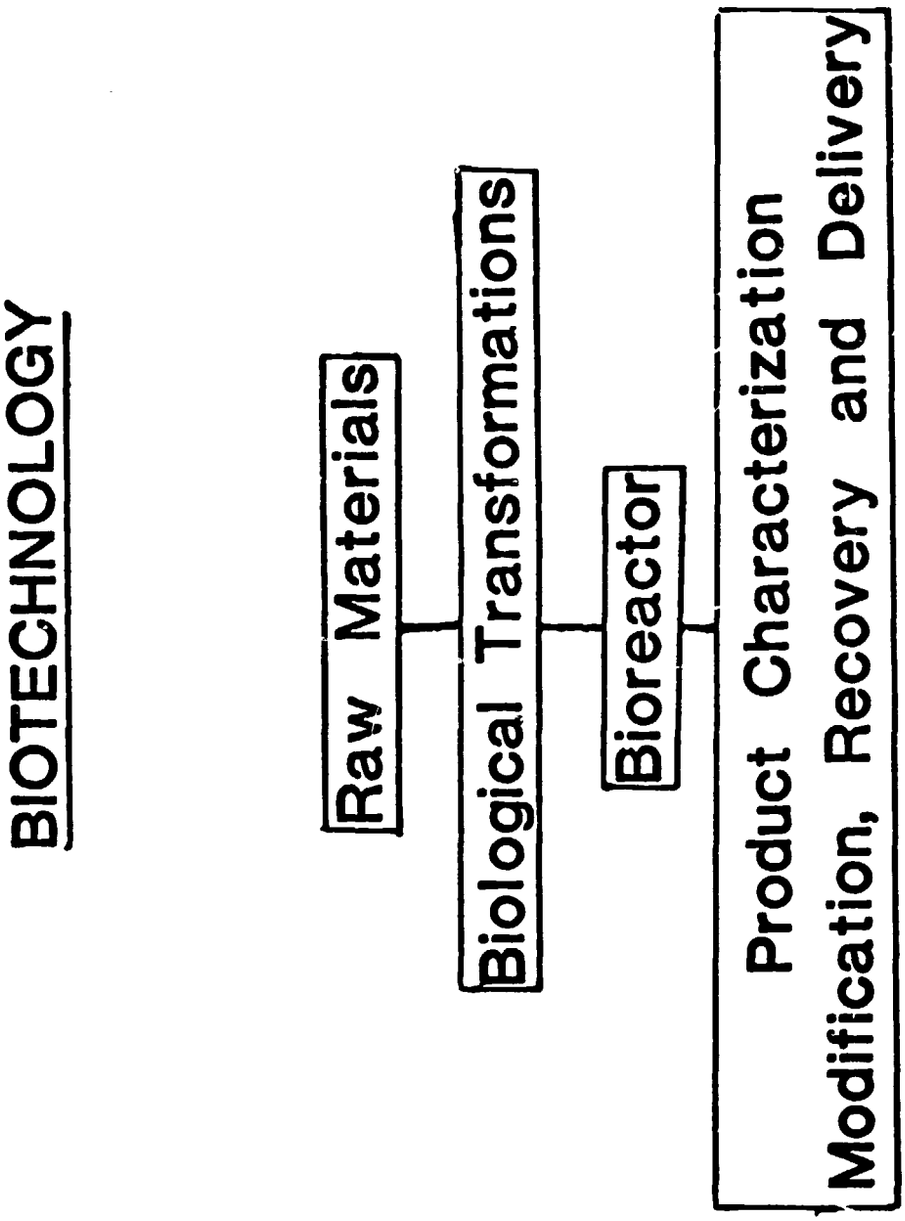

Figure II. Lactobacillus program.

Current Objectives and Status of Lactobacillus Project

• Lactobacillus Industrial strains Natural isolates	• Lack of basic knowledge
• General Microbiology	• Carbohydrate utilization • Mutagenic procedures
• Cloning Systems	• Plasmid isolation • Plasmid characterization • Chimeric vectors Transformation
• Applied Problems	• Control of post-acidification Rates of acid as f(T) Growth as f(T) • Control of rheology • Flavor generation

Development of Recombinant DNA Systems for Industrial Microorganisms

A. Vectors

cloning sites, origin of replication, selectable markers

B. Transformation systems

natural competence, cation treatment, protoplasts, liposomes

Figure III. Development of a rDNA system.

Figure IV. Restriction map of pLZ64.

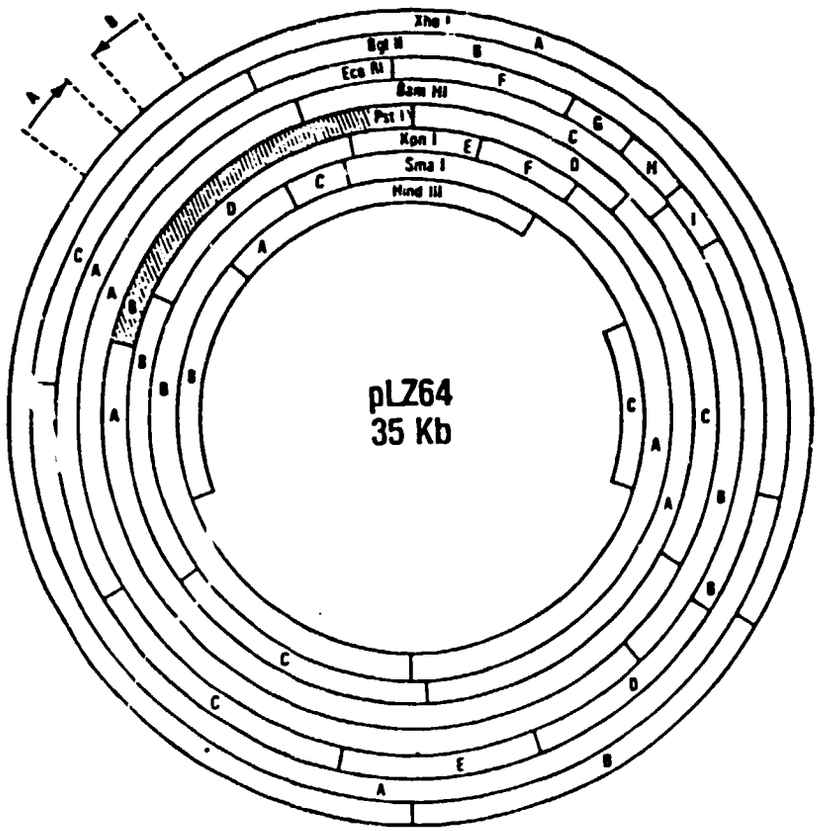

Figure V. Interspecies relationship between Lactobacillus species. (Adapted from Kandler, 1984).

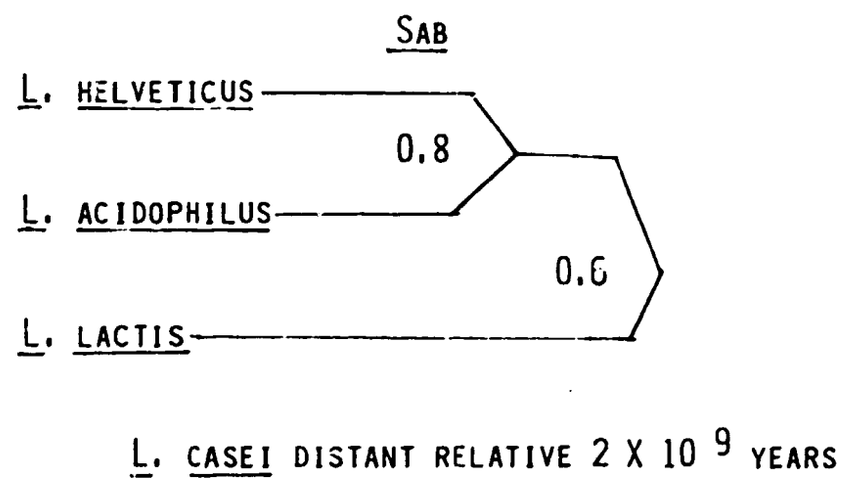

Figure VI. Restriction map of pBSNH692B.

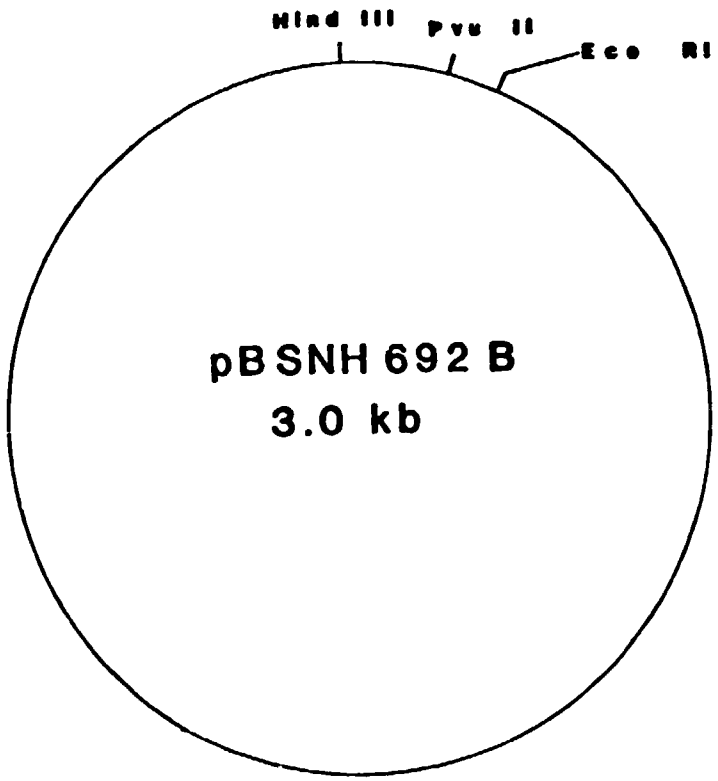

Figure VII. Strategy for construction of pLBC104EM.

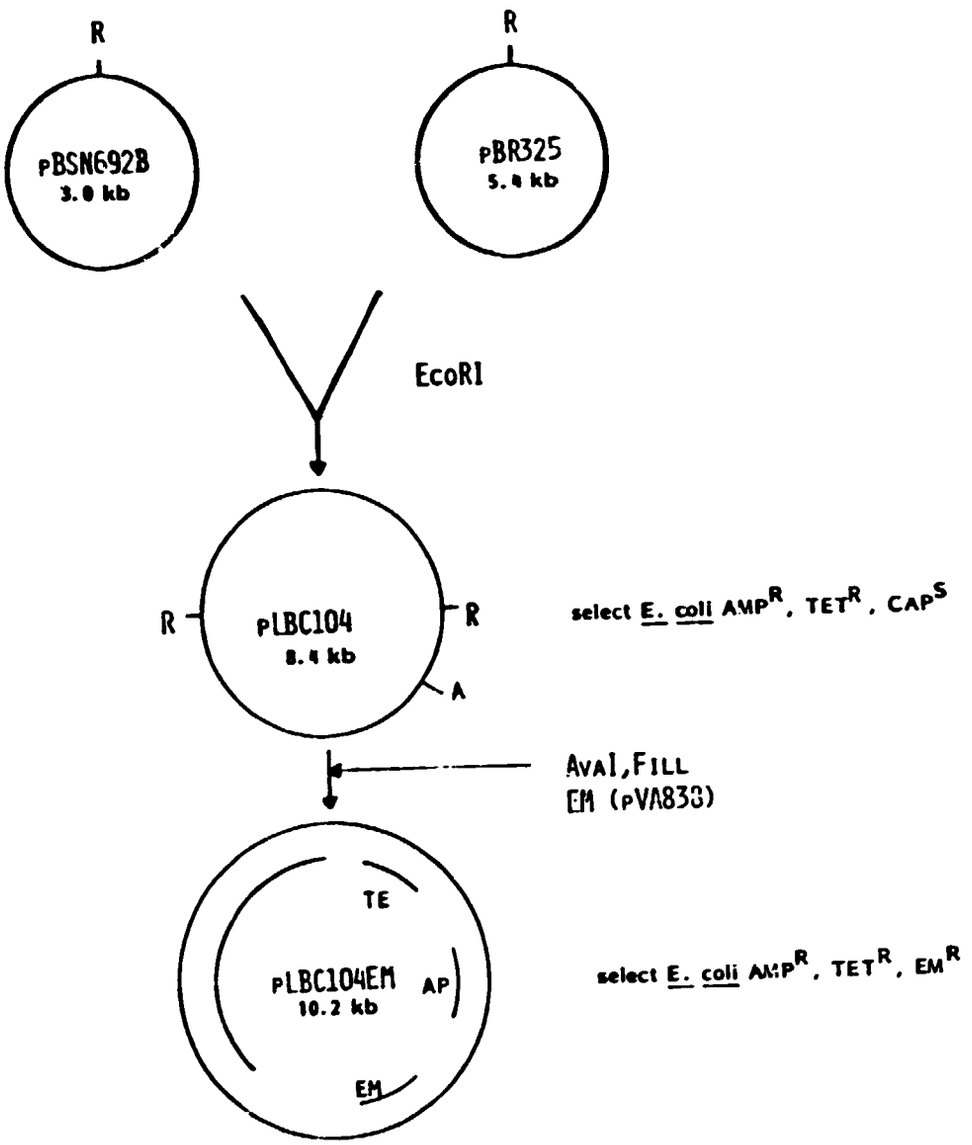

Figure VIII. Strategy for construction of pLBCH692EM.

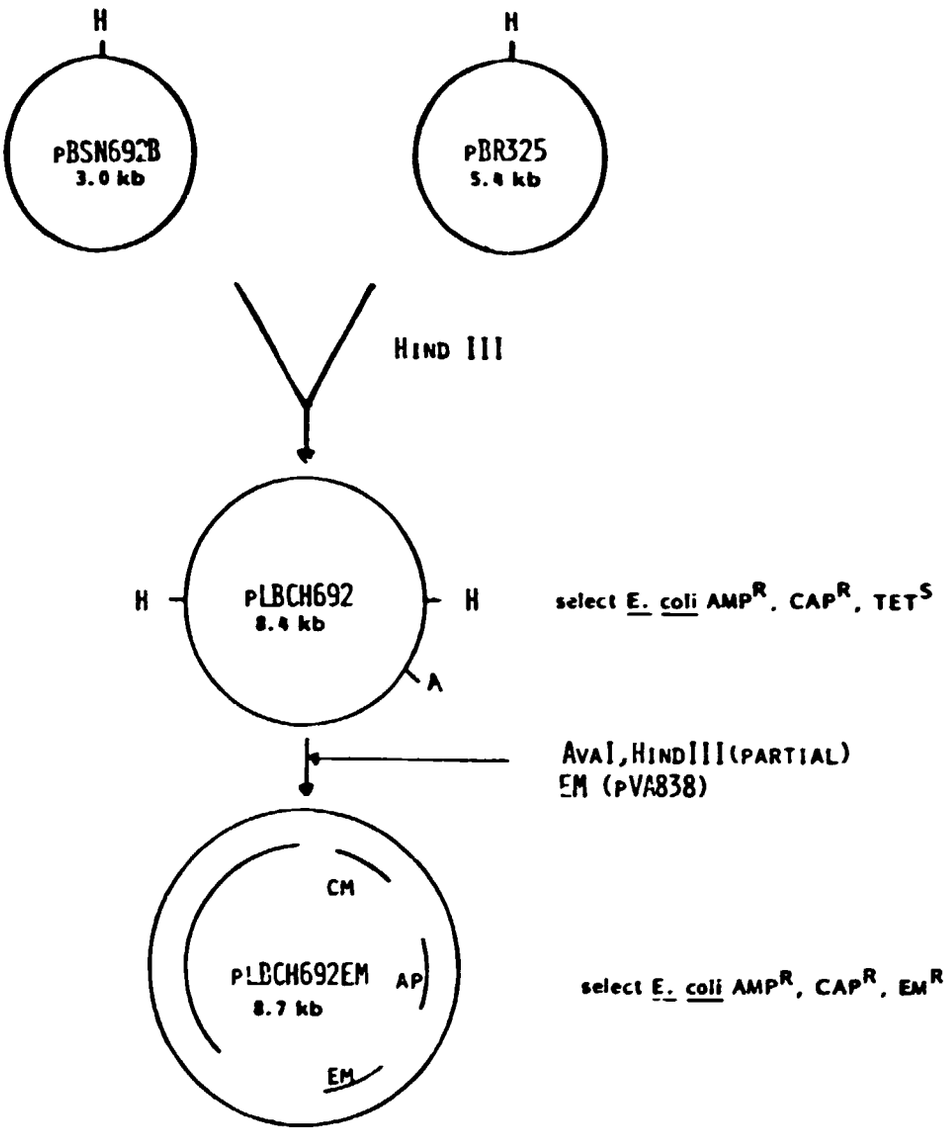

Figure IX. Restriction map of pLBC104EM.

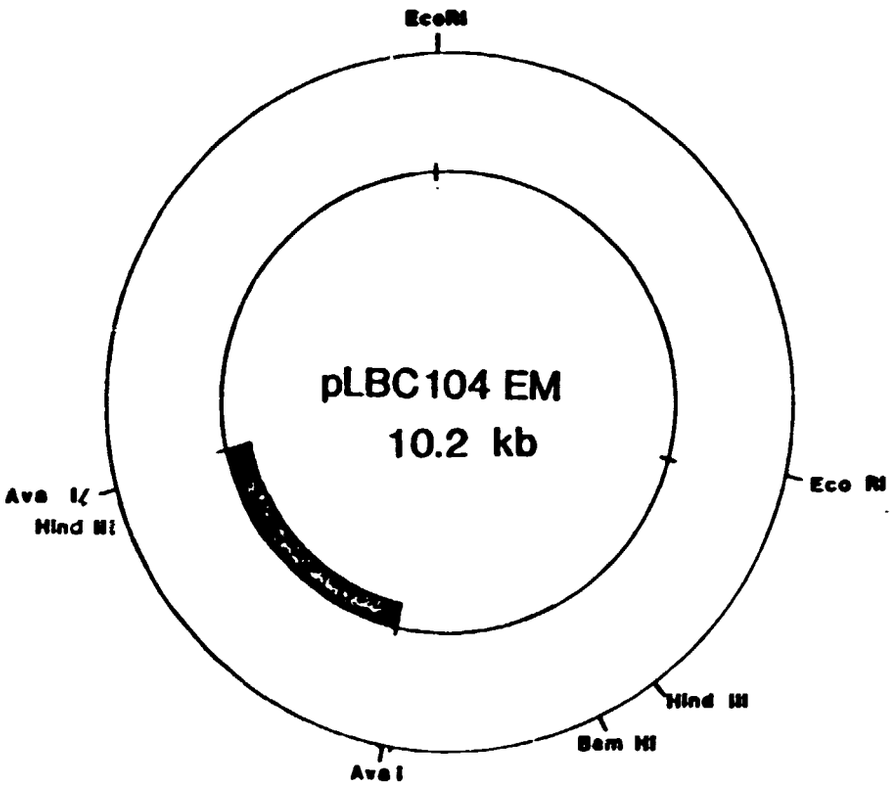

Figure X. Restriction map of pLBC692EM.

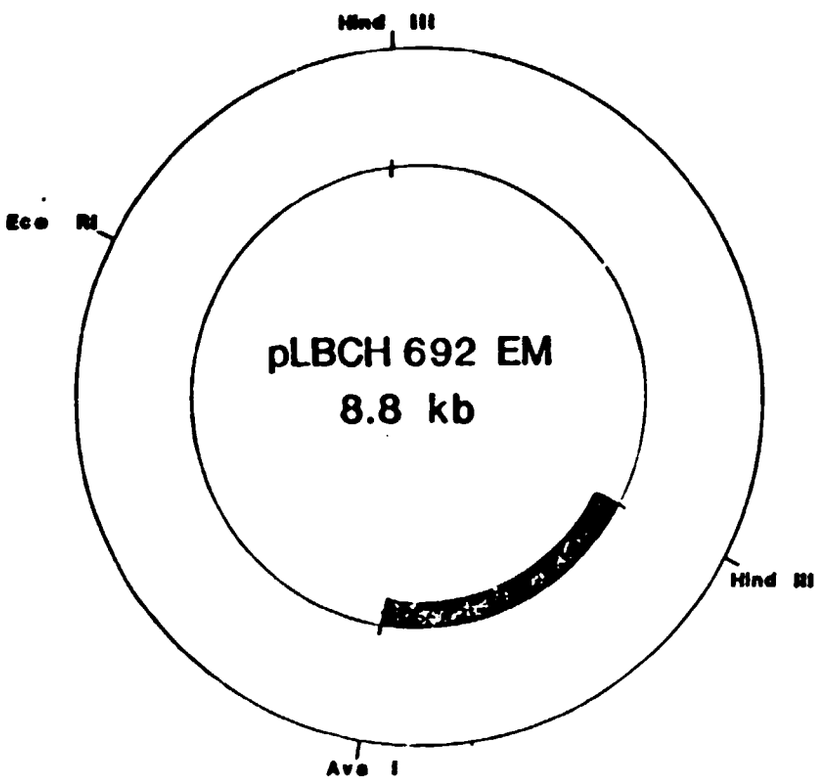

Figure XI. Lactobacillus transformation protocol.

TRANSFORMATION PROTOCOL FOR LACTOBACILUS

STAGE		PARAMETER
GROWTH	*	GROWTH IN MRS BROTH
WASH	*	RAFFINOSE
PROTOPLAST	*	LYSOZYME & MUTANOLYSIN
TRANSFORMATION	*	DNA (0 ^{o}C FOR 15 MIN.) PEG-6000 + HEAT-SHOCK (37 ^{o}C, 2 MIN.)
SELECTION	*	RECOVERY MEDIA WITH & WITHOUT ANTIBIOTICS