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Construction of food-grade mutants of lactic acid bacteria

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Summary — Selection of reliable starter strains is an important objective for dairy industries. The present industrial approach is to isolate a strain at random by screening a collection of natural isolates. More recent advances in genetic technology may give rise to more direct strategies for strain selection. To this end, we have constructed genetic tools based on two natural processes: homologous recombination and transposition. Using these tools, we can: i) identify and modify genes involved in a bacterial process; ii) construct food-grade mutants, ie, genetically modified strains with no trace of foreign DNA; and iii) extend this modification to other related bacteria. The engineered strain has the advantage that its improved properties are well defined and can be reproduced. Food-grade mutants represent a real potential to answer some of the industrial problems concerning starter technology.

lactic acid bacteria / food-grade mutant / transposition / homologous recombination / genetic modification

Résumé — Les mutants alimentaires de bactéries lactiques: une méthode à exploiter. *La sélection de ferments lactiques de qualité est une préoccupation constante des industriels laitiers. À l'heure actuelle, l'approche industrielle consiste à rechercher les nouvelles souches dans une collection de souches provenant d'isolats naturels. L'amélioration des connaissances concernant les bactéries lactiques permet désormais de proposer une alternative à cette technique de criblage d'une collection. Il s'agit de la modification génétique dirigée des souches au moyen d'outils génétiques. Les outils génétiques permettent d'utiliser des processus cellulaires naturels, la transposition et la recombinaison homologue, afin de modifier précisément le génome bactérien. Ces outils permettent i) d'identifier et de modifier les gènes impliqués dans une fonction bactérienne, ii) de générer des mutants alimentaires c'est-à-dire des souches génétiquement modifiées ne comportant aucune trace d'ADN étranger et iii) de réaliser la même modification dans plusieurs souches proches afin de leur conférer un caractère d'intérêt. Le mutant alimentaire porte une modification génétique parfaitement contrôlée qui peut être reproduite. L'utilisation de mutants alimentaires nous semble présenter un réel intérêt pour répondre à certains problèmes industriels et obtenir des produits plus attractifs.*

bactérie lactique / mutant alimentaire / transposition / recombinaison homologue / modification génétique

INTRODUCTION

Milk fermentation is both an industry and a biological science. Although the bacteria used for fermentation contribute little to raw material costs, they are implicated in problems which may have heavy economic consequences. Knowledge of bacteria has gradually increased over the years, allowing the selection of 'reliable' starter strains. Initially, lactic acid bacteria (LAB) naturally present in foods were used for fermentation. As bacteriological methods improved, the types of LAB implicated in a particular production were identified. This allowed industrial fermentation to use characterized types of bacteria which could be cultured in the laboratory. To avoid fermentation problems, criteria were defined to select 'reliable' starter strains among the natural LAB population. For example, these strains should: i) give a good product; ii) survive during storage; and iii) resist phage attack. To date, starter selection is empiric: strains are screened for the desired characteristics, and those which best meet these requirements are retained. This approach to find strains with improved characteristics is fruitful, but the genes conferring the useful properties have not been identified. Therefore, if the same property is desired in another strain, it is necessary to repeat the whole screening process.

In the last decade, the genetic knowledge of LAB has flourished: molecular biology and biochemical studies have elucidated part of the cheese-making process and genetic tools have been developed. It is thus possible to propose another method for the selection of starter strains: a strain with desired characteristics may be isolated by a directed genetic approach. The genetic approach is based on the observation that bacteria naturally gain, eliminate and modify genetic information. Some of these natural genetic modifications are the result of transposition or homologous recombination events. These cellular processes can be used in the laboratory to specifically modify strains. Therefore, a bacterial strain modified in the laboratory may be identical to a strain isolated at random.

The genetic tools developed thus far for lactococcal strains relied only on homologous recombination and allowed modification of characterized genes. Therefore the construction of food-grade mutants was limited to the modification of previously identified genes. So far, some 150 lactococcal genes have been identified. They are implicated in such varied processes as casein degradation, sugar utilization, peptide uptake and degradation, bacteriocin production, and phage resistance. Genetic modification of these genes may lead to improved starter strains with increased phage resistance or better growth. Genetic studies may also provide answers for other industrial problems such as long-term survival or stress survival but, as a first step and prior to any genetic modification, these genes must be identified. To do this, we developed a tool to perform transposon mutagenesis (Maguin et al, 1996). This technique can first, lead to a food-grade mutant with a new property and second, allow us to identify genes involved in a given process (stress survival, for example). Once a gene has been identified by transposition it can be modified in other strains using homologous recombination techniques (Biswas et al, 1993). In both cases (transposition or homologous recombination), the final engineered strain is obtained via natural cellular processes and does not contain any trace of foreign DNA: it is a food-grade mutant. The engineered strain has the advantage that the improved property is well defined and can be reproduced.

CHOICE OF THE DELIVERY VECTOR

To make use of genetic tools, it is first necessary to introduce DNA into the strain to be modified. Two types of delivery vector can be

used: a non-replicative (suicide) plasmid or a replication thermosensitive (Ts) replicon. The use of a non-replicative vector is restricted to efficiently transformable strains. Since transposition and homologous recombination are low frequency events, a large population of transformed cells is needed to select these rare events. A Ts replicon allows modification of strains, even if they are poorly transformable. A single transformed bacterial cell, grown at the permissive temperature for replication, generates a large population of bacteria carrying the plasmid. The transposition or recombination events can then be identified from among a large population at the non-permissive temperature for replication. Considering that most LAB, particularly the industrial strains, are poorly transformable, we isolated a Ts plasmid named pG+host (Maguin et al, 1992). pG+host replication has been tested in numerous Gram-positive bacteria among which are *Lactococcus lactis*, *Streptococcus thermophilus*, *Enterococcus faecalis*, *Lactobacillus fermentum* (M Fons, personal communication) and *Lb acidophilus* (H Boot, personal communication).

USE OF TRANSPOSITION TO ASSOCIATE A USEFUL PROPERTY WITH A GENETIC MODIFICATION

Transposition is the process by which genetic mobile elements insert themselves into the DNA of a cell. If a mobile element transposes randomly, its DNA integration site varies from cell to cell. Transposition can thus generate a population of mutants among which new phenotypes can be screened or selected. The mobile element is then used as a tag to identify the genetic modification which leads to the new phenotype (Berg et al, 1989). Numerous transposable elements have already been identified in LAB (Romero and Klaenhammer, 1993; Gasson and Fitzgerald, 1994). The insertion sequence IS*S1* is widespread among lactococci and has been thoroughly characterized (Polzin and Shimizu-Kadota, 1987; Haandrikman et al, 1990; Cluzel et al, 1991; Huang et al, 1992). Using non-replicative vectors, it was shown that IS*S1* and the iso-IS*S1* element IS*946* (Romero and Klaenhammer, 1990) transpose randomly into the chromosome of *L lactis* strains (Romero and Klaenhammer, 1992; Le Bourgeois et al, 1992; Dinsmore et al, 1993). However in these systems, use of IS*S1* is limited to strains with high transformation frequencies. To develop an efficient transposon mutagenesis system for lactococcal strains, we cloned the IS*S1* element on pG+host (Maguin et al, 1992) which encodes an antibiotic (Ab) resistance marker (Maguin et al, 1996). The resulting plasmid named pGh:IS*S1* (fig 1A), replicates at 30°C. Above 37°C, plasmid replication is inactive: the plasmid is lost and cells become Ab-sensitive unless IS*S1* transposition mediates integration of the whole pGh:IS*S1* into the chromosome. Since IS*S1* transposes randomly into the chromosome, the mutants are distinct (fig 1B). A large population of mutants can therefore be obtained by a simple incubation at 37°C with Ab. Bacteria with desired properties can then be identified from among the mutated bacterial population.

To test out the transposition system in the lactococcal strain MG1363, we selected mutated strains with increased resistance to acid pH at 37 °C. Transposition occurred in about 5% of the cells containing pGh:IS*S1*. Among the mutants, 0.2% were more resistant to acid pH than the wild-type strain. The transposition system has been successfully tested in *L lactis* subsp *lactis* and subsp *cremoris*, *S thermophilus* and *E faecalis*. In view of the good transposition activity observed in these different bacteria, as well as the broad host range thermosensitivity of pG+host, the pGh:IS*S1* transposon may have broad application in

LAB. Thus using this type of approach, generation of a food-grade mutant and characterization of the mutation are feasible by means of the pGh:IS*S1* system.

ISOLATION OF FOOD-GRADE MUTANTS

Transposon mutagenesis by pGh:IS*S1* results in mutated strains containing a transposed structure in its chromosome comprised of pG+host flanked by two IS*S1* elements (fig 2A). Deletion of the foreign DNA, ie, pG+host, is necessary to generate a food-grade mutant. Homologous recombination between the duplicated IS*S1* sequences will lead to excision of pGh:IS*S1*. After pGh:IS*S1* excision, the mutated strain is Ab-sensitive and contains only one IS*S1* element in the mutated gene (fig 2B). Since IS*S1* is a natural lactococcal IS element, the excised mutant is by definition, food-grade. Although homologous recombination happens naturally in bacteria at low frequency (Leenhouts et al, 1991a, b), the presence of the active pG+host replicon stimulates the recombination process (Noirot et al, 1987) and thus facilitates the isolation of food-grade mutants (Biswas et al, 1993). Thus, by incubation of a mutant culture at 30 °C, pG+host replicates, and stimulates the recombination event between the two IS*S1* sequences. This simple treatment generates food-grade mutants at high frequencies (fig 2B; Maguin et al, 1996).

CHARACTERIZATION OF THE IS*S1* TARGET

A food-grade mutant can be obtained without any characterization of the transposon

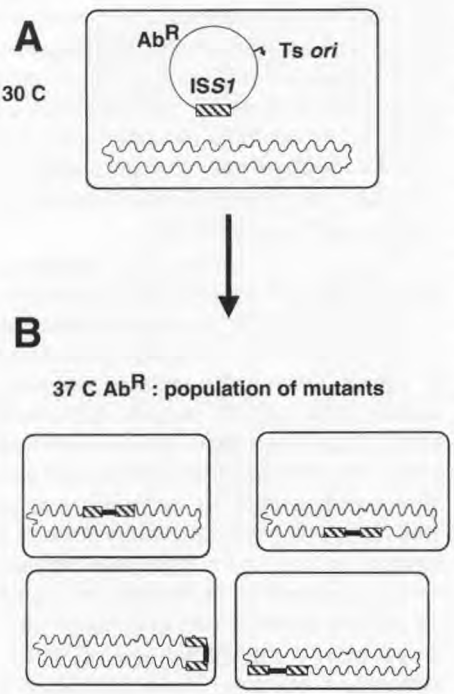

Fig 1. pGh:IS*S1* mutagenesis system. **A.** Schematic representation of a bacteria containing the pGh:IS*S1* plasmid. The plasmid is constituted of the pG+host replicon (Ts *ori),* an antibiotic-resistant gene (Ab^R) and the IS*S1* element. At 30 °C, the plasmid replicates in the cell. **B.** At 37 °C, plasmid replication is inactive, and the plasmid is lost unless IS*S1* transposition occurred. IS*S1* transposition results in chromosomal integration of duplicated IS*S1* flanking the pG+host plasmid. Since IS*S1* transposes at random, the integration site of the transposed structure varies from cell to cell. Symbols: wavy line, chromosome; black line, pGh+host DNA; hatched box, IS*S1*.
*Système de mutagenèse par le pGh:IS*S1. ***A****. Représentation d'une bactérie contenant le plasmide pGh:IS*S1*. Le plasmide se compose du réplicon pG+host (Ts* ori*), d'un gène de résistance à un antibiotique (*Ab^R*) et de l'élément mobile IS*S1*. À 30 °C, le plasmide se réplique dans les cellules.* ***B.*** *À 37 °C, la réplication plasmidique est inactive et le plasmide est perdu sauf si la bactérie subit un événement de transposition. La transposition de l'IS*S1 *aboutit à l'intégration dans le chromosome du plasmide pG+host encadré par des IS*S1 *dupliquées. La transposition de l'IS*S1 *se fait au hasard, le site d'intégration de la structure transposée varie d'une cellule à l'autre. Symboles : ligne courbe : chromosome, ligne noire : ADN pG+host, boîte hachurée : IS*S1.

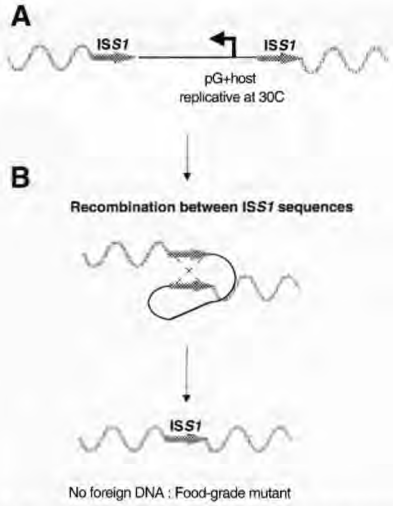

Fig 2. Isolation of food-grade mutants via transposition and homologous recombination. **A.** Representation of the mutated chromosome. IS*S1* elements are duplicated on both sides of the pG+host plasmid. At 30 °C, pG+host replication is activated (black arrow) and stimulates recombination between duplicated IS*S1* sequences. **B.** Homologous recombination between the two IS*S1* sequences (hatched line) leads to excision of the pGh:IS*S1* plasmid, which is subsequently lost by plating at the nonpermissive temperature (37 °C). After recombination, the mutated strain contains only one IS*S1* element (a natural lactococcal sequence) and no foreign DNA and is thus food-grade. Symbols: wavy grey line, chromosomal DNA; arrows, IS*S1* element; black line, pG+host DNA.
Obtention de mutants alimentaires par les processus de transposition et de recombinaison homologue. ***A.*** *Représentation de la structure chromosomique d'un mutant obtenu par transposition de l'IS*S1*. L'élément IS*S1 *encadre le plasmide pG+host. À 30 °C, la réplication du pG+host est activée (flèche noire) et stimule la recombinaison homologue entre les séquences IS*S1 *dupliquées.* ***B.*** *La recombinaison homologue entre les deux séquences IS*S1 *(traits pointillés) aboutit à l'excision du plasmide pGh:IS*S1 *qui est ensuite perdu lorsque la souche est étalée à température nonpermissive (37 °C). Suite à l'événement de recombinaison, la souche mutée contient seulement un exemplaire de l'IS*S1 *(séquence originaire d'un lactocoque) et aucune trace d'ADN étranger. De ce fait, la souche est un mutant alimentaire. Symboles : ligne courbe grise: ADN chromosomique, flèches : élément IS*S1*, ligne noire : ADN correspondant au pG+host.*

target. However, identification of the targeted gene gives the information necessary to modify related strains in the same way. To identify the alteration in an IS*S1*-mutated strain, we used the mutant in which pG+host is present in the transposed structure (fig 3). The pGh:IS*S1* plasmid contains unique restriction sites on both sides of the IS*S1* element (fig 3). Digestion of chromosomal DNA of a mutated strain by one of the unique restriction enzymes generates a fragment containing the pGh:IS*S1* plasmid plus one chromosomal junction (fig 3). After circularization, this fragment replicates as a plasmid in *E coli*. The region corresponding to the IS*S1* target can then be sequenced and analyzed by comparison with data banks.

This type of analysis was used to identify 30 IS*S1*-mutated genes which conferred acid pH-resistance to *L lactis* strain MG1363. Among these mutants, three revealed genes previously described in *L lactis*, 11 genes had homologues in other bacteria with determined functions, three genes had homologues of unknown functions, and ten mutants revealed new potential genes that had not been previously identified elsewhere. Identifying the targeted genes by sequence homology is often very informative. For example, one of the acid pH-resistant mutants is affected in a gene homologous to *ahrC* of *Bacillus subtilis*. The *ahrC* gene of *B subtilis* encodes a protein which regulates both arginine biosynthesis and degradation pathways (Baumberg and Klingel, 1993). Arginine degradation leads to different products including NH_3 which contributes to neutralization of an acid environment (Abdelal, 1979; Marquis et al, 1987). We propose that the *L lactis* mutant affected in *ahrC* is deregulated in arginine synthesis and/or degradation. This type of mutant could neutralize its acidic environment and thus be less sensitive to external pH.

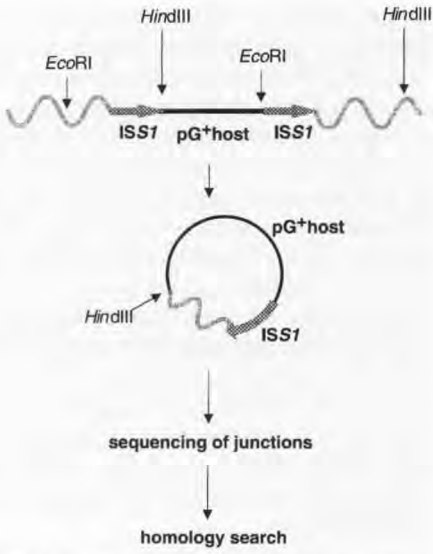

Fig 3. Cloning of IS*S1*-chromosome junctions. Representation of the chromosomal structure of an IS*S1* mutated strain. Unique restriction sites (*Hin*dIII and *Eco*RI) are present on either side of the pG+host plasmid. Digestion of the mutated chromosome by *Hin*dIII generates a fragment constituted of pG+host and the right IS*S1*-chromosome junction. After ligation, the plasmid is circularized and can transform an *E coli* strain. The sequence of the junction can be determined with primers corresponding to IS*S1* or pG+host sequences respectively. Digestion of the chromosomal DNA by *Eco*RI, followed by the same procedure as above, is performed to clone the left IS*S1*-chromosome junction. Symbols: wavy grey line, chromosomal DNA; arrows, IS*S1* element; black lane, pG+host DNA.

*Clonage des jonctions entre l'IS*S1 *et le chromosome. Représentation de la structure chromosomique d'un mutant obtenu par transposition de l'IS*S1. *Les sites de restriction* Hin*dIII et* Eco*RI sont uniques dans la structure transposée et se situent de part et d'autre du plasmide pG+host. La digestion par* Hin*dIII de l'ADN chromosomique du mutant produit un fragment constitué du pG+host et de la jonction droite IS*S1*-chromosome. Après circularisation, ce fragment s'établit comme un plasmide chez* E coli. *La jonction est alors séquencée au moyen d'amorces correspondant à la séquence de l'IS*S1 *ou à celle du pG+host. La jonction gauche IS*S1*-chromosome est obtenue en appliquant la même procédure après digestion par* Eco*RI de l'ADN chromosomique du mutant. Symboles : ligne courbe grise : ADN chromosomique, flèches : IS*S1*, ligne noire : pG+host.*

MODIFICATION OF A KNOWN GENE TO GET A FOOD-GRADE MUTANT

By taking advantage of natural bacterial recombination functions, a known gene on the chromosome can be replaced by a 'modified' copy of the gene which is presented on a plasmid (fig 4). This method, referred to as replacement recombination, can be used to introduce a gene from a related bacteria into the strain, or to modify (increase, decrease or eliminate) the expression of a gene. The modified strain can be a food-grade mutant if the modification introduced does not include foreign DNA. In replacement recombination, the plasmid used can be either non-replicative (Leenhouts et al, 1991a, b) or Ts (Bhowmik et al, 1993; Biswas et al, 1993). Our results indicate that a pG+host delivery vector is much more efficient than a non-replicative vector (Biswas et al, 1993). Therefore, using pG+host, the procedure to obtain food-grade mutants does not require a selective marker in the modified gene and can be applied to various strains. Once again, strains obtained with this procedure may be identical to natural isolates. Gene replacement is now widely used in research laboratories. This technique of gene modification is often used in lactococcal strains, and applications to other LAB increased in the last years. The most spectacular extension of this technique concerns the lactobacilli species (Kammerer, this issue): in the last 2 years, replacement recombination was used in *Lb plantarum* (Leer et al, 1993; Ferain et al, 1994; Fitzsimons et al, 1994 ; Hols et al, 1994), *Lb acidophilus* (van der Vossen et al, 1994), and *Lb helveticus* (Bhowmik and Steele, 1994).

CONCLUSION

The procedures of gene replacement and transposition mutagenesis are easy to use: they are based on antibiotic selection and

different growth temperatures of the culture. Therefore, genetic engineering does not require any fancy or expensive material. The major roadblock in the development of genetic tools is the introduction of DNA into the strain to be modified. The efficiency of the transformation process can dictate the choice of the delivery vector: poorly transformable strains can be modified using conditionally replicative vectors, but not suicide vectors. Use of a replication Ts plasmid also increases the chances of isolating a particular food-grade mutant. The potential benefits of food-grade mutants justify the initial investment of adapting or developing a conditional delivery vector in an organism of interest. Food-grade mutants isolated in the laboratory are the result of precise genetic modifications which are obtained by natural cellular processes (transposition or homologous recombination) and therefore may be identical to natural strains.

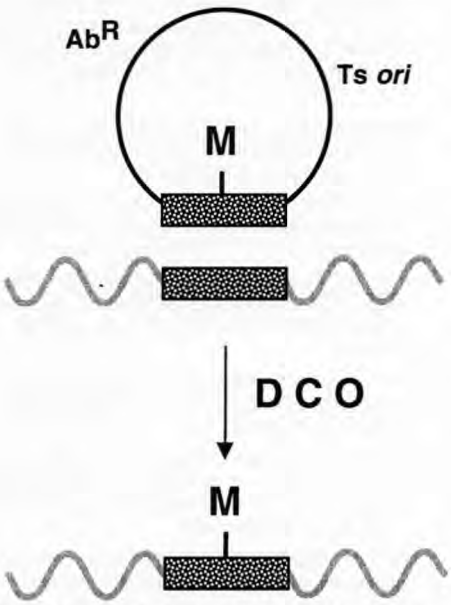

Fig 4. Gene replacement via homologous recombination. The plasmid (black circle) carries a chromosomal fragment (dotted box) with a modified gene (capital M). The unmodified homologous region (dotted box) is present on the chromosome (wavy line). A double recombination event results in integration of the modified gene in the chromosome and the loss of the delivery vector. There is no foreign DNA in the modified strain, thus making it a food-grade mutant.
Remplacement de gène par recombinaison homologue. Le plasmide (ligne noire) porte un fragment d'ADN chromosomique (rectangle à points blancs) comportant un gène modifié (M majuscule). La région homologue non modifiée est présente sur le chromosome (ligne courbe). Un double événement de recombinaison conduit à l'intégration du gène modifié dans le chromosome et à la perte du plasmide : il n'y a plus d'ADN étranger dans la souche modifiée qui est de ce fait un mutant alimentaire.

Manufacturers are interested in producing a reliable safe product, and at the same time want to improve techniques to overcome problems they encounter during fermentation. A genetic approach is a powerful means to attain these goals. Thus far however, genetic modifications are restricted to research purposes. By working together, researchers and manufacturers can define the real problems involved in fermentation, and provide solutions which do not involve the transfer of foreign genes, but rather a rearrangement of genes already present in the lactic acid bacterial repertoire.

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