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Genetics – Chromosome mapping

Molecular genetics in *Streptococcus thermophilus*: from transformation to gene expression

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Summary — *Streptococcus salivarius* subsp *thermophilus (S thermophilus)* is a homofermentative, thermophilic lactic acid bacteria, used in dairy starter cultures. Despite its widespread and long-term use, its molecular biology and genetics have only recently started to be investigated. We report here the isolation and characterization of a cryptic, 3-kb plasmid which was converted into an *E coli* – *S thermophilus* shuttle vector. Using this and other plasmids, transformation was optimized and used to integrate non-replicative plasmids into the bacterial genome. Resulting cointegrates were able to amplify. Resolution of the cointegrates was used for gene-replacement by introducing an *in vitro* generated deletion into a genomic located structural gene. By the same mechanism, a heterologous, promoter-less marker gene was inserted onto the genome between the permease and β-galactosidase gene of the lactose operon. It was thereafter expressed as a functional part of the operon and followed lactose regulation. The control region of the lactose operon was investigated by analyzing promoter up and down mutants.

***Streptococcus salivarius* subsp *thermophilus* / molecular genetics / transformation / genomic integration / gene expression**

Résumé — Génétique moléculaire de *Streptococcus thermophilus* : de la transformation à l'expression des gènes. Streptococcus salivarius *subsp* thermophilus (S thermophilus) *est une bactérie lactique thermophile au métabolisme homofermentaire. Elle intervient dans la composition de nombreux levains utilisés en industrie agroalimentaire. Bien qu'ayant été utilisées depuis des centaines d'années, les recherches en matière de biologie moléculaire et de génétique sont relativement récentes. Nous reportons ici l'isolement et la caractérisation d'un plasmide cryptique de 3 kb ainsi que son utilisation pour l'élaboration d'un vecteur navette pouvant se répliquer chez* S thermophilus *comme chez* E coli. *En partant de cette base et en utilisant d'autres plasmides, la transformation de* S thermophilus *a été optimisée, puis utilisée pour l'intégration de plusieurs plasmides non réplicatifs. Des amplifications, suivies de réarrangements des séquences intégrées, ont été observées. Ces mécanismes furent alors utilisés pour introduire* in vitro *des délétions contrôlées dans un gène structural situé sur le chromosome de* S thermophilus. *De la même manière, un gène marqueur dépourvu de promoteur a été introduit dans l'opéron lactose, entre les séquences codant pour la perméase et la β-galactosidase. Nous reportons ici l'expression de ce gène au sein de l'opéron; il est régulé par le système de régulation propre à l'opéron. La sélection et le séquençage de promoteurs forts et faibles est sur le point de permettre l'analyse des séquences régulatrices.*

Streptococcus salivarius *subsp* thermophilus / *génétique moléculaire / transformation / intégration chromosomique / expression de gènes*

INTRODUCTION

Streptococcus salivarius subsp *thermophilus (S thermophilus)* is an important microorganism for the fermentation of food. It is predominantly used for the production of fermented milk products such as yoghurt and cheese. Only recently has progress been made in the genetics of this organism. Several gene transfer techniques such as conjugation (Gasson and Davies, 1980; Romero *et al,* 1987), transformation (Mercenier *et al,* 1988; Somkuti and Steinberg, 1988) and transfection (Mercenier *et al,* 1989) have been reported for this species. This enabled the examination and use of already existing bacterial plasmids as cloning vectors (Somkuti and Steinberg, 1988; Mercenier *et al,* 1989) as well as a beginning in designing new vector systems (Slos *et al,* 1991). Although very little is known about transcriptional and translational control regions in *S thermophilus* (Mercenier and Lemoine, 1989; Mercenier, 1990), expression of some heterologous genes, delivered and maintained on plasmids, has been reported (Somkuti *et al,* 1991). However, expression levels are not predictable and often low or not detectable (Mercenier and Lemoine, 1989; Mercenier, 1990).

Plasmids are not *a priori* segregated in a stable way and may be lost under non-selective growth conditions. This may in particular be true for plasmid systems which are genetically engineered and carry heterologous DNA. Selection applied for ensuring plasmid maintenance in most cases make use of marker genes referring resistance to antibiotics. Although very convenient for laboratory experiments, such a selection system cannot be applied in food production. To date, a food grade gene transfer system for *S thermophilus* has not been reported. It is in light of these interests that we started our own work on the molecular biology of *S thermophilus*. In this article, we present an overview of some recent progress from our laboratory on plasmid development, transformation, genomic integration and gene expression.

PLASMIDS

S thermophilus is a species which seems to be naturally poor in plasmids. However, several small cryptic plasmids were identified from different strains (reviewed by Mercenier; 1990). Six *S thermophilus* production strains from our collection were analyzed for the presence of natural plasmids by agarose gel electrophoresis. Only one strain showed the presence of a 3-kb plasmid DNA, named pCRN201. A restriction map of the plasmid was determined (fig 1) and showed several unique restriction sites. In order to identify the region essential for replication in *S thermophilus*, several different subclones of pCRN201 were constructed in *E coli* and tested for replication in *S thermophilus*. The potential region of the replication origin is indicated in figure 1. Unique restriction sites not located within the origin of replication could now be used as convenient cloning sites. Suitable *E coli – S thermophilus* shuttle vectors, *eg*

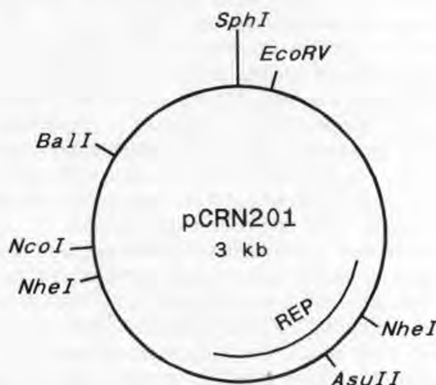

Fig 1. Restriction map of plasmid pCRN201.

pDP160 which has pJDC9 (Chen and Morrison, 1988) inserted at the *Sph*I site of pCRN201, were constructed and are in use in our laboratory.

TRANSFORMATION

Plasmids used to transform *S thermophilus* were pDP160, pVA838 (Macrina *et al*, 1982) and pNZ12 (Simons and de Vos, 1987). They all replicate in both *E coli* and *S thermophilus*, and their antibiotic resistance markers, either erythromycin or chloramphenicol, are functional in the 2 host systems for appropriate selection. As the transformation method we used electroporation. The protocols proposed by Somkuti and Steinberg (1988) and Mercenier (1990) gave insufficient transformation frequencies with our production strains. Hence, we optimized the conditions for one of our strains, ST11, by applying a mathematical iteration process of multifactorial experimental plans (Cochran and Cox, 1957). We now routinely obtain transformation frequencies of 10^4 – >10^5 transformants per µg DNA, depending on the choice of plasmid and mode of selection (Marciset and Mollet, unpublished results). The frequencies are now suitable for direct cloning and genome integration experiments. It is worth mentioning that the individual methods for high rate transformation are very strain-specific and have to be optimized for each new specific strain. However, transformation frequencies are usually high enough to successfully transfer plasmids to different strains.

GENOMIC INTEGRATION

In order to investigate integration of DNA into the bacterial genome, we transformed ST11 with *E coli* based plasmid DNA, which cannot autonomously replicate in *S thermophilus*. The plasmids, derivatives of pUC19 (Yanisch-Perron *et al*, 1985) and pGEM5 (Promega, USA), carry the erythromycin resistance gene from pVA838 and contain DNA inserts of between 1.55–3.2 kb, homologous to the lactose permease and β-galactosidase locus of the genome (Herman and McKay, 1986; Poolman *et al*, 1989; Schroeder *et al*, 1991). After transformation, erythromycin-resistant colonies appeared on selective agar plates at a frequency of between 1–10^2 transformants per µg DNA. Genomic DNA analysis, using Southern blot hybridization techniques, confirmed that the transformants had the *E coli* plasmid integrated into the genome and maintained it by passive replication. All analyzed insertions, without exception, recombined into the genome at their homologous DNA stretch and formed classical cointegrates, whereby the homologous DNA region flank the integrated plasmid. Upon stringent selection on erythromycin, amplification of the cointegrates occurred, thus giving rise to an increased copy number of the integrated plasmid, *ie* the antibiotic resistance gene. A schematic representation of the recombination events is shown in figure 2.

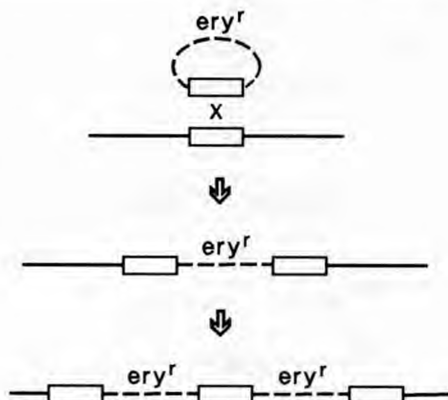

Fig 2. Plasmid integration and amplification. Boxes represent stretches of homologous DNA. Plasmid vector sequences with the erythromycin resistance gene (*ery*^{r}) are indicated as broken lines.

The flanking repeats of the cointegrates were not only able to amplify the integrated plasmids, but also to resolve the cointegrates upon release of the selective pressure. This was shown by integrating plasmids containing *in vitro* generated deletions within the β-galactosidase gene. Appropriate resolution of the corresponding cointegrates resulted in a perfect replacement of the wild-type gene by its modified version (see fig 3). The modified strains were now again erythromycin-sensitive. They grew as white colonies on appropriate X-gal plates and were unable to ferment lactose. Southern blot analysis verified the presence of the genetic modifications and the absence of plasmid DNA.

INTEGRATIVE GENE EXPRESSION

The method of genomic cointegrate formation and resolution gives us not only the possibility to delete or replace DNA sequences, but also to very specifically integrate heterologous genes. Therefore, it should be possible to integrate a gene of interest into a vital operon of the *S thermophilus* genome in such a way as to preserve the correct functioning, *ie* transcription and translation, of the operon and to have the heterologous gene as an integrative, functional part of the operon. The gene should be placed in front of an essential cistron of the operon. Thus, selective pressure on the essential gene during cell growth would ensure genetic maintenance and expression of the integrated gene. According to which operon is chosen as carrier system, different levels of expression and possibilities of regulation can be adopted.

In order to demonstrate the feasibility of such a procedure a marker gene, the promoter-less *cat* gene isolated from pNZ12, was inserted precisely between the lactose permease and β-galactosidase gene of an integration plasmid. The plasmid was then used to replace the original lactose operon of ST11 with the new construction by making use of the above-described 2-step recombination processes. Correct isolates were identified and named ST11-Cat. The new gene organization of the modified op-

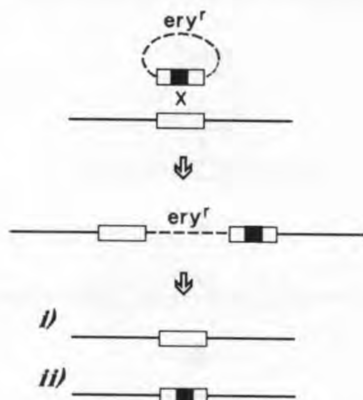

Fig 3. Gene replacement. Symbols are as in figure 2. A black box within the homologous DNA indicates the *in vitro* generated deletion. Resolution of the cointegrate results either in wild-type configuration (i) or gene-replacement (ii).

ST11:

ST11-Cat:

Fig 4. Lactose operon of ST11 and ST11-Cat. Lactose permease and β-galactosidase genes are marked with *lacS* and *lacZ*, respectively, the chloramphenicol acetyl transferase gene with *cat*. Promoter (●) and terminator (▲) are indicated.

eron (verified by Southern blot analysis) is shown in figure 4. Growth of ST11-Cat is now resistant to chloramphenicol: expression and regulation of the *cat* gene was shown to be parallel to that of the ß-galactosidase gene, which is vital for growth of ST11-Cat on lactose, *ie* milk. Analysis of independent colonies after growth in milk for > 100 generations indicated that the *cat* gene was durably maintained.

GENE EXPRESSION

The 2 modified ST11 strains produced from the integration techniques are now being used to study gene expression in this organism.

The ST11 strain with the integrated *cat* gene has been treated with mutagens and colonies selected which are capable of growth in the presence of increasing concentrations of chloramphenicol. Those mutants which also have a concomitant increase of ß-galactosidase activity are analysed for mutations within the promoter region of the lactose operon (Constable and Mollet, unpublished results). Similarly, mutations are being analysed which cause a decrease in the expession of the 2 genes.

Using the β-galactosidase minus ST11 host strain, work is ongoing on the characterization of other important signal sequences involved in gene expression. Promoter probe vectors have been constructed which use the homogenic, promoter-less β-galactosidase gene as a marker. This has advantages over the more commonly used antibiotic resistance genes for promoter identification in that it is a homogenic gene, eliminating problems associated with codon usage and toxicity. Furthermore, its expression levels can be directly assayed by using chromogenic substrates.

CONCLUSIONS

In this article, we showed that the basic tools and techniques for applying molecular genetics in *S thermophilus* have been developed to a great extent. Specifically, it is now possible to efficiently transform selected strains with well characterized plasmids, to integrate and amplify plasmids on the bacterial genome and to engineer, replace or introduce new genes directly on the genome.

The isolation and characterization of *bona fide S thermophilus* plasmids gives us the possibility to develop them into easy, efficient food-grade transformation vectors. Studies to integrate a homogenic expression system onto such a plasmid for controlled gene expression are in progress in our laboratory.

As transposition in *S thermophilus* has not yet been reported, cointegrative plasmid integration may serve as an alternative method for integrating DNA into the bacterial chromosome and disrupting genomic genes or gene structures. For these purposes, the homologous target sequences present on the integration plasmids need not *a priori* be identified. In fact, plasmids carrying genomic DNA from shotgun cloning can be used for quasi-random integration.

We have shown that genes can be integrated specifically into functional operons and, hence, be controllably expressed. This provides several important advantages over the more traditional plasmid transformation and expression systems: i) promoter and regulator regions do not have to be cloned and engineered; ii) the gene transfer system is absolutely food-grade; iii) integration of the gene is stable; and iv) selection for maintenance and expression of the gene is indirect, *ie via* selection for the downstream placed cistron within the operon. In particular, integration of a gene

into the lactose operon in front of the ß-galactosidase gene ensures its expression and maintenance during growth of the strain in its natural habitat, milk.

Integrative expression of an antibiotic resistance marker gene, *eg* against chloramphenicol, under lactose operon regulation provides us with an ideal system to genetically investigate the involved control elements. In the same manner, analysis of the control region of other known genes can be achieved. Subsequent identification of sequences directing expression of the plasmid located β-galactosidase gene will complement the above-determined data.

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