Lait (1993) 73, 145-151

Genetics - Chromosome mapping

Efficient system for genetic modification of lactic bacteria: construction of food grade strains

I Biswas, P Duwat, SD Ehrlich, A Gruss *,
T Hege, P Langella, Y Le Loir, E Maguin

Laboratoire de Génétique Microbienne, INRA, 78352 Jouy-en-Josas cedex, France

Summary — The construction of recombinant strains of lactic bacteria has become an important objective for many researchers. New strains may resolve some of the pre-existing problems in industrial fermentation, and may offer new approaches to meeting medical and pharmaceutical needs. Such an approach is limited by technical problems of manipulation of the lactic bacteria. We have therefore focused on the methodology for the isolation of recombinant strains. Here, we describe the development of a broad host range thermosensitive (Ts) vector, pG⁺host, which allows efficient integration of DNA by single and double crossing over into the chromosome of a variety of Gram-posltive bacteria. pG⁺host has been adapted with an origin of transfer which allows it to be mobilized *in trans* by a conjugative helper plasmid derived from broad host range plasmid pIP501. We describe a method, using pG⁺host, which permits the construction of food grade recombinant strains, *ie,* the integration of foreign DNA (or other modifications) without leaving a selectable marker behind. Using this method, 1–40 % of a bacterial population underwent replacement recombination in the absence of selection for the replacing DNA. With selection, 50–98 % of chromosomal replacements were selected. Possible desirable features of recombinant lactic strains are discussed.

thermosensitive plasmid / homologous recombination / double crossing over / pG⁺host

Résumé — Système efficace de remplacement de gènes chez les bactéries lactiques. *L'amélioration des souches de bactéries lactiques par manipulation génétique est devenue un objectif essentiel pour de nombreux chercheurs. En effet, la construction de souches recombinantes pourrait résoudre certains problèmes liés aux fermentations industrielles et offrir aux bactéries lactiques de nouvelles applications dans les domaines médicaux et pharmaceutiques. Nous décrivons un plasmide thermosensible (pG⁺host) à large spectre d'hôtes qui facilite la construction de souches recombinantes pG⁺host permet l'intégration d'ADN par simple ou double* crossing over *(dco) dans le chromosome de diverses bactéries à Gram positif. Le procédé de dco introduit uniquement l'ADN étranger ou la modification chromosomique d'intérêt, le reste du matériel génétique de la cellule étant inchangé. Pour cette raison, les bactéries modifiées par ce procédé répondent aux normes en vigueur pour une utilisation agroalimentaire. Dans* L lactis, *nous avons obtenu 1 à 40% de bactéries modifiées par dco sans sélectionner le nouveau gène et 50 à 98% de dco en le sélectionnant. Un dérivé de pG⁺host mobilisable par le système de conjugaison de pIP501 a aussi été construit, il permet d'envisager le transfert du pG⁺host dans des bactéries non transformables, puis d'y appliquer nos méthodes de modifications chromosomiques.*

plasmide thermosensible / recombinaison homologue / double* crossing over / *pG⁺host

* Correspondence and reprints

INTRODUCTION

Bacteria are the least expensive component of the fermentation process yet seem to cause the majority of problems with economic consequences, of which bacteriophage and bacterial contamination are the most cited. Improvement of the bacterial properties should have the most effect and at the lowest cost in the long run. The advanced level of molecular biology makes the 'bacterial' approach attractive for solving fermentation problems, and will probably create new ideas regarding the uses of these bacteria. There is still some groundwork to do, since most bacteria which have good cheesemaking properties are not well characterized and their genetics are not at all developed. The development of bacterial strains with defined properties will greatly help resolve the types of problems presently encountered. To start with, we are trying to modify the bacterial strains which are already used for fermentation. The types of genes which may be interesting to establish in these bacteria include the bacteriocins (to combat toxic bacterial contaminants), bacteriophage resistance genes, and novel proteases (for taste). For this purpose we have developed some genetic tools which allow us to transfer foreign DNA into different organisms, and stably integrate this DNA in the chromosome. We combined a temperature-sensitive plasmid (Maguin *et al,* 1992) with an origin of conjugational transfer (Langella *et al,* 1992) to make a cloning vector. This vector can be readily transferred between lactic strains. If a fragment of chromosomal DNA is present on this plasmid, we can select for stable integration in the chromosome of the information carried by the vector (Biswas *et al,* 1992).

RESULTS

Isolation of a thermosensitive broad host range plasmid and its adaptation as a delivery vector

A broad host range plasmid, pGK12 (Kok *et al,* 1984), was chosen to isolate a thermosensitive (Ts) mutant. This plasmid, a recombinant of pWV01 (Leenhouts *et al,* 1991) and is referred to here as a rcr plasmid (te Rielle et al, 1986; Gruss and Ehrlich, 1989). Plasmid DNA was submitted to *in vitro* hydroxyalamine mutagenesis. After electrotransformation into *L lactis* subsp *lactis* strain IL1403, colonies were screened for growth in selective media at 28 °C, but not at 37 °C. Upon screening > 5 000 colonies, we isolated a single Ts plasmid (Maguin *et al,* 1992). This plasmid (pVE6002, now named pG+host), was modified for cloning purposes. The most practical derivative designated pG+ host5 (described in Biswas *et al,* 1992 and in the *Appligene* catalogue) contains the minimal replicon of pBR322, a multicloning site, and the Em^r gene of pE194 (fig 1). The pBR322 origin facilitates cloning in *E coli,* since cells can be maintained at 37 °C. At this temperature, the Ts origin is off (pBR322 origin is active) and growth of *E coli* is better. In Gram-positive bacteria, only the thermosensitive origin is active, and cells are maintained at 28–30 °C.

Integration of the vector into the chromosome by single cross-over (SCO) homologous recombination

The strategy for homologous integration into the chromosome (fig 2) makes use of pG+ host thermosensitivity: a pG+host

plasmid containing a random chromosomal fragment is established in a lactic strain (this need not be an efficient step). The strain is grown overnight at 28 °C in the presence of Em, then diluted at least 100-fold in the same media and grown at 28°C for 2–2.5 h (early log phase). The culture is shifted to 37.5 °C for 3 h (between 6–9 generations) to lower the plasmid copy number per cell. The sample is then diluted and plated at 37 °C on M17 Em plates to detect integration events, and at 28 °C on non-selective media to determine the viable cell counts. Frequency of integrations per cell was estimated as the ratio of Em^r resistant cells at 37 °C to the number of viable cells at 28 °C. For subsequent use, the integrants obtained at 37°C are grown at 37.5 °C in M17 media containing Em.

We determined the integration frequencies of 14 random chromosomal fragments 1 kb in length, cloned onto pG+ host. Ten of these plasmids integrated with frequencies of between 10^2–10^4. Physical analyses of integrants by chromosome mapping, restriction digestion and Southern hybridization using the vector DNA as probe, confirmed that integration occurred *via* SCO. The high frequency integration in *L lactis* using pG+ host demonstrates the efficiency of this system. Since we use a broad host range delivery vector, the technology is readily applied for use in a variety of Gram-positive organisms.

Four plasmids integrated at very low frequencies which proved to occur at non-specific sites. The absence of homologous integration with these plasmids may indicate that gene disruption would have been lethal to the cell.

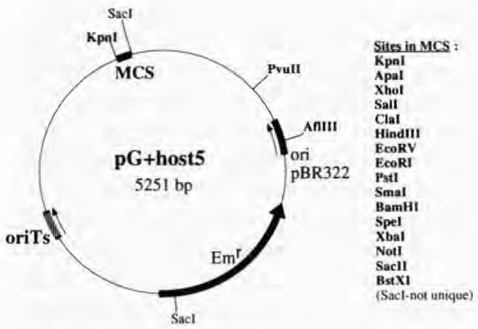

Fig 1. Thermosensitive vector pG+host5. Plasmid pG+host5 (5251 bp) contains thermosensitive broad host range replication origin (oriTs, hatched box), and origin of replication from pBR322 (ori pBR322, grey box) which facilitates cloning in *E coli*. pG+host5 contains a multicloning site (MCS) from pBluescrlpt (Stratagene) for convenient cloning. It encodes the erythromycin resistance *(Em^r)* gene from pE194 (black arrow), which can be selected in *E coli* and in numerous Gram-positive bacteria. Unique restriction sites in the MCS are shown at right.

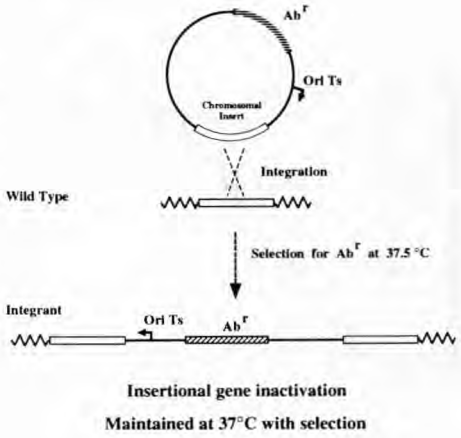

Fig 2. Strategy for chromosomal gene inactivation by SCO homologous integration A pG+host plasmid carrying a chromosomal insert (white box) is first established in the cell at 28°C with antibiotic (Ab). Integration occurs *via* SCO between regions of homology on plasmid and chromosome, and plasmid integrants are selected at 37.5°C with antibiotic. Integration will result in a gene inactivation if the homologous segment is the internal part of a gene. The integrated structure is stably maintained at 37°C with antibiotic. Symbols: black line, plasmid backbone; hatched box, antibiotic resistance gene *(Ab^r);* white box, homologous segment; wavy line, flanking chromosome; Ori Ts, pG+host replication origin.

The relationship between the length of homology and integration frequency was also examined. A nested set of DNA fragments of the sequenced *ilv* operon of *L lactis* strain IL1403 (Godon and Goupil, personal communication; Godon *et al,* 1992) were cloned in pG+host5 (fig 1) and integration frequencies of these clones were determined. We found a log-linear relationship of integration frequency *versus* homology with 2.5–0.5 kb homologous regions. Recombination frequncies appeared to reach saturation for fragment lengths > 2.5 kb (fig 3). The maximum integration frequency, 9 x 10^2, was observed with 3.4 kb homology. Chromosome mapping confirmed homologous integration by SCO.

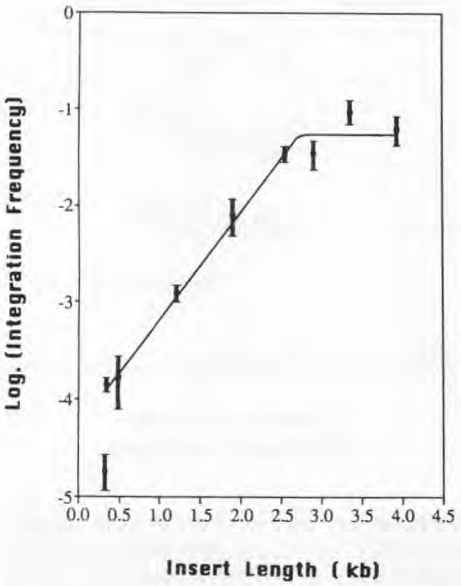

Fig 3. Log-linear relationship between integration frequency and insert homology : integration frequencies were measured with pG+host5 containing nested DNA fragments of between 330–3949 bp of the lactococcal *ilv* operon (9 were tested). For insert between 356–2552 bp, a log-linear relationship is observed. Insert sizes > 2 552 bp do not show a significant increase in integration frequency. Highest frequencies were observed with a the 2 904 bp insert, while the lowest were obtained with a 330 bp insert.

Our experiments show that homologous integration can be an efficient process, which is proportional to the length of homology. It is unnecessary to utilize extended regions of homology for integration, as homology of 3.2 kb give maximal integration frequencies. As little as 500 bp (or less) is enough to obtain integration by SCO. This system is useful for gene inactivation, and the recovery of chromosomal DNA sequences surrounding the site of plasmid integration.

Method of replacement (double-crossing over) recombination into the chromosome using the Ts vector

In the food industry, strain modification is hampered by the need for a clean gene replacement method which does not leave markers behind. This event requires a double cross-over event of high efficiency. We saw a simple way to adapt pG+host for this purpose (Biswas *et al,* 1992, fig 4) : we constructed a pG+host containing a chromosomal (homologous) segment interrupted by a foreign gene. One could also interrupt the segment by a deletion or mutation. In a first step, a marker on the vector could be used to select integration of the Ts plasmid into the chromosome at high temperature. Once integrated, we knew from previous reports (Noirot *et al,* 1987; Hamilton *et al,* 1989; Petit *et al,* 1992) that if the plasmid replicon in the chromosome is activated by a temperature shift-down, homologous recombination is stimulated in its proximity. In this case recombination would occur between the duplication generated by the plasmid integration. When cointegrates generated at 37 °C are subsequently grown at 28 °C, the permissive tempera-

ture for plasmid replication, a second recombination deletion event occurs between chromosomal duplications; the free plasmid should be regenerated in the cell. The chromosomal deletion will either give the desired chromosomal replacement or will restore the original chromosomal structure (fig 4). Using pG+host, we based our strategy for obtaining double cross-over replacement recombination on these data.

The protocol used was the following: a strain carrying the plasmid of interest (Tet^r gene in the chromosomal insert and Em^r on the vector) was grown overnight at 37 °C with Em or Tet to obtain a population of integrants. This mixed population was then incubated at 28 °C without antibiotic selection to allow stimulation of recombination *via* plasmid replication. Cells are then plated at 37°C with or without selection for Tet. In the case where there is a selectable marker in the replacing DNA (Tet^r as shown in fig 4), the replacement event was judged by the percentage of Em^s cells among the Tet^r; 50–98% replacement events were obtained depending upon the construct and the experiment. In a mock experiment in which no selection was imposed for the replacing DNA, we plated cells on non-selective plates, and then screened colonies for the presence of the Tet^r gene. For 2 different constructs, 1–7% and 10–40% of colonies underwent replacement recombination without any direct selection for the event. These results show the feasibility of our system in obtaining food grade recombinant strains which contain no deleterious or extra DNA beside the specific modification desired.

We and our colleagues have already used the system described here to construct mutations in the lactic and other Gram-positive bacteria. We have recently obtained a *recA* derivative of lactococcal straln MG1363 using this method (Duwat, personal communication). Aside from its potential uses in fundamental analyses of

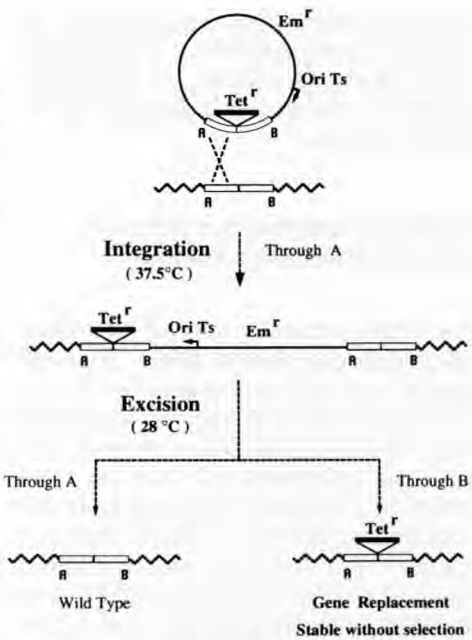

Fig 4. General strategy for replacement recombination: the pG+host plasmid carries a chromosomal DNA fragment (white box) interrupted by a marker (here represented by Tet^r). The initial recombination event occurs by SCO integration (shown here through region A) when liquid cultures are maintained overnight at 37.5°C with selection for the plasmid antibiotic marker (Em^r). Cultures are then diluted and shifted to 28°C. At this temperature, replication of the integrated pG+host (Ori Ts) is activated, and recombination between duplicated sequences is stimulated; this recombination event leads to plasmid excision. Plasmid excision through A restores the parental chromosomal structure, while excision through B produces a gene replacement event. If the initial integration had occurred at B, excision at A would result in gene replacement. Cultures are plated at 37°C, with or without selection for the replacing fragment (Tet^r). If no selection is applied, screening for gene replacement can be performed (we found between 1–40% gene replacement without selection at this step). Symbols: black line, plasmid backbone; wavy line, chromosome; white boxes, regions of homologous chromosomal fragment between the plasmid and the chromosome; hatched box, Em^r marker; black box, Tet^r marker; Ori Ts, pG+host replication origin.

the lactic bacteria, this recA strain has potential industrial application: a *recA* strain is genetically more stable, and may be resistant to induction of bacteriophage by agents of stress.

Further modifications of pG⁺host: use of a mobilization system

The origin of transfer (*oriT*) of streptococcal conjugative plasmid pIP501 (Horodniceanu *et al,* 1976) has been recently localized to a 2.2-kb DNA fragment and cloned onto pG⁺host5 (Langella submitted). This construct (pG⁺host5::*oriT*) can be transferred by conjugative mobilization at high frequencies between *L lactis* strains, if transfer *(tra)* functions are provided *in trans* by a pIP501 derivative. pIP501 also transfers from streptococcal donor strains to a wide variety of streptococci (Horodniceanu *et al,* 1976; Bougueleret *et al,* 1981; Buu-hoi *et al,* 1984; Horaud *et al,* 1985; Langella and Chopin, 1989) and other Gram-positive bacteria such as staphylococci (Engel *et al,* 1980; Schaberg *et al,* 1982), lactobacilli (Gibson *et al,* 1979; West and Warner, 1985; Langella and Chopin, 1989), *Listeria* spp (Buu-hoi *et al,* 1984), pediococci (Gonzalez and Kunka, 1983) and *Leuconostoc* spp (Pucci *et al,* 1988). The development of a recombinant plasmid transfer system based on pIP501 is potentially useful particularly since many Gram-positive bacteria are poorly transformable (Mercenier and Chassy, 1988). Experiments are currently in progress to determine the host range of pG⁺host: :*oriT* mobilization by pIP501.

CONCLUSIONS

The TspG⁺ host plasmid allows the introduction of genetic modificatlons in the chromosomes of numerous Gram-positive bacteria. The DCO procedure we describe enables the construction of food grade recombinant lactic strains, which contain no unwanted genetic information. Of course, the same technology is applicable to strains of medical importance, in the construction of live vaccine strains.

We are presently developing a system with pG⁺host for transposon mutagenesis, with promising results. These tools should simplify genetic characterization of many Gram-positive bacteria.

REFERENCES

Biswas I, Maguin E, Ehrlich D, Gruss A (1993) High efficiency gene inactivation and replacement system for Gram-positive bacteria. *J Bacteriol* 175 (in press)

Bougueleret L, Bieth G, Horodniceanu T (1981) Conjugative R plasmids in group C and G streptococci. *J Bacteriol* 145, 1102-1105

Buu-hoi A, Bieth G, Horaud T (1984) Broad-host-range of streptococcal macrolide resistance plasmids. *Antimicrob Agents Chemother* 25, 289-291

Engel H, Soedirman N, Rost J, van Leeuwen W, van Embden JDA (1980) Transferability of macrolide, lincosamide, and streptogramin resistances between group A, B and D streptococci, *Streptococcus pneumoniae,* and *Staphylococcus aureus. J Bacteriol* 142, 407-413

Gibson EM, Chace N, London S, London J (1979) Transfer of plasmid-encoded antibiotic resistance from streptococci to lactobacilli. *J Bacteriol* 137, 614-619

Godon JJ, Chopin MC, Ehrlich SD (1992) Branched-chain amino acid biosynthesis genes in *Lactococcus lactis. J Bacteriol* 174, 6580-6589

Gonzalez C, Kunka BS (1983) Plasmid transfer in *Pediococcus* spp: intergeneric and intrageneric transfer of pIP501. *Appl Environ Microbiol* 46, 81-89

Gruss A, Ehrlich D (1989) The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol Rev* 53, 231-241

Hamilton C, Aldea M, Washburn B, Babitzke P (1989) New method of generating deletions and gene replacement in *Escherichia coli*. *J Bacteriol* 171, 4617-4622

Horaud T, Bouguenchaud C, Pepper K (1985) Moleculer genetics of resistance to macrolides, lincosalides and streptogamin B (MLS) in streptococci. *Antimicrob Agents Chemother* 10, 795-801

Horodniceanu T, Bouanchaud D, Bieth G, Chabbert Y (1976) R plasmids in *Streptococcus agalactiae* (group B). *Antimicrob Agents Chemother* 10, 795-801

Kok J, van der Vossen J, Venema G (1984) Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microbiol* 48, 726-731

Langella P, Chopin A (1989) Conjugal transfer of plasmid pIP501 from *Lactococcus lactis* to *Lactobacillus delbrueckii* subsp *bulgaricus* and *Lactobacillus helveticus*. *FEMS Microbiol Lett* 60, 149-152

Leenhouts K, Kok J, Venema G (1991) Lactococcal plasmid pWV01 as an integration vector for lactococci. *Appl Environ Microbiol* 57, 2562-2567

Maguin E, Duwat P, Hege T, Ehrlich SD, Gruss A (1992) New thermosensitive plasmid for Gram-positive bacteria. *J Bacteriol* 174, 5633-5638

Mercenier A, Chassy B (1988) Strategies for the development of bacterial transformation systems. *Biochimie* 70, 503-517

Noirot P, Petit MA, Ehrlich SD (1987) Plasmid replication stimulates DNA recombination in *Bacillus subtilis*. *J Mol Biol* 196, 39-48

Petit MA, Mesas J, Noirot P, Morel-Deville F, Ehrlich SD (1992) Induction of DNA amplification in the *Bacillus subtilis* chromosome. *EMBO J* 11, 1317-1326

Pucci M, Monteschio M, Kemker C (1988) Intergeneric and intrageneric conjugal transfer of plasmid-encoded antibiotic resistance determinants in *Leuconostoc* spp. *Appl Environ Microbiol* 54, 281-287

Schaberg D, Clewell D, Glatzer L (1982) Conjugative transfer of R-plasmids from *Streptococcus faecalis* to *Staphylococcus aureus*. *Antimicrob Agents Chemother* 22, 204-207

te Riele H, Michel B, Ehrlich SD (1986) Are single stranded circles intermediates in plasmid DNA replication? *EMBO J* 5, 631-637

West C, Warner P (1985) Plasmid profiles and transfer of plasmid-encoded antibiotic resistance in *Lactobacillus plantarum*. *Appl Environ Microbiol* 50, 1319-1321