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CA 2321645 C 2007/02/20

(11)(21) **2 321 645**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 1999/12/28
(87) Date publication PCT/PCT Publication Date: 2000/07/06
(45) Date de délivrance/Issue Date: 2007/02/20
(85) Entrée phase nationale/National Entry: 2000/08/28
(86) N° demande PCT/PCT Application No.: FR 1999/003297
(87) N° publication PCT/PCT Publication No.: 2000/039312
(30) Priorité/Priority: 1998/12/29 (FR9816529)

(51) Cl.Int./Int.Cl. *C12N 15/74* (2006.01),
C12N 1/21 (2006.01), *C12N 15/87* (2006.01)
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(54) Titre : SEQUENCES D'ADN CONTENANT UN MECANISME DE TRANSFERT PAR CONJUGAISON
(54) Title: DNA SEQUENCES CONTAINING A CONJUGATIVE TRANSFER MECHANISM

(57) **Abrégé/Abstract:**

The invention relates to a DNA sequence capable of being transferred by conjugation, which comprises the sequence SEQ ID No 2. The invention further relates to the use of this DNA sequence and to a method of carrying it out.



ABSTRACT

The invention relates to a DNA sequence capable of being transferred by conjugation, which comprises the sequence SEQ ID No 2. The invention further relates to the use of this DNA sequence and to a method of carrying it out.

DNA sequences containing a conjugative transfer mechanism

The present invention relates to novel DNA sequences capable of being transferred by conjugation, to plasmids containing these sequences, to bacteria containing these DNA sequences or these plasmids, and to the use of these
5 bacteria.

Lactic acid bacteria are involved in the production and preservation of a large number of food products, such as cheese, butter, yogurt, sausage or sauerkraut, among which dairy products are of particular importance. The transformation of milk by lactic acid bacteria is being carried out in ever-larger
10 vats. An understanding of the mechanisms of transfer of genetic material is essential for improving the strains of lactic acid bacteria used in these fermentations. The different mechanisms of transfer of genetic material are transformation, transduction, protoplast fusion and conjugation.

Transformation consists in causing genetic material to enter a bacterium by
15 natural competence, conversion of the cells to protoplasts or electroporation of the cells.

Transduction consists in causing genetic material to enter a bacterium by means of a bacteriophage vector.

Protoplast fusion consists in converting two types of cells to protoplasts so
20 that, after contact, genetic material passes from one strain into the other strain.

Conjugation consists in bringing two types of cells into contact so that genetic material passes from one strain into the other strain by virtue of natural conjugative genes.

Two distinct cases are possible: either the plasmid possesses all the genes
25 involved in conjugation and said plasmid itself passes into the receptor strain, or the plasmid possesses only the genetic components sufficient for its transfer, the other components being present e.g. on another plasmid (Mobilization of the relaxable *Staphylococcus aureus* plasmid pC221 by the conjugative plasmid pG01 involves three pC221 loci, S.J. Projan and G.L. Archer, J. Bacteriol. 1989; 171:
30 1841-1845). The latter situation has an advantage: if the plasmid is transferred to a receptor strain without the conjugative plasmid, it will be unable to be transferred again, its dissemination thereby being prevented. This technique of conjugative plasmid transfer also has the advantage of introducing genetic material into strains in which this is difficult using the transfer techniques described above.

A few studies have already made it possible to demonstrate conjugative systems in lactic acid bacteria:

- Genetic analysis of regions of the *Lactococcus lactis* subsp. *lactis* plasmid pRS01 involved in conjugative transfer, D.A. Mills, C.K. Choi, G.M. Dunny and
5 L.L. McKay, Applied and Environ. Microbiol. 1994; 60 (12): 4413-4420;

- Splicing of a group II intron involved in the conjugative transfer of pRS01 in lactococci, D.A. Mills, L.L. McKay and G.M. Dunny, J. Bacteriol. 1996; 178 (12): 3531-3538.

In the present patent application, a DNA sequence is described which
10 comprises at least one conjugative transfer mechanism; this DNA sequence comprises a functional part of 5333 bp present in the strain *Lactococcus lactis* FL877 deposited on 30 September 1998 in the CNCM (Collection Nationale de Cultures de Microorganismes) under no. I-2082.

This DNA sequence of 5333 bp (SEQ ID No : 1) was isolated from the
15 plasmids contained in the strain FL877.

More precisely, the fragment of 5333 base pairs (bp) was isolated by total digestion, with the restriction enzyme *EcoRI*, of the plasmids contained in the strain *Lactococcus lactis* FL877. This fragment carries one or more conjugative transfer mechanisms and is capable of being transferred to another strain,
20 especially another strain of *L. lactis*, for example from the strain MG1363 (GASSON M.J., J. Bacteriol. 1983; 154: 1-9). This fragment also carries a system of functional replication in *L. lactis*.

From the nucleotide sequence SEQ ID No : 1, the Applicant then isolated a DNA sequence of 2590 bp, which alone confers on a plasmid the property of being
25 transferred to another strain by conjugation, especially from the strain MG1363.

This sequence of 2590 bp (SEQ ID No : 2) can be obtained by the PCR (polymerase chain reaction) method with the aid of appropriate oligonucleotides.

Thus, according to a first feature, the present invention relates to a nucleic acid sequence capable of being transferred by conjugation, which comprises the
30 sequence SEQ ID No : 2, its complementary strand or any sequence derived from said sequence or from its complementary strand by virtue of the degeneracy of the genetic code.

The invention preferably relates to a nucleic acid sequence capable of being transferred by conjugation, said sequence being selected from:

35 a) the nucleotide sequence of 5333 bp (SEQ ID No : 1) or its comple-

mentary strand;

b) any sequence hybridizing with the sequence a) under strict conditions; or

c) sequences derived from the sequences a) and b) by virtue of the degeneracy of the genetic code.

5 The invention relates more particularly to a nucleic acid sequence selected from:

a) the nucleotide sequence of 2590 bp (SEQ ID No : 2) or its complementary strand;

b) any sequence hybridizing with the sequence a) under strict conditions; or

10 c) sequences derived from the sequences a) and b) by virtue of the degeneracy of the genetic code.

According to the present invention, "hybridizing under strict conditions" is understood as meaning hybridization under the following stringency conditions: 42°C in a 20 mM sodium phosphate buffer (pH 6.5) containing 50% of formamide, 15 5 x SSC, 1 x Denhardt's, 0.1% of SDS and 100 µg/ml of RNA, and then washing at 60°C in a buffer containing 0.1 x SSC and 0.1% of SDS.

The invention further relates to DNA sequences which have a high degree of homology with the above DNA sequences. A high degree of homology means a homology (ratio of the identical nucleotides to the total number of nucleotides) of 20 at least 70%, preferably of at least 80% and particularly preferably of at least 90% of the nucleotide sequences when they are aligned according to the maximum homology by the optimum sequence alignment method of Needleman and Wunsch, J. Mol. Biol. 1970; 48: 443-453. This method is used especially in the UWGCG software of the University of Wisconsin: Devereux et al., Nucl. Ac. Res. 1984; 12: 25 8711-8721 - option GAP.

The invention further relates to plasmids transformed with one of the DNA sequences according to the invention. These plasmids can be e.g. plasmid pLDP1 (PREVOTS F. et al., FEMS Microbiol. Lett. 1996; 142: 295-299) and plasmid pLAB510 derived from pPF107-3 (PREVOTS F. et al., FEMS Microbiol. Lett. 30 1998; 159: 331-336), into which one of the DNA sequences according to the invention has been cloned by the conventional techniques well known to those skilled in the art.

The invention further relates to bacteria, especially lactic acid bacteria, preferably belonging to the species *Lactococcus lactis*, which contain at least one 35 DNA sequence or one plasmid as defined above.

These bacteria can be used for the conjugative transfer of genetic material, especially genetic material of industrial interest, to a strain of industrial interest. The conjugative transfer mechanism can be carried by a plasmid or by another part of the bacterial genome.

5 In particular, these bacteria can be used for the conjugative transfer of properties such as phage resistance, the ability to ferment lactose, proteolysis, peptidolysis and bacteriocin production, and of genes coding for proteins of pharmaceutical interest, to strains of industrial interest, particularly in the dairy industry, but also in the pharmaceutical industry.

10 The strains of industrial interest which can advantageously receive genetic material with the aid of the DNA sequences according to the invention, or a plasmid containing them, are e.g. the strains *L. lactis* ssp *lactis* and *L. lactis* ssp *cremoris*.

The invention therefore also relates to these strains of industrial interest
15 into which said genetic material has been integrated.

The invention will be understood more clearly with the aid of the Examples below, which include experimental results and a discussion thereof. Some of these Examples relate to experiments performed for the purpose of carrying out the invention, while others are Examples of implementation of the invention, which
20 are of course given purely by way of illustration.

A large part of all the techniques described in these Examples, which is well known to those skilled in the art, is described in detail in the work by Sambrook, Fritsch and Maniatis entitled: "Molecular Cloning; a laboratory manual", published in 1989 by Cold Spring Harbor Press, New York (2nd edition).

25 The following description will be understood more clearly with the aid of Figures 1 and 2 below, which show:

Figure 1: Subcloning of internal fragments of plasmid pLAB500

The following abbreviations are used in this Figure:

Rep+ or -: fragment replicative (+) or non-replicative (-) in MG1363
30 Rel+ or -: relaxation (+) or non-relaxation (-) in MG1363
Mob+ or -: high efficacy (+) or low efficacy (-) of transfer of the plasmid from MG1363 to LM2301

In addition, the numbers above the sequences represent the base position on plasmid pLAB500.

35 Figure 2: Kinetics of transfer of plasmid pLAB500

Example 1: Sequence of the 5333 bp fragment

The PCR (polymerase chain reaction) technique, described e.g. in the work by Maniatis cited above, makes it possible to amplify a DNA fragment located between 2 suitably chosen oligonucleotides. This amplified DNA can easily be
 5 ligated to itself if restriction sites are provided by the oligonucleotides. In fact, the sequences of these oligonucleotides can contain, at their 5' end, a heterologous part of the DNA to be amplified, consisting e.g. of 10 to 12 base pairs, six of which constitute a restriction site.

The following oligonucleotides were used to construct an erythromycin
 10 resistance gene flanked on either side by an *EcoRI* restriction site:

oligonucleotide 1:

TACATACGCGTCTCATATATACTTTAGATTG (SEQ ID No : 3)

oligonucleotide 2:

TACATACGCGTGACTTAGAAGCAAACCTTAAG (SEQ ID No : 4)

15 oligonucleotide 3:

TTAAATGATCAGAGCTCCACCGCGGTGGCGG (SEQ ID No : 5)

oligonucleotide 4:

TATTTTGATCAGAACAAAAGCTGGGTACCGG (SEQ ID No : 6)

oligonucleotide 5:

20 ATTTATGATCATTTCAGTCGGGAAACCTGT (SEQ ID No : 7)

oligonucleotide 6:

AATTTTGATCAAGTATACCTAATAATTTATC (SEQ ID No : 8)

oligonucleotide 7:

TATGTGAATTCGACTTAGAAGCAAACCTTAAG (SEQ ID No : 9)

25 oligonucleotide 8:

TATGTGAATTCAGTATACCTAATAATTTATC (SEQ ID No : 10)

Using oligonucleotides 5 and 1, a 1.1 kb fragment containing the origin of replication could be amplified by PCR from plasmid pRC1 (LE BOURGEOIS P. et al., Gene 1992; 111: 109-114).

30 Using oligonucleotides 2 and 6, a 1 kb fragment containing the erythromycin resistance gene could be amplified by PCR from plasmid pRC1.

These two fragments were digested with *BclII* and *MluI* and ligated and were then used to transform *E. coli* TG1 (GIBSON T.J., Studies on the Epstein-Barr genome, PhD thesis, Cambridge University, Cambridge, UK, 1984) to give
 35 plasmid pRC1int.

Using oligonucleotides 3 and 4, a 0.3 kb fragment containing several unique restriction sites could be amplified by PCR from plasmid pRC1. This fragment was digested with *Bcl*I and then ligated to plasmid pRC1int, itself digested with *Bcl*I. This novel plasmid, containing the erythromycin resistance gene, an origin of functional replication in *E. coli* but not in *L. lactis*, and several unique restriction sites, was called pRC1N.

Using oligonucleotides 7 and 8, a 939 bp fragment containing the erythromycin resistance gene was amplified by PCR from plasmid pRC1N. This fragment was subsequently digested with *Eco*RI and then ligated to all the plasmids of the strain *L. lactis* FL877, which had previously been extracted and digested with the restriction enzyme *Eco*RI. The strain MG1363 was transformed by this ligation mixture. All the resulting erythromycin resistant clones were used as the donor strain in a conjugation experiment (PREVOTS F. et al., FEMS Microbiol. Lett. 1994; 117: 7-14) with the receptor strain LM2301 (WALSH P.M. et al., J. Bacteriol. 1981; 146: 937-944), which is resistant to streptomycin and sensitive to erythromycin. The transconjugants are selected for their resistance to these two antibiotics. The plasmids which had been transferred to this strain LM2301 by conjugation were extracted and used to retransform the strain MG1363. Plasmid pLAB500 of 6.3 kb was isolated in this way; it was capable of replicating in both the strains LM2301 and MG1363 and capable of being transferred from the strain MG1363 to the strain LM2301 with a high efficiency.

After digestion with the restriction enzyme *Eco*RI, this plasmid gives 2 fragments of 1 kb and 5.3 kb. The first fragment contains the erythromycin resistance gene. The last fragment of 5333 bp was entirely sequenced by the method of Sanger et al. (PNAS - USA 1977; 14: 5463).

Analysis of the sequence showed that the 5333 bp fragment possesses 5 complete open reading frames of more than 150 bp (Fig. 1).

Example 2: Reduction of the size of plasmid pLAB500

The PCR technique was applied in order to determine which of the open reading frames are involved in conjugation and which are involved in replication of the plasmid.

9 oligonucleotides, each comprising an *Eco*RI or *Hind*III restriction site, were synthesized for this purpose. These oligonucleotides have the following sequences:

oligonucleotide 9:

TATAAGAATTCCGCTCGTGTCTCGTCGCACC (SEQ ID No : 11)

oligonucleotide 10:

TATAAGAATTCCATAAATCTACTCTATGC (SEQ ID No : 12)

5 oligonucleotide 11:

TATAAGAATTCGTAGATGAGATTTTAAAGC (SEQ ID No : 13)

oligonucleotide 12:

TATTAGAATTCTATTTAGGGGTATGTAAC (SEQ ID No : 14)

oligonucleotide 13:

10 TATTAGAATTCCGTGATTTCTTTAGTGCTGTC (SEQ ID No : 15)

oligonucleotide 14:

TATATAAGCTTAGTATACCTAATAATTTATC (SEQ ID No : 16)

oligonucleotide 15:

TATATAAGCTTCACTTCGCAAAATTCGCG (SEQ ID No : 17)

15 oligonucleotide 16:

TATATAAGCTTGAGGGTCGAGCTTGAGCG (SEQ ID No : 18)

oligonucleotide 17:

TATATAAGCTTCACTCTTTATATGCTAATAC (SEQ ID No : 19)

20 Using oligonucleotides 14 and 15, a DNA fragment of 2846 bp, containing the erythromycin resistance gene and ORF E in the form of a *HindIII*-*HindIII* fragment, could be amplified by virtue of the restriction sites provided by the oligonucleotides, enabling this fragment to be ligated to itself.

Likewise, a DNA fragment of 4808 bp, containing the erythromycin gene and ORF B, C, D and E, could be amplified using oligonucleotides 14 and 16.

25 Finally, a DNA fragment of 5169 bp, containing the erythromycin gene and ORF B, C, D and E, could be amplified using oligonucleotides 14 and 17.

These DNA fragments were amplified by PCR from a preparation of plasmid pLAB500 with the enzyme ELONGASE® (BRL).

30 The PCR products were purified by extraction with phenol/chloroform, precipitated with ethanol, digested with the restriction enzyme *HindIII* and ligated to themselves. Cloning of these fragments onto themselves yielded plasmids pLAB501, pLAB502 and pLAB503. These plasmids were constructed in the strain MG1363. Their ability to replicate in this strain indicates that ORF E codes for a protein involved in replication of the plasmid. Moreover, over 230 amino acids,
35 this protein shows a homology of 21.7% with the protein RepE involved in

replication of the F factor of *E. coli*.

These plasmids were then tested for their ability to be transferred to the strain LM2301 by conjugation. Plasmids pLAB502 and pLAB503 have a comparable transfer efficiency to that of pLDP1, the negative control. It can therefore be said that these plasmids do not possess all the DNA necessary for their conjugative transfer. On the other hand, pLAB501 has a comparable conjugative transfer efficiency to that of pLAB500, so all the DNA necessary for the conjugative transfer of pLAB500 must be present in pLAB501.

10 Example 3: Subcloning of pLAB500 fragments into plasmid pLDP1

A fragment containing ORF C and D in the form of an *EcoRI-EcoRI* fragment of 2068 bp could be amplified by PCR using oligonucleotides 9 and 12.

A fragment containing ORF C in the form of an *EcoRI-EcoRI* fragment of 1201 bp could be amplified by PCR using oligonucleotides 9 and 13.

15 A fragment containing ORF B and C in the form of an *EcoRI-EcoRI* fragment of 2136 bp could be amplified by PCR using oligonucleotides 11 and 13.

A fragment containing ORF B, C and D in the form of an *EcoRI-EcoRI* fragment of 2602 bp could be amplified by PCR using oligonucleotides 10 and 12.

20 A fragment containing ORF B and C in the form of an *EcoRI-EcoRI* fragment of 1735 bp could be amplified by PCR using oligonucleotides 10 and 13.

After digestion with the enzyme *EcoRI*, each of these fragments was cloned into pLDP1 to give plasmids pLAB504, pLAB505, pLAB506, pLAB507 and pLAB508 respectively. Among these plasmids, only pLAB507 was transferred with a significant efficiency, which was better than that of the other plasmids but nevertheless not as good as that of pLAB500. It can therefore be said that ORF B, C and D are necessary for conjugative transfer.

Example 4: Subcloning of a pLAB500 fragment into pLAB510

The following oligonucleotides were used in this subcloning:

30 oligonucleotide 18:

AGGTTTCCCGACTGGAAATG (SEQ ID No : 20)

oligonucleotide 19:

TACGTGAATTCAGTTTAAATCAATCTAAAG (SEQ ID No : 21)

oligonucleotide 20:

35 TACGTGGATCCATCGGCATAATCGTTAAAAC (SEQ ID No : 22)

oligonucleotide 21:

TACGTGAATTCAGAAGAACCCTTAAC (SEQ ID No : 23)

Plasmid pLDP1 is transferred by conjugation from MG1363 to LM2301 with a low but non-zero efficiency. To eliminate the contribution of this plasmid in the transfer, the PCR fragment obtained with oligonucleotides 10 and 12 was cloned into another plasmid.

The erythromycin resistance gene was first amplified by PCR from plasmid pRC1N and oligonucleotides 18 and 19, and then digested with the restriction enzymes *Bam*HI and *Eco*RI. Using oligonucleotides 20 and 21, a replicative fragment of plasmid pPF107-3 (GenBank access number: Y12675) was amplified from the total DNA of the strain I-942 (deposited in the CNCM on 12 April 1990) and then digested with the restriction enzymes *Bam*HI and *Eco*RI. These two PCR fragments were ligated and used to transform MG1363. The novel plasmid obtained, called pLAB510, was digested with *Eco*RI and then ligated to the fragment obtained with oligonucleotides 10 and 12 by PCR amplification from plasmid pLAB500, and digested with *Eco*RI. This ligation was used to transform MG1363. This novel plasmid, pLAB511, was tested for its ability to be transferred from MG1363 to LM2301 (Table 1). It can be seen that this novel plasmid pLAB511 is indeed transferable between these two strains by conjugation.

20

Table 1: Conjugative transfer efficiency

Plasmid	ORF	Efficiency	Relative efficiency
pLAB500	A,B,C,D,E	8.3×10^{-4}	100
pLAB501	B,C,D,E	7.1×10^{-4}	85
pLAB502	B,C,D,E	1.1×10^{-7}	0.01
pLAB503	E	$<5 \times 10^{-9}$	<0.0006
pLAB504	C,D	1×10^{-7}	0.01
pLAB505	C,D	5.7×10^{-8}	0.007
pLAB506	B,C	5.7×10^{-8}	0.007
pLAB507	B,C,D	4.5×10^{-5}	5.4
pLAB508	B,C	4.2×10^{-7}	0.05
pLDP1	/	9.2×10^{-8}	0.01
pLAB510	/	$<5 \times 10^{-9}$	<0.0006
pLAB511	B,C,D	1.3×10^{-3}	157

Efficiency = ratio of the number of transconjugants to the number of receptor cells.

Relative efficiency = ratio of the efficiency of conjugation for a given plasmid to the efficiency of conjugation for pLAB500 x 100.

5 ORF = complete open reading frames contained in the plasmid.

Example 5: Demonstration of the relaxation conferred by different plasmids

Relaxation (formation of open circular forms of plasmids from supercoiled forms of plasmids) is involved in the transfer of certain plasmids (Plasmid-protein relaxation complexes in *Staphylococcus aureus*, R. Novick, J. Bacteriol. 1976; 127: 1177-1187). From comparisons with computerized data banks (GenBank), the protein deduced from ORF C shows a homology of 35.2% with Rlx, a relaxase from *Staphylococcus aureus*. It can therefore be assumed that this protein deduced from ORF C is also a relaxase. Plasmids pLAB500 to pLAB508, pLAB510, 10 pLAB511 and pLDP1 were therefore extracted and deposited on agarose gel. After migration, among all these plasmids extracted from MG1363, only pLAB503, 15 pLAB510 and pLDP1 do not have an open circular form, so ORF C is indeed involved in the relaxation of plasmids.

20 Example 6: Kinetics of pLAB500 transfer

Conjugations between MG1363 containing pLAB500 and LM2301 were performed at different times in order to evaluate the point at which transfer took place. It is found (cf. Table 2 below and Figure 2) that conjugation has already started after 1 hour and that the peak of transfer efficiency is reached after about 4 25 hours. Conjugations every 10 minutes between 0 and one hour also showed that no conjugation took place before one hour.

Table 2: Kinetics of pLAB500 transfer

Conjugation time (hours)	Efficiency of conjugation
0	$<1.1 \times 10^{-8}$
1	6.6×10^{-5}
2	9.8×10^{-5}
3	1.7×10^{-4}
4	3.4×10^{-4}
5	2.7×10^{-4}
6	2.2×10^{-4}
7	6.7×10^{-5}
8	4.2×10^{-5}
9	3.0×10^{-5}
29	7.9×10^{-5}

Efficiency of conjugation = ratio of the number of transconjugants to the
5 number of receptor cells.

SEQUENCE LISTING

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<400> 13
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<210> 14
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<210> 23
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<220>
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sequence:oligonucleotide

<400> 23
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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isolated nucleic acid sequence capable of being transferred by conjugation, which comprises the sequence SEQ ID No: 2 or its complementary strand.
2. The isolated nucleic acid sequence according to claim 1 which is:
 - a) the nucleotide sequence of 5333 bp (SEQ ID No: 1) or its complementary strand;
 - b) any sequence hybridizing with the sequence a) under the following stringency conditions: 42°C in a 20 mM sodium phosphate buffer (pH 6.5) containing 50% of formamide, 5 x SSC, 1 x Denhardt's, 0.1% of SDS and 100 µg/ml of RNA, and subsequent washing at 60°C in a buffer containing 0.1 x SSC and 0.1% of SDS.
3. The isolated nucleic acid sequence according to claim 1 which is:
 - a) the nucleotide sequence of 2590 bp (SEQ ID No: 2) or its complementary strand;
 - b) any sequence hybridizing with the sequence a) under the following stringency conditions: 42°C in a 20 mM sodium phosphate buffer (pH 6.5) containing 50% of formamide, 5 x SSC, 1 x Denhardt's, 0.1% of SDS and 100 µg/ml of RNA, and subsequent washing at 60°C in a buffer containing 0.1 x SSC and 0.1% of SDS.
4. A plasmid comprising the sequence according to any one of the claims 1 to 3.
5. The plasmid according to claim 4 which is selected from plasmid pLDP1 or plasmid pLAB510.
6. A bacterium which contains the sequence according to any one of claims 1 to 3 or the plasmid according to claim 4 or 5.
7. The bacterium according to claim 6 which is a lactic acid bacterium.
8. The lactic acid bacterium according to claim 7 which belongs to the species *Lactococcus lactis*.

9. Use of the bacterium according to any one of claims 6 to 8 for the conjugative transfer of genetic material to a strain of industrial interest.
10. Method of transferring genetic material to a strain of industrial interest, which consists in transferring the genetic material by conjugation from the bacterium according to any one of claims 6 to 8 to said strain of industrial interest.

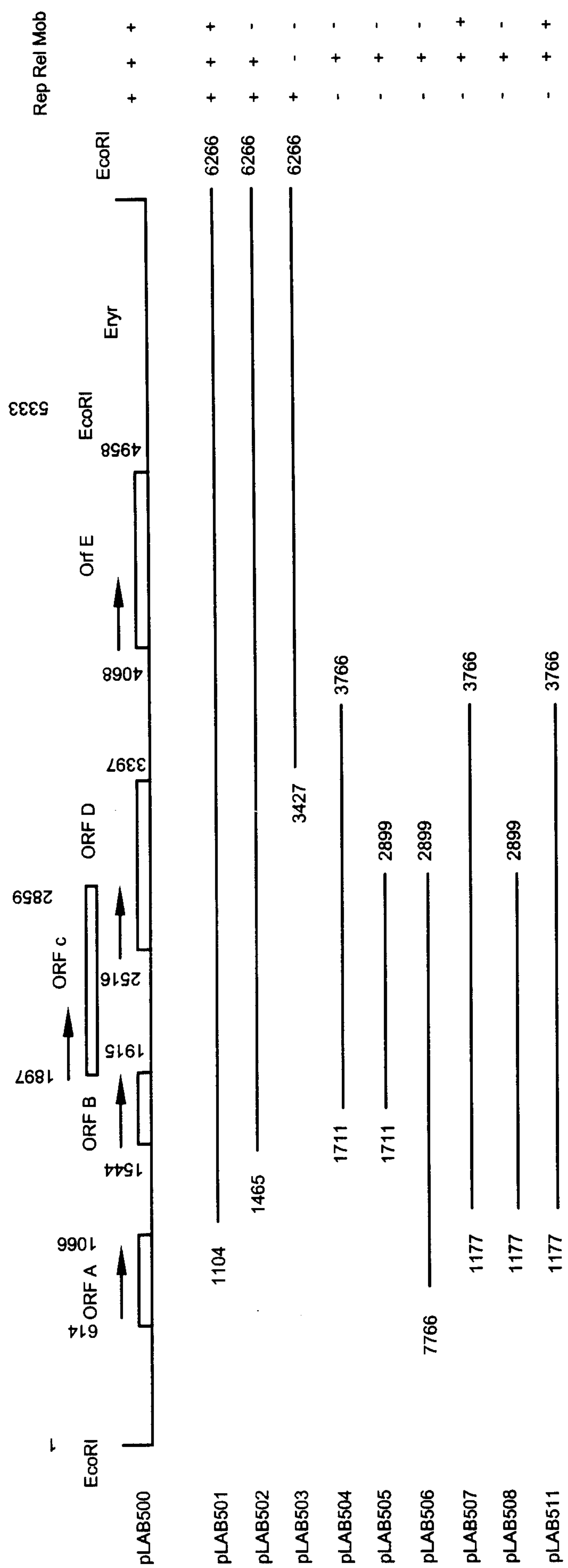


FIG.1

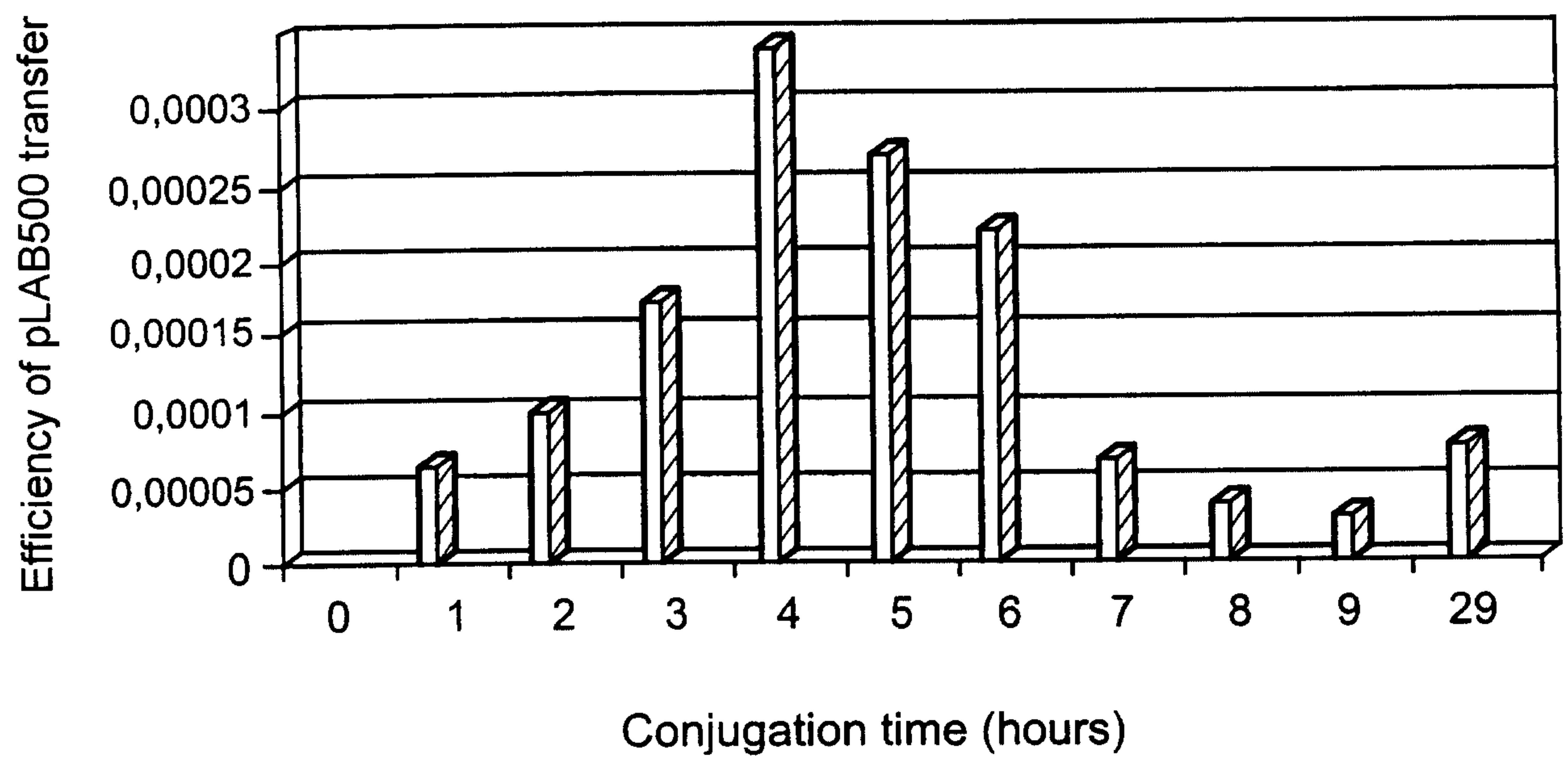


FIG.2