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NOTICE OF ENTITLEMENT

(To be filed before acceptance)

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being the applicant in respect of Application No. 68486/94, state the following:-

Part 1 - *Must be completed for all applications*

The person nominated for the grant of the patent has entitlement from the actual inventors by virtue of a contract of employment.

Parts 2 & 3 - *not applicable*

Part 4 - *Must be completed for PCT applications*

The person nominated for the grant of the patent has entitlement from the applicant of the application listed in the declaration under Article 8 of the PCT by virtue of a contract of employment.

The basic application listed in the declaration under Article 8 of the PCT is the first application made in a Convention country in respect of the invention.

Part 5 - *not applicable*

Part 6 - *Must be completed if the application relates to a microorganism and relies on Section 6 of the Act*

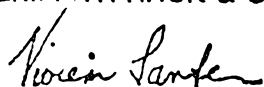
The person nominated for the grant of the patent is the depositor of the deposit as listed hereafter

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(54) Titre: CLONAGE ET EXPRESSION DU GENE DE L'ENZYME MALOLACTIQUE DE *LACTOCOCCUS LACTIS*

(57) Abstract

The invention relates to the production of a DNA fragment comprising the gene of the malolactic enzyme of *Lactococcus lactis*, and to the cloning and expression of said gene in *E. coli*, and in yeasts, particularly of the genus *Saccharomyces* and *Schizosaccharomyces*.

(57) Abrégé

L'invention est relative à l'obtention d'un fragment d'ADN comprenant le gène de l'enzyme malolactique de *Lactococcus lactis*, et au clonage et à l'expression de ce gène chez *E. coli* et chez des levures, en particulier des genres *Saccharomyces* et *Schizosaccharomyces*.

CLONING AND EXPRESSION OF THE GENE FOR THE MALOLACTIC
ENZYME OF *LACTOCOCCUS LACTIS*

The present invention relates to the cloning of the structural gene for the malolactic enzyme of
5 *Lactococcus lactis*, to its complete nucleotide sequence and to the deduced protein sequence, as well as to DNA fragments carrying the sequence encoding this gene. It also relates to the expression of this gene in various prokaryotic or eukaryotic hosts, and in particular in
10 *Saccharomyces* and *Schizosaccharomyces*.

Malolactic fermentation, a secondary fermentation which is observed during the process of wine-making in addition to the alcoholic fermentation performed by yeasts, consists in the degradation, by
15 certain lactic acid bacteria, of the malic acid present in wines, to lactic acid and CO₂. The bacterial species naturally present in the fermentative flora and which are responsible for the malolactic fermentation belong to the genera *Lactobacillus*, *Leuconostoc* and
20 *Pediococcus*.

This reaction has two main advantages:

- It leads to a deacidification of the wine by conversion of a diacid (L-malate) to a monoacid (L-lactate), the consequences of which are advantageous
25 at the organoleptic level, for the majority of red wines and some white wines,

- It permits the microbiological stabilization of the wine, and therefore a better preservation, by using a fermentable substrate, which prevents subsequent fermentations by other strains of undesirable
30 microorganisms. Finally, malolactic fermentation can have an influence on the quality of the wine from the aromatic point of view.

This reaction poses, however, a certain number
35 of problems because of its slowness and its uncontrollable character.

Malolactic fermentation occurs spontaneously only at the end of the natural wine-making process; indeed, the pH, the ethanol content and the presence

SO₂ in the wine considerably slow down the growth of the lactic acid bacteria naturally present in the fermenting mass and which are responsible for the malolactic fermentation. In order to try to accelerate this
5 fermentation, the technique most commonly used lies in the addition, to the fermenting medium, of an inoculum of lactic acid bacteria.

However, this technique does not always resolve the problems of implantation of the lactic acid
10 bacteria because of the difficulty which they have in adapting to the wine medium. Moreover, the introduction of lactic acid bacteria amounts to adding, in addition to the malolactic enzyme whose activity is desired, numerous other enzymes whose action on the numerous
15 substrates present in the wine can result in a development of the organoleptic qualities of the product which is very difficult to control.

It would therefore be desirable to have the possibility of carrying out the malolactic fermentation
20 so as to arrive rapidly, and while removing uncontrollable variables, at an improvement of the product obtained.

With the aim of better controlling the malolactic fermentation, it has been proposed to clone the
25 genes for the malolactic enzymes of lactic acid bacteria, and to introduce and express them in the microorganism which is normally involved in the manufacture of wine, such as *S. Cerevisiae*.

WILLIAMS et al. [App. Environ. Microbiol. 47:
30 288-293 (1984)], as well as European Application 103300 describe the cloning of a 5 kb DNA fragment carrying the gene for the malolactic enzyme *L. delbrueckii* into *S. Cerevisiae* and into *E. coli*.

The gene for the malolactic enzyme of *L. oenos*
35 has also been cloned into *E. Coli* [LAUTENACH et al., Microbiol., 39: 29-39 (1984)].

However, the strains of microorganisms transformed express the gene only at a very low level,



insufficient to permit its use in the manufacture of wine.

In addition, in both cases, an instability of the DNA cloned into *E. Coli* was observed, even going as far as bringing about the complete loss of the gene.

Moreover, a malolactic enzyme, having an activity of a level comparable to that of the malolactic enzyme of bacteria of the genera *Leuconostoc*, *Pediococcus* and *Lactobacillus* mentioned above, have recently been purified from *Lactococcus lactis*. It is a protein of about 230 kDa, consisting of subunits of about 56 kDa. Its pHi. is about 4.3, and its Km for malic acid is about 10 to 12 mM. Its N-terminal sequence has also been determined [RENAULT, Malolactic fermentation: Genetics and Genetic Engineering, GIM90, 6th International Symposium on Genetics of Industrial Microorganisms, Strasbourg (1990)].

From a purified preparation of this malolactic enzyme, the Inventors obtained preparations of polyclonal antibodies specifically directed against this enzyme. A *Lactococcus lactis* DNA library was then prepared in the vector λ gt11, in *E. coli*. The screening of this library with the antibodies obtained made it possible to clone DNA fragments, which represent between the whole lot 4.5 kb of the same region of genomic DNA of *Lactococcus lactis*, containing the malolactic gene, and a fragment of the gene for malate permease, these genes being organized into operon. The Inventors identified a 2684 bp DNA fragment including the entire gene for the malolactic enzyme, and determined the sequence of this fragment, which is represented in the sequence listing in the annex under the number SEQ.ID.NO.1. In addition, the expression of the malolactic gene has been obtained in various prokaryotic and eukaryotic hosts. Thus, the introduction of the *Lactococcus lactis* malolactic gene into *E. coli*, under the control of its own regulatory elements as well as the expression of the said gene in



yeast under the control of yeast regulatory elements have been achieved.

The subject of the present invention is a nucleic acid fragment, characterized in that it comprises the sequence of the gene for the malolactic enzyme of *Lactococcus lactis*.

By "sequence of the gene for the malolactic enzyme of *Lactococcus lactis*", there is understood in particular the coding sequence stretching from nucleotides 466 to 2085 (inclusive) of the sequence SEQ. ID. NO 1 represented in the sequence listing in the annex, as well as any sequence which, taking into account the degeneracy of the genetic code, encodes the same polypeptide; this definition also comprises the sequences carrying minor modifications intended to permit a better expression of the gene in a determined host.

The invention also encompasses nucleic acid fragments comprising a sequence homologous or complementary to the sequence of the gene for the malolactic enzyme of *Lactococcus lactis*, as defined above, or to a fragment of at least 10-mer, preferably of at least 20-mer, of the said sequence, and which can be used in particular as probes for the location of the said gene and/or as primers for its amplification.

The subject of the invention is also expression cassettes, comprising the sequence of the gene for the malolactic enzyme of *Lactococcus lactis*, under the control of sequences capable of regulating the expression of the said gene.

By "sequences regulating the expression of a gene", there is understood sequences of the promoter and terminator type which are active in the host in which it is desired to obtain the expression of the said gene. The promoters and terminators of various genes can be used, associated in various combinations.

Among the sequences which can be used to obtain the expression, in yeast, of the gene for the malolactic enzyme of *Lactococcus lactis*, there may be



mentioned, as non-limitative example, the promoters and terminators, known per se, of the genes for alcohol dehydrogenase I (ADHI), for phosphoglycerate kinase (PGK), and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The expression cassettes in accordance with the invention can be carried by plasmids, or integrated into the chromosomal DNA of the host yeast.

The subject of the invention is also recombinant vectors characterized in that they comprise at least one DNA fragment containing the sequence of the gene for the malolactic enzyme of *Lactococcus lactis*, as defined above.

According to a preferred embodiment of the present invention, the said vectors are expression vectors, comprising an expression cassette as defined above.

According to a preferred embodiment of the present invention, the said vectors comprise regulatory sequences which are active in the yeast.

Advantageously, the vectors in accordance with the invention are shuttle vectors, also possessing a bacterial replication origin and a selectable marker in a bacterium (for example, gene for resistance to an antibiotic).

These vectors can be selected on the basis of the nature and the strength of the regulatory elements which enter into the expression cassette. The promoters and terminators described above, or any other sequence which makes it possible to control and regulate the expression of a gene in yeast, can be chosen.

Another criterion for the choice of vectors lies in the number of copies thereof, which is determined by the choice of the replication origin.

By way of example, a choice can be made between the following vectors:

- Replicative vector (YEp) at high copy number, possessing as replication origin in yeast, a portion of the endogenous 2μ plasmid.



- Replicative vector (YRp) at high copy number, possessing as replication origin, a chromosomal ARS sequence.

- Linear replicative vector (YLp) at high copy number, possessing telomer sequences as replication origin.

- Replicative vector (YCp) at low copy number, possessing a chromosomal ARS sequence and a centromere sequence.

Preferably, the vectors in accordance with the invention also contain selectable markers in yeast, such as markers for auxotrophy: URA₃, LEU₂, HIS₃, TRP₁, ADE, and the like and/or markers for resistance to antibiotics (G418, hygromycin B, chloramphenicol, phleomycin), to herbicides (sulphometuron-methyl), to copper, and the like.

Vectors in accordance with the invention, carrying the gene encoding the malolactic enzyme of *Lactococcus lactis* can be introduced into any yeast strain, by various transformation techniques.

Among the most common transformation techniques which can be used, there may be mentioned the protoplasts technique, the technique of permeabilization to lithium salts and electroporation.

In all cases, the process for this construction comprises the following steps:

- construction of an expression cassette comprising the gene for the malolactic enzyme and the regulatory elements of variable strength;
- introduction of this cassette either as single copy, or as multiple copies into the yeast.

The choice can also be made to integrate the gene for the malolactic enzyme of *Lactococcus lactis*, equipped with its control sequences, into the genome of the yeast, in which case an integrative vector (YIp) possessing no replication origin in the yeast will be chosen for example; it is also possible to integrate this gene using other techniques, for example co-transformation.



It is possible to modulate the level of expression of the gene encoding the malolactic enzyme of *Lactococcus lactis*, by adjusting especially the number of copies of the gene which are introduced into the yeast, and/or the strength of the regulatory elements associated therewith.

The subject of the present invention is also strains of transformed eukaryotic or prokaryotic cells, characterized in that they contain at least one heterologous DNA fragment carrying at least one copy of a gene encoding the malolactic enzyme of *Lactococcus lactis*, under the control of sequences regulating the expression of the said gene in the said cell.

According to a preferred embodiment of the present invention, the said cells are yeast cells.

The yeast strains in accordance with the invention find numerous applications in the food industry and in particular in the field of oenology for carrying out malolactic fermentation.

According to a preferred arrangement of this embodiment, the said yeasts belong to the genus *Sacharomyces*.

According to another preferred arrangement of this embodiment, said yeasts belong to the genus *Schizosaccharomyces*.

Schizosaccharomyces is sometimes used in oenology for its capacity to degrade malate. However, its use is limited because this degradation which is carried out using the malic enzyme does not produce lactate, but ethanol and CO₂, which results in a substantial deacidification, which may have negative influences on the characteristics of the wine.

Strains of *Schizosaccharomyces* obtained in accordance with the invention and expressing the gene for the malolactic enzyme, carry out the malolactic fermentation with degradation of L-malate to L-lactate, and thus have the advantage of permitting a much less substantial deacidification than that which results from the malate/ethanol conversion.



These *Schizosaccharomyces* strains can have numerous uses in oenology; they can for example be used:

- in co-culture with an oenological strain of *Saccharomyces cerevisiae*;
- as immobilized cells.

The present invention will be understood more clearly with the aid of the additional description below, which refers to examples describing the cloning of the gene for the malolactic enzyme of *Lactococcus lactis* and its expression in bacteria and yeasts.

- General methods

Malolactic enzyme was purified as described by RENAULT (1990, communication cited earlier); cf. also C. ROULLAND, (DEA in oenology-ampelography, September 1988, University of Bordeaux II).

The general techniques for manipulation of nucleic acids and molecular cloning to which reference is made in the examples below are those described by SAMBROOK et al. [Molecular Cloning: A Laboratory Manual (second edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (1989)].

EXAMPLE 1 - PURIFICATION OF MALOLACTIC ENZYME

The extraction procedure is briefly recalled below:

Strain - culture conditions

Lactococcus lactis (IL 1441) is cultured in the following medium:

	Yeast extract	6 g
30	Tryptone	2 g
	Glucose	15 g
	DL-malic acid	15 g
	K ₂ HPO ₄	0.5 g
	KH ₂ PO ₄	0.5 g
35	MgSO ₄	0.4 g
	NaCl	0.02 g
	FeSO ₄	0.004 g
	MnSO ₄	0.02 g
	H ₂ O	qs 1 l



The pH is adjusted to 5%. The sterile medium is inoculated with 10% (v/v) of a preculture.

- Preparation of a crude extract and assays

5 The cells are cultured in 3 litres of the above medium at 28°C, for 24 hours, harvested by centrifugation at 1000 g for 10 min at 4°C, and washed twice with 0.9% NaCl.

10 The pellet is taken up in 30 ml of phosphate buffer (0.1 M, pH 6.0). The cells are ground by ultrasound for 6 min at 0°C. After centrifugation (30,000 g, 15 min, at 4°C) the supernatant constitutes the crude extract.

The proteins are assayed by BCA Protein Assay Reagent (PIERCE).

15 The malolactic activity is measured by the decarboxylation of malic acid monitored using a CO₂ electrode (EISCHWEILER).

- Purification of the enzyme

20 After treatment with DNase I (1 mg/ml and 15 min of incubation at room temperature), the crude extract is subjected to a fractional precipitation with ammonium sulphate (35% saturation, then 70%).

25 The supernatant from the precipitation at 70% has no activity. The pellet, taken up in 2 ml of 0.1 M phosphate buffer, pH 6.0, and which contains all the malolactic activity, is deposited onto a filtration column on ACA 34 gel (ULTROGEL; fractionation range from 20,000 to 350,000) 40 cm in length and 2.6 cm in diameter, previously equilibrated with elution buffer:
30 0.01 M phosphate buffer, pH 6.0, 0.1 M KCl. The elution is performed with a flow rate of 18 ml/h. The volume of fractions collected is 2.4 ml.

35 Under these conditions, the most active fractions correspond to fractions 50 to 54. They are combined for an additional purification by isoelectric focusing.

The isoelectric focusing is performed on a 110 ml LKB column. The pH gradient is formed by means of the ampholines used at 2% and is stabilized by a glycerol



density gradient which is established during the filling of the column. The most active fraction which focuses at pH 4.3, the pHi of the protein, is recovered and then undergoes an additional step of purification
5 by ion-exchange chromatography (SEPHAROSE Q anion-exchange column, PHARMACIA).

The elution is performed by a gradient from 0 to 0.5 M NaCl, in a 50 mM Tris-Hcl buffer, pH 7.6, 0.1 mM EDTA, 1 mM β -mercaptoethanol.

10 The fraction containing the malolactic activity is eluted at an NaCl concentration of 0.33 M. It is this fraction which is used for the production of antibodies.

15 **EXAMPLE 2: PRODUCTION OF POLYCLONAL ANTIBODIES DIRECTED AGAINST THE MALOLACTIC ENZYME OF LACTOCOCCUS LACTIS:**

The antibodies were obtained by injecting into two rabbits a portion of the purified preparation of malolactic protein according to the following procedure:

20 - Subcutaneous injection of 2 ml of solution of malolactic protein (100 μ g in 0.3 M NaCl buffer) supplemented with 2 ml of complete Freund's adjuvant.

- Second injection 40 days later, with the same quantity of malolactic protein and 2 ml of incomplete
25 Freund's adjuvant.

The blood of the two rabbits is recovered 12 days after the 2nd injection, left for 24 h at 4°C to allow coagulation, centrifuged at 10,000 g for 10 min at 4°, and the serum recovered is aliquoted and stored
30 at -20°C.

The antibodies to malolactic enzyme were then tested by ELISA test carried out on both sera (called 120 and 119) obtained from rabbit serum according to the procedure described by SAMBROOK et al. [(1989)].
35 This analysis made it possible to determine that the optimal dilution for the use of the antibodies was 1/1000th.



The specificity of the antibodies was then determined by immunoelectrophoretic transfer (Western blotting).

5 The proteins from a crude extract of *Lactococcus lactis* (prepared as described in Example 1), on the one hand, and from the purified preparation of malolactic enzyme, on the other hand, were separated by SDS-PAGE electrophoresis and transferred onto nitro-cellulose membrane.

10 The polyclonal antibodies obtained are then bound to the membrane and revealed by anti-rabbit antibodies coupled to alkaline phosphatase.

The serum 120 recognizes, on the crude extract and on the purified fraction, a predominant band of
15 molecular weight of about 60 kD corresponding to the malolactic enzyme, and several contaminant bands of lower molecular weight.

On the other hand, the serum 119 recognizes on the purified fraction, as well as on the crude extract,
20 a single band, corresponding to the malolactic enzyme; this highly specific serum was therefore used for the subsequent experiments.

EXAMPLE 3 : CONSTRUCTION OF A LIBRARY FOR EXPRESSION OF GENOMIC DNA OF LACTOCOCCUS LACTIS IN E. COLI

25 a) Extraction of *Lactococcus lactis* DNA

The *Lactococcus lactis* genomic DNA library was prepared in the vector λ gt11.

The genomic DNA of *Lactococcus lactis* IL 1441 was, in a first stage, extracted and purified. For
30 that, 500 ml of a culture in exponential phase are centrifuged at 5000 g for 10 min. The pellet is taken up in 5 ml of TE (10 mM Tris, 1 mM EDTA, pH 8) containing 150 mM NaCl and 1 mg/ml of lysozyme, and incubated for 10 min at 37°C. 1 ml of SDS, 25% in EDTA, 0.1 M, pH 8,
35 are added and the mixture is incubated for 15 min at 60°C. The DNA is purified by two extractions with phenol-chloroform, followed by two others with chloroform-isoamyl alcohol (24:1). A final purification is carried out on caesium chloride gradient in the



presence of ethidium bromide. The ethidium bromide is extracted with isoamyl alcohol. After dilution with 2 volumes of water, the DNA is precipitated with 6 volumes of ethanol. The precipitate is recovered using
5 a rod and dissolved in 1 ml of TE. The DNA concentration in solution was determined ($1 \mu\text{g}/\mu\text{l}$) by measurement of the OD at 260 nm.

b) Digestion of the *Lactococcus lactis* DNA

125 μg of *Lactococcus lactis* DNA are partially
10 digested with two units of enzyme AluI and two units of HaeIII for 35 min at 37°C so as to generate blunt ends. The digestion is stopped by addition of EDTA to 0.1 M final.

The digested DNA is precipitated with 1/20 vol
15 of 3 M sodium acetate and 2 volumes of ethanol. After centrifugation, the DNA is taken up in 300 μl of TE and the fragments separated on sucrose gradient for 15 hours at 25,000 rpm.

Fractions of 0.5 ml are harvested and analysed
20 on 0.8% agarose gel. The fraction containing fragments of 1.5 and 4 Kb is dialysed against TE for 4 hours.

c) Ligation of the digested DNA into the vector $\lambda\text{gt}11$

The vector $\lambda\text{gt}11$ (derived from bacteriophage λ)
25 was chosen to prepare the expression library. The principle of the preparation of the library consists in inserting DNA fragments into the 3' region of the phase encoding the β -galactosidase gene, so as to express hybrid proteins under the control of the
30 β -galactosidase promoter.

A kit for cloning into $\lambda\text{gt}11$ (AMERSHAM) was used according to the procedure recommended by the supplier.

1 μg of (blunt-ended) DNA digested and dialysed
35 as described above was inserted into the vector $\lambda\text{gt}11$ according to the following principle:

The DNA is ligated to dephosphorylated adaptors having a blunt end and another EcoRI cohesive end. After removal of the free adaptors by purification on



exclusion column, the "adapted" DNA fragments are phosphorylated and ligated to the arms of the vector which were also digested with EcoRI and dephosphorylated.

5 The *in vitro* packaging of the phage particles is then carried out. The library thus obtained was titrated by infection of the bacterial strain *E. coli* 1090 [hSd ($r^{-}km^{+}k$) lac U169, ProA⁺, lon⁻, araD139, StrA, SupF, trpC22: Tn10 (pMC9)] with an aliquot frac-
10 tion of the phage suspension, and the transformants selected on LB medium (bactotryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) + ampicillin (50 µg/ml) at 43°C (to induce bacterial lysis). The lysis plaques are obtained after 4 h of incubation.

15 The library thus obtained represents about 3 times the *Lactococcus lactis* genome.

EXAMPLE 4 : SCREENING OF THE GENOMIC DNA LIBRARY

a) Plating of the library and preparation of the filters

20 75,000 lysis plaques were obtained by plating the library on LB+ ampicillin + MgCl₂ medium and incubation for 3 h 30 min at 43°C (conditions for non-expression). The plates are covered with nitrocellulose filters (C extra, AMERSHAM), impregnated with 10 mM
25 IPTG and incubated for 4 h at 37°C, so as to induce the expression.

 The filters are rinsed in TNT (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20), and then incubated with gentle stirring for 30 min at room temperature in
30 the same buffer.

 In order to limit the detection of "false positive" clones which can hybridise in a non-specific manner during the screening of the library, a second series of filters, constituting duplicates of the first
35 series, were deposited on the plates, incubated and treated in the same manner.

b) Depletion of the serum 119

 The immunological screening of the library is carried out with the aid of the serum 119 previously



depleted against the *E. coli* proteins. The depletion of the serum is performed as follows:

A culture of *E. coli* Y1090 is carried out in 100 ml of LB + ampicillin for 24 h at 37°C. The culture is centrifuged at 5000 g for 10 min at 4°C. The pellet is taken up in 3 ml of buffer (50 mM Tris, 10 mM EDTA, pH 8), and the suspension frozen at -20°C and thawed (several times). The cells are completely lysed by sonication at 0°C (six times 20 seconds), then centrifuged at 12,000 g for 10 min at 4°C. The lysate is recovered and incubated for 4 h at room temperature in the presence of 1 ml of antibody 119 diluted 1/10th in TNT buffer + 1% gelatin + 3% BSA + 5% powdered skim milk.

The depleted serum is stored at 4°C in the presence of sodium azide (0.05%).

c) Immunological screening of the library

The nitrocellulose filters are then incubated in the presence of the depleted serum 119 as described above, and the revealing of the bound antibodies is carried out with the aid of anti-rabbit antibodies coupled to alkaline phosphatase, as previously described.

Only the clones showing a positive signal on the two filters are taken into consideration. 24 positive clones were thus selected.

In order to verify the specificity of the signal obtained, the positive clones were subcloned and a second detection with the aid of antibodies was carried out. Around each positive lysis plaque, an agarose carrot is removed and incubated in 500 µl of SM buffer (NaCl 6 g/l, MgSO₄ 7H₂O 2 g/l, Tris base 6 g/l, gelatin 0.01%, pH 7.5), for 2 hours at 4°C in order to allow diffusion of the phage particles. This suspension is again used to infect *E. coli* 1090 and the mixture plated on LB + ampicillin + MgCl₂ plate as above, so as to obtain well isolated lyses plaques. A second screening by the serum 119 carried out as above made it possible to eliminate 14 clones out of the 24 tested,



which no longer exhibited significant signals. The other 10 clones, very clearly positive were selected.

d) Characterization of the recombinant clones

5 The phage DNA from the 10 positive clones was extracted and analysed. A well isolated positive lysis plaque (subclone) was systematically used as starting material.

10 The DNA is extracted from confluent lysis plaques on LB + ampicillin plates. The plates are covered with 5 ml of SM buffer and stirred at 4°C for 2 hours. The suspension is recovered, supplemented with few drops of chloroform and centrifuged at 4000 g for 10 min at 4°C in order to remove the bacterial debris. The DNA is extracted from the phage suspension thus
15 purified.

The first analysis consisted in amplifying, by PCR, the inserts carried by the recombinant phages in order to determine their size.

20 Two oligonucleotides [λ gt11 Primer (forward) 24-MER and λ gt11 Primer (reverse) 24-MER, company BIOLABS] corresponding to β -galactosidase sequences surrounding the EcoRI cloning site were used as primers for amplification of the inserts.

25 Analysis of the amplification products on agarose gel (1.6%) made it possible to determine that the 10 λ gt11 clones selected had inserts of between 2 and 3.5 kb in size.

30 In order to establish the restriction map of the cloned DNA fragments, the inserts of the λ gt11 vectors were subcloned into the plasmid pUC18 [YANISH-PERRON et al. Gene No. 33, p. 103-119, (1985)]. It was thus possible to obtain the subcloning of 6 inserts.

35 The restriction map of the 6 inserts was prepared; 5 of the 6 inserts have a common region, suggesting that these 5 inserts come from the same genomic fragment. This was verified by Southern hybridization of restriction fragments of *Lactococcus lactis* genomic DNA separated on agarose gel and



transferred onto Nylon membrane, with the inserts radioactively labelled with ^{32}P .

The restriction maps of 4 of these 5 inserts are represented in Figure 1.

5 The sizes of the inserts represented are 3 kb, 2.7 kb, 2 kb and 3 kb. As these inserts overlap, the total size of the cloned region is 4.5 kb.

 The 6th clone, which has an insert having a restriction map without any similarity with that of the
10 other 5 clones, comes from a different genomic DNA fragment.

 In order to determine whether one of the two types of cloned DNA fragments contained the 5' region of the gene encoding the malolactic enzyme of
15 *Lactococcus lactis*, a Southern hybridization of the inserts separated on agarose gel and transferred onto nylon membrane, with a probe of oligonucleotides derived from the protein sequence of the NH_2 -terminal end of the malolactic enzyme (P. RENAULT, communication
20 cited earlier) was carried out. The probe chosen is a mixture of oligonucleotides deduced from the sequence including the first 6 amino acids:

ATG(A,C)G(G,A,T,C)GC(G,A,T,C)CA(T,C)GA(G,A)AT

 The 5 inserts coming from the same DNA fragment
25 hybridize with the probe; which proves that the 5' end of the gene encoding the malolactic enzyme of *Lactococcus lactis* is contained in the 5 cloned inserts. On the other hand, no hybridization was detected on the 6th clone.

30 e) Sequence of the clone P153A

 The nucleotide sequence of one of the 5 clones P153A having a 2.7 kb insert was determined by dideoxy-termination method in an Applied Biosystem sequencer. The total sequence of the insert is represented in the
35 annex, under the identification number SEQ ID NO 1, as well as the amino acid sequence which is deduced therefrom. The coding region of the malolactic gene is 1620 nucleotides. The first 20 amino acids deduced from the nucleotide sequence obtained are identical to the



protein sequence determined from the purified enzyme, with the exception of the 14th amino acid (lysine instead of cysteine determined from the purified enzyme). This open reading frame is capable of encoding
5 a protein of 53 kD, this is completely compatible with the size of the malolactic enzyme of *Lactococcus lactis* estimated by gel filtration and SDS-PAGE electrophoresis (about 60 kD).

The sequence SEQ ID NO 1 comprises, in addition,
10 465 nucleotides upstream of the codon for initiation of translation. This sequence comprises the entire promoter of the gene for the malolactic enzyme. The *Lactococcus lactis* promoters have a size of the order of 150 nucleotides upstream of the initiation codon.
15 Furthermore, the signals characteristic of initiation of transcription of the *Lactococcus lactis* genes are present.

Downstream of the open reading frame, 596 nucleotides were sequenced, and another open reading
20 frame of 581 nucleotides was detected in 3' of the malolactic gene in another reading frame.

The determination of homologies with the genes or known proteins was carried out by comparison of the nucleotide sequence SEQ ID NO 1, and the protein
25 sequences deduced with databases.

- As regards the open reading frame of 1620 nucleotides, very substantial amino acid and nucleotide conservations were detected with various malic enzymes, which are responsible for the decarboxylation of malate
30 to pyruvate.

Moreover, a consensus sequence for binding of NAD was found.

These homologies are perfectly consistent with the function of the malolactic enzyme, which uses, as
35 malic enzyme, malate as substrate, and requires NAD for the conversion of the substrate to lactate.

- As regards the beginning of the reading frame identified downstream of the malolactic gene, amino acid homologies, which were also substantial (of the



order of 40%), were identified with citrate permeases. It appears extremely probable that this open reading frame may correspond to the 5' region of a malate permease.

5 It therefore appears that the structural gene encoding the malolactic enzyme, and a portion of the malate permease gene are present on the DNA fragment sequenced, and that these genes are organized into operon.

10 EXAMPLE 5 : EXPRESSION OF THE MALOLACTIC GENE IN E. COLI

The expression of the malolactic gene was studied in *E. coli*, in various transformants of the bacterial strain DH5 α : p153A (clone sequenced), p5A, 15 p191A (see restriction maps, Figure 1).

The transformants as well as the control strain DH5 α transformed with pUC18, were inoculated into a range of culture media at increasing malate concentration: 3 ml of M9 medium (Na₂HPO₄, 7H₂O 6 g/l, KH₂PO₄ 20 3 g/l, NaCl 0.5 g/l, NH₄Cl 1 g/l, glucose 4 g/l), supplemented with thiamine 1 mg/l, aspartic acid 20 mg/l and ampicillin 20 mg/l, and containing 0; 0.3; 0.5 or 1% malate (w/v). The medium is buffered to pH 6.5 by the addition of citric acid.

25 The cultures were incubated at 37°C, with stirring, for 48 h.

As control, *Lactococcus lactis* was cultured in 8 ml of M9 medium containing 5 g/l of "YEAST EXTRACT" and the same malate range. The cultures were incubated 30 at 28°C, for 48 h, under anaerobic conditions.

The cultures were then centrifuged for 10 min at 4000 g. The supernatants were recovered and tested for the presence of L-lactate. The L-lactate assay was performed with the aid of a kit (BOEHRINGER), according 35 to the procedure described by the supplier.

The results, presented in Table I below (expressed in g/l) show a very clear production of lactate in the culture supernatant of the transformants p153A and p191A, whereas no trace of lactate is



detected in the supernatant of the control strain (*E. coli* DH5 transformed with pUC18). Another transformant, p5A, which was tested under the same conditions, does not, on the other hand, produce lactate. This is explained by the fact that the malolactic gene carried by the recombinant vector p5A has a 3' end from which 200 nucleotides have been truncated. This region therefore seems to be essential for the malolactic activity.

In the absence of malate, there is no significant production of lactate in the culture supernatant of the transformants p153A and p191A, nor of the control strain transformed with pUC18. This is explained by the fact that *E. coli* does not produce L-lactate under these culture conditions.

In the presence of malate, no production of lactate is observed for the control strain. On the other hand, as regards the transformants p153A and p191A, a significant production of lactate is observed in the presence of 0.1, 0.3 and 1% malate.

The level of lactage produced is quite low when it is compared with that produced by the control strain *L. lactis* tested under the same conditions and starting with the same malate concentrations. However, it should be noted that the production of L-lactate in *L. lactis* corresponds to the L-lactate produced by malolactic fermentation, and also to a high proportion of the L-lactate produced by degradation of sugars, via L-LDH which converts pyruvate to lactate.

Moreover, it is difficult to determine the portion of malate which is really available for the malolactic fermentation. Indeed, under the culture conditions used for *E. coli*, a significant portion of the malate can be used via the tricarboxylic cycle.



TABLE I

Bacterial strain	% malate in culture medium			
	0	0.3	0.5	1
<i>E. coli</i> DH5 α (pUC18)	0.001	0.017	0.008	0.015
<i>E. coli</i> (p153A)	0.015	0.12	0.23	0.31
<i>E. coli</i> (p191A)	0.017	0.17	0.15	0.2
<i>Lactococcus lactis</i>	2.6	5.09	8.5	-

EXAMPLE 6 : EXPRESSION OF THE MALOLACTIC GENE IN YEAST

In order to carry out the expression of the malolactic gene cloned into yeast, the coding region was placed under the control of yeast regulatory elements (promoters and terminators), in yeast/*E. coli* shuttle vectors.

a) Introduction of the malolactic gene into the multicopy plasmid pVT10-U

The expression plasmid pVT100-U was used. This plasmid contains the replication origin of yeast 2 μ , the selectable marker URA3 and the strong ADH regulatory elements (alcohol dehydrogenase I promoter and terminator), as well as the bacterial elements (replication origin and the gene for resistance to ampicillin).

This plasmid has been described by VERNET et al [Gene 52; 225-233, (1987)].

The coding region of the malolactic gene was amplified by PCR starting with the plasmid p153A, with the aid of primers consisting of oligonucleotides derived from the sequence of the malolactic gene. In order to facilitate the cloning, the introduction of restriction sites XhoI upstream and XbaI downstream of the coding region was carried out during the amplifications.

The oligonucleotides used as primers are:

- an oligonucleotide which makes it possible to isolate the coding region at the level of the codon for initiation of translation ATG (primer 1) and containing a XhoI site.



- On the 3' side, the oligonucleotide chosen (primer 3') is located a few nucleotides downstream of the codon for termination of translation.

The sequences as well as the exact positions of the primers used are indicated in Figure 2.

The amplification was carried out in the following manner:

	primer 1	4 μ l (30 pmol)
	primer 3	4 μ l (30 pmol)
10	Taq buffer 10X	10 μ l
	Taq polymerase (5 μ / μ l)	0.5 μ l
	dNTP 2 μ M	10 μ l
	MgCl ₂ 25 mM	6 μ l
	p153A	4 μ l (40 ng)
15	H ₂ O	62 μ l

Amplification conditions: 30 seconds at 94°C, 30 seconds at 40°C, 1 min at 72°C for 30 cycles.

The size of the amplified fragments was checked by analysing an aliquot on an agarose gel.

100 ng of the amplified fragments were digested with XhoI and XbaI and ligated to 50 ng of pVT100-U vector previously digested with XhoI and XbaI and dephosphorylated.

The vector pVT100-U obtained, called pM1, is represented in Figure 3.

b) Transformation of the yeast

The yeast strain *Saccharomyces cerevisiae* SCV5M was transformed with the vector pM1.

The strain SCV5M was deposited on 18 June 1992 at the Collection Nationale de Cultures de Microorganismes, held by Institut Pasteur, under the number I-1222. It is a laboratory strain which is haploid, *ura*⁻, *MATa*, derived from an oenological strain.

The transformation method used is that of lithium acetate described by ITO et al. [J. Bacteriol. No. 153, pp. 163-168, (1983)].



c) Production of malolactic fermentation in yeast

Two of the transformants obtained, called ScV5M/pMIa and ScV5M/pMIb were tested for their capacity to carry out the malolactic fermentation (conversion of malate to lactate); these transformants were cultured under the following conditions:

- on the one hand, on minimum synthetic medium YNB (6.7 g/l "yeast nitrogen base without amino acid" (DIFCO), 20 g/l glucose) containing 0, 0.6 or 1% malate, buffered either to pH 3, or to pH 6 with citric acid (6.3 g/l).

- On the other hand, on synthetic medium mimicking the composition of a grape must, [SABLAYROLLES et BARRE, Sciences des Aliments, No. 6, p. 373-383 (1986)], buffered to pH 3.3.

The two media used do not contain uracil, which ensures a selection pressure for the plasmids.

A transformant of ScV5M by the plasmid pVT100-U containing no insert was used as negative control.

CULTURE AND ASSAY CONDITIONS

8 ml of medium are inoculated with the yeast strains (transformants and control). The growth is carried out in sterile tubes of 10 ml, at 28°C, for 60 h, without stirring.

The cultures are centrifuged for 5 min at 4000 g and the supernatant tested for the production of L-lactate using a Boehringer kit, as indicated in Example 5.

RESULTS

The results obtained are presented in Table II.

In the minimum synthetic medium YNB (Table II), in the absence as in the presence of malate (0.3 and 1%), no trace of L-lactate is detected with the control yeast (ScV5M/pVTU). This is completely consistent with the fact that *S. cerevisiae* is incapable of carrying out the conversion of malate to lactate, and that, on the other hand, under anaerobic conditions, it produces



only traces of lactate from glucose, mainly in the form of D-lactate.

5 The transformed strains pM1a and pM1b, in the absence of malate, produce traces of L-lactate of the order of about fifty to about one hundred milligrams. It is probable that this slight production results from the conversion, by the malolactic enzyme, of a portion of the endogeneous yeast malate, metabolized from glucose. In the presence of malate in the culture
10 medium (0.6 and 1% of malate), and in YNB media buffered to pH 3, or in synthetic broth (pH 3.3, 3 g/l of malate), a very significant production of L-lactate (from 0.5 to 0.7 g/l for the clone pM1a under these experimental conditions) is observed.

15 The highest production is observed in synthetic broth whose composition is very similar to that of the grape musts (0.7 g/l of L-lactate, equivalent to 7.7 $\mu\text{mol/ml}$).

20 Under the same culture conditions (YNB) but at pH 6, the production of L-lactate is very low, of the same order of magnitude as in the absence of malate. The difference in production of L-lactate according to the pH of the culture medium could be explained by a problem of permeation of the malate in *S. cerevisiae* at
25 pH 6. At pH 3 or 3.3, a large portion of the malate (pK 3.5) present in non-dissociated form can enter inside the cell by mere diffusion. On the other hand, at pH 6, the malate, mainly in dissociated form, would enter less easily inside the cell.

30 The recombinant strains therefore degrade malate to lactate by malolactic fermentation, and with a higher efficiency at acidic pH, therefore under conditions similar to those in oenology.



TABLE II: ASSAY OF LACTATE IN *S. CEREVISIAE*

	Yeast strain	L-lactate produced (g/l)	L-lactate produced ($\mu\text{mol/ml}$)
5	ScVRM/pVT100-U		
	- in (pH = 3):		
	0% malate	2.4×10^{-3}	0.02
	0.6% malate	7×10^{-3}	0.07
	1% malate	9×10^{-3}	0.1
10	- in (pH = 6):		
	0% malate	3×10^{-3}	0.03
	0.6% malate	6×10^{-3}	0.06
	1% malate	10×10^{-3}	0.11
	- Synthetic broth (pH = 3.3)	9×10^{-3}	0.1
15	ScV5M/pM1a		
	- in (pH = 3):		
	0% malate	0.15	1.66
	0.6% malate	0.4	4.4
	1% malate	0.52	5.7
20	- in (pH = 6):		
	0% malate	0.1	1.1
	0.6% malate	0.07	0.77
	1% malate	0.05	0.55
25	- Synthetic broth (pH = 3.3)	0.7	7.7
	ScV5M/pM1b		
	- IN (Ph = 3):		
	0% malate	0.15	1.66
	0.6% malate	0.40	4.4
30	1% malate	0.48	5.3
	- in (pH = 6):		
	0% malate	0.11	1.22
	0.6% malate	0.08	0.88
	1% malate	0.056	0.62
35	- Synthetic broth (pH = 3.3)	0.51	5.66



The same experiment was carried out on synthetic broth, and the production of lactate measured after culturing for 10 days; 1.5 g/l (equivalent to 17 μ mol/ml) of lactate were produced under these conditions.

EXAMPLE 7: EXPRESSION OF THE MALOLACTIC GENE IN SCHIZOSACCHAROMYCES POMBE

The expression of the malolactic gene was also studied in *Schizosaccharomyces pombe*. For this purpose, the coding region of the malolactic gene was placed under the control of an *S. pombe* promoter, in an *S. pombe*/*E. coli* shuttle vector.

a) Introduction of the malolactic gene into the multicopy plasmid pART1

The expression plasmid pART1 was used. This plasmid contains the yeast replication origin ARS1, the selectable marker LEU2 and the *S. pombe* alcohol dehydrogenase promoter, as well as bacterial elements (replication origin and gene for resistance to ampicillin).

This plasmid has been described by McLeod et al., (EMBO J. 6; 729-736, 1987).

The coding region of the malolactic gene was amplified by PCR starting with the plasmid p153A, with the aid of primers consisting of oligonucleotides derived from the sequence of the malolactic gene. In order to facilitate the cloning, the introduction of BamHI restriction sites upstream and SacI downstream of the coding region was carried out during the amplifications.

The oligonucleotides used as primers are:

- an oligonucleotide which makes it possible to isolate the coding region at the level of the codon for initiation of translation ATG (primer 12) and containing a BamHI site

- on the 3' side, the oligonucleotide chosen (primer 13) is located a few nucleotides downstream of



the codon for termination of translation and has a SacI site.

The sequences as well as the exact positions of the primers used are indicated in Figure 2.

5 The amplification was carried out in the following manner:

primer 12	4 μ l (20 pmol)
primer 13	4 μ l (20 pmol)
Taq buffer 10X	10 μ l
10 Taq polymerase (5 U/ μ l)	0.5 μ l
dNTP 2 μ M	10 μ l
mgCl ₂ 25 mM	6 μ l
p153A	10 μ l (100 ng)
H ₂ O	65.5 μ l

15 Amplification conditions: 30 seconds at 94°C, 30 seconds at 37°C, 1 min at 72°C for 30 cycles.

The size of the amplified fragments was checked by analysing an aliquot on agarose gel.

20 100 ng of the amplified fragments were digested with BamHI and SacI and ligated to 50 ng of vector pART1 previously digested with BamHI and SacI and dephosphorylated.

The vector obtained, called pD4, is represented in Figure 4.

25 **b) Transformation of *S. pombe***

An *S. pombe* strain auxotrophic for leucine was transformed with the vector pD4 and with the vector pART1. It is the laboratory strain leu1-32h+, which was supplied by J.KOHLI, Institute of General Microbiology, 30 Raltzerstrasse 4, CH-3012, Bern, Switzerland.

The method of transformation used is that of transformation with lithium acetate adapted from Ito et al. (Journal of Bacteriology 153, 163-168, 1983).

35 **c) Production of malolactic fermentation in *S. pombe***

One of the transformants obtained, called Sp/D42 was tested for its capacity to carry out the malolactic fermentation (conversion of malate to



lactate). The transformant was cultured under the following conditions:

- in minimum synthetic medium YNB containing 50 g/l of glucose and 5.5 g/l of L-malate, buffered either to pH 3.5 or to pH 6. This medium does not contain leucine, which ensures a selection pressure for the plasmids introduced.

A transformant of the same strain by the plasmid pART1 not containing insert was used as negative control.

8 ml of medium are inoculated with the yeast strains (transformant and control). The growth is carried out in sterile tubes of 10 ml, at 40°C, for one week, without stirring.

The cultures are centrifuged for 5 min at 4000 g and the supernatant tested for the production of L-lactate using a BOEHRINGER kit as indicated above. The degradation of L-malate was studied by assaying the L-malate residual in the culture medium with the aid of a BOEHRINGER kit.

The results obtained are presented in Table III below.

Under the various culture conditions tested, the control yeast Sp/pART1 produces only traces of L-lactate (of the order of 20 to 40 mg/l). Indeed, like *S. cerevisiae*, *S. pombe* is incapable of converting malate to lactate, and, on the other hand, produces only traces of lactate under anaerobic conditions from glucose.

The strain transformed with the malolactic gene (Sp/D42), in the absence of malate, produces very low quantities of L-lactate, of the order of 170 to 300 mg/l. This slight production very probably results from the conversion, by the malolactic enzyme, of a portion of the yeast endogenous malate, metabolised from glucose. This was also observed with the *S. cerevisiae* transformants (Table II). In the presence of 5.5 g/l of L-malate in the culture medium, a very high production of L-lactate is observed for the



transformant Sp/D42, from 2.4 to 3 g/l according to the pH of the culture medium. The highest production is observed at acidic pH, therefore under pH condition [sic] similar to those in oenology. The production
5 obtained at pH 6 is also very high, compared to that obtained for the *S. cerevisiae* transformants at the same pH.

On the other hand, in the presence of 5.5 g/l of L-malate and at acidic pH, the transformant Sp/D42
10 is capable of degrading practically all the L-malate of the medium (at pH 3.5, only 150 mg/l of L-malate were not degraded) and furthermore, of converting more than 80% of the malate present to L-lactate (3.05 g/l).

TABLE III: ASSAY OF LACTATE IN *S. POMBE*

15

Yeast strains	L-lactate produced (g/l)	Residual L-malate (g/l)
Sp/pART1 - in (pH = 3.5) 0% malate 0.55% malate - in (pH = 6) 0% malate 0.55% malate	not detected 0.04 0.02 0.03	1.4 4.6
Sp/D42 - in (pH = 3.5) 0% malate 0.55% malate - in (pH = 6) 0% malate 0.55% malate	0.17 3.05 0.3 2.4	0.15 1.5

20

25

30



SEQUENCE LISTING

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(ii) TITLE OF THE INVENTION: CLONING AND EXPRESSION
OF THE GENE FOR THE MALOLACTIC ENZYME OF
LACTOCOCCUS LACTIS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER-READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible



(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version
#1.25 (EPO)

(vi) DATA FOR THE PRIOR APPLICATION:

(A) FILE NUMBER: FR 93 06003
(B) DATE FILED: 18-MAY-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2684 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Lactococcus lactis

(ix) ADDITIONAL CHARACTERISTIC:

(A) NAME/KEY: CDS
(B) LOCATION: 466..2085
(D) OTHER INFORMATION: /product = "MALOLACTIC
ENZYME"

(ix) ADDITIONAL CHARACTERISTIC:

(A) NAME/KEY: -35_signal
(B) LOCATION: 392..397

(ix) ADDITIONAL CHARACTERISTIC:

(A) NAME/KEY: -10_signal
(B) LOCATION: 416..421

(xi) DESCRIPTION OF THE SEQUENCE: SEQ ID NO: 1:



TTTTGTTGAA AAAATTTCTA ATCAAATTAT TAACCTAAAA GATACATAAA TTTAAAAAAT	60
AAAAGTAGAG TGATTTTACT CTACTTTTTT AGAATACTTT TATATAATAG GAAATATGAA	120
TAAAGCAAAG CGCACAAATTT TGGTTTTATT TAAAAAATG GATACCTTAG ATACACAACC	180
ACCATTGACA AAAAATCTTA ATCCTAAATT GGTGAAACC CTGATAAATT AGGGATAGTA	240
ATAGGAGGAG GACAGTTTAT CATTTAATAG TTATAAGCTA ATTTTACTA CCATTTCTTT	300
GATTAATATC ATCTATTTTT ATATAGAGAC TTTTAAATAA ACATTGACAT TATTTATGCG	360
TTATAAATTA AATTTATCAA CACTAAGGAA TTTGACTATA ACGATAAAAG AAGTTTATAG	420
TAATAAGTA ATAACATTAA TTATAATTTT AATGGAGGTT GTACG ATG CGT GCA	474
Met Arg Ala	
1	
CAT GAA ATT TTA AAC AAT CCT TTT TTA AAT AAA GGA ACA GCT TTT ACT	522
His Glu Ile Leu Asn Asn Pro Phe Leu Asn Lys Gly Thr Ala Phe Thr	
5 10 15	
ATG AAA GAC CGT CAA GAA TTG GGG TTG ATT GGT CTT CTT CCA CCA ACT	570
Met Lys Asp Arg Gln Glu Leu Gly Leu Ile Gly Leu Leu Pro Pro Thr	
20 25 30 35	
GTT CAA ACA ATT GAG GAA CAA GCT GAA CAA ACT TAC GAA CAA TAT TTG	618
Val Gln Thr Ile Glu Glu Gln Ala Glu Gln Thr Tyr Glu Gln Tyr Leu	
40 45 50	
ACA AAA CCA TCT GAT TTA GAA AAA CGT CAT TTC TTG ATG GAA ATT TTT	666
Thr Lys Pro Ser Asp Leu Glu Lys Arg His Phe Leu Met Glu Ile Phe	
55 60 65	
AAT ACA AAC CGT ACT TTG TTT TAC TAC TTA TTC AAC AAA CAT ATT GTA	714
Asn Thr Asn Arg Thr Leu Phe Tyr Tyr Leu Phe Asn Lys His Ile Val	
70 75 80	
GAA TTT AAT CCA GTT GTT TAT GAT CCA ACA ATT GCT GAT ACA ATT GAA	762
Glu Phe Asn Pro Val Val Tyr Asp Pro Thr Ile Ala Asp Thr Ile Glu	
85 90 95	
AAC TAC AGT CAT TTG TTC GTA GAT CCA CAA TAT GCT GCT TAT CTT GAT	810
Asn Tyr Ser His Leu Phe Val Asp Pro Gln Tyr Ala Ala Tyr Leu Asp	
100 105 110 115	
ATT AAC CAC CCT GAA AAC ATT ACT GAA ACA TTG AAA AGT GCA GCA GGT	858
Ile Asn His Pro Glu Asn Ile Thr Glu Thr Leu Lys Ser Ala Ala Gly	
120 125 130	
GAC AGA GAA ATT CGT CCT ATT GTT GTA ACT GAT GCT GAA GGA ACC CTT	906
Asp Arg Glu Ile Arg Pro Ile Val Val Thr Asp Ala Glu Gly Thr Leu	
135 140 145	
GGT ATT GGA GAC TGG GGA ACT CAA GGT GTT GAT ATC TCA GTT GGT AAA	954
Gly Ile Gly Asp Trp Gly Thr Gln Gly Val Asp Ile Ser Val Gly Lys	
150 155 160	
TTA ATG ATT TAT ACA GCC GCA GCA GGT ATT GAT CCA GCG TCT GTA CTT	1002
Leu Met Ile Tyr Thr Ala Ala Ala Gly Ile Asp Pro Ala Ser Val Leu	
165 170 175	
CCA GTT GTT ATT GAT GCA GGG ACA AAT AGG AAA GGA CTT TTA GAA GAT	1050
Pro Val Val Ile Asp Ala Gly Thr Asn Arg Lys Gly Leu Leu Glu Asp	
180 185 190 195	



CAT TTG TAT CTT GGA AAT CAT CAA GAA CGT ATT TAC GGT GAT CAA TAC His Leu Tyr Leu Gly Asn His Gln Glu Arg Ile Tyr Gly Asp Gln Tyr 200 205 210	1098
TAC AGT TTC GTC GAT CAA TTT GTA GAA ACT GCA GAA TCA ATT TTC CCT Tyr Ser Phe Val Asp Gln Phe Val Glu Thr Ala Glu Ser Ile Phe Pro 215 220 225	1146
AAA TTG TAC CTT CAC TGG GAA GAT TTC GGA CGT TCA AAT GCT GCA ACA Lys Leu Tyr Leu His Trp Glu Asp Phe Gly Arg Ser Asn Ala Ala Thr 230 235 240	1194
ATT TTA AAT AAC TAC AAA ACA AAA ATC CCA ACA TTT AAT GAT GAC ATT Ile Leu Asn Asn Tyr Lys Thr Lys Ile Pro Thr Phe Asn Asp Asp Ile 245 250 255	1242
CAA GGA ACT GGT ATT GTT GTT TTA GGT GGT ATT TTC GGA TCA CTT GAC Gln Gly Thr Gly Ile Val Val Leu Gly Gly Ile Phe Gly Ser Leu Asp 260 265 270 275	1290
ATT ACA GGT GAA AAA TTA ACT GAT CAA GTA TAT CTT TGC TAT GGT GGT Ile Thr Gly Glu Lys Leu Thr Asp Gln Val Tyr Leu Cys Tyr Gly Gly 280 285 290	1338
GGT TCA GCC GGT GCA GGG ATT GCT GGT CGT GTT CAT GCT GAA ATG GTT Gly Ser Ala Gly Ala Gly Ile Ala Gly Arg Val His Ala Glu Met Val 295 300 305	1386
AGT GAA GGT CTT TCT GAA GAA GAA GCT TAC AAA CAT TTC TTC ATG ATT Ser Glu Gly Leu Ser Glu Glu Glu Ala Tyr Lys His Phe Phe Met Ile 310 315 320	1434
GAT CAA CAA GGT TTA CTT TTT GAT GAT ATG GAA GAC CTT ACA CCA GCT Asp Gln Gln Gly Leu Leu Phe Asp Asp Met Glu Asp Leu Thr Pro Ala 325 330 335	1482
CAA AAA CCA TTT GCT AAA AAA CGT GCT GAT TAT AAA GAT GCT GGA GAT Gln Lys Pro Phe Ala Lys Lys Arg Ala Asp Tyr Lys Asp Ala Gly Asp 340 345 350 355	1530
ATG ACT GAC CTT CTT AAC GTT GTT AAG ACA GTA AAA CCA ACT ATT TTA Met Thr Asp Leu Leu Asn Val Val Lys Thr Val Lys Pro Thr Ile Leu 360 365 370	1578
GTA GGA ACT TCA ACT AAT CCA GGT GCC TTT ACA AAA GAA GTT GTT GAA Val Gly Thr Ser Thr Asn Pro Gly Ala Phe Thr Lys Glu Val Val Glu 375 380 385	1626
GCA ATG TGT GCT AAT ACA GAA CGC CCA GTA ATC TTC CCT ATC TCA AAT Ala Met Cys Ala Asn Thr Glu Arg Pro Val Ile Phe Pro Ile Ser Asn 390 395 400	1674
CCA ACT AAA AAA ATG GAA ACT ACA GCT GAA CAA GTT ATT GAG TGG TCT Pro Thr Lys Lys Met Glu Thr Thr Ala Glu Gln Val Ile Glu Trp Ser 405 410 415	1722
GAT GGA AAA GCT TTT GTC GCT ACT GGT GTT CCT TCA GGA ACA ATC AGC Asp Gly Lys Ala Phe Val Ala Thr Gly Val Pro Ser Gly Thr Ile Ser 420 425 430 435	1770
TAC AAA GGT GTT GAT TAT CAA ATT GGT CAA GCA AAT AAC TCA CTT ATC Tyr Lys Gly Val Asp Tyr Gln Ile Gly Gln Ala Asn Asn Ser Leu Ile 440 445 450	1818
CAC CCA GGT TTG GGC TTA GGA ATG TTG GCA TCT GAA GCA AAA CTT TTG His Pro Gly Leu Gly Leu Gly Met Leu Ala Ser Glu Ala Lys Leu Leu 455 460 465	1866



ACA GAT GAA ATG ATC GGT GCA GCT GCA CAT TCA TTG AGC GGT TTA GTA Thr Asp Glu Met Ile Gly Ala Ala Ala His Ser Leu Ser Gly Leu Val 470 475 480	1914
GAT CCA GGT AAA CCA GGT GCT CCT GTT CTT CCT CCA TTT GAA TTT GTT Asp Pro Gly Lys Pro Gly Ala Pro Val Leu Pro Pro Phe Glu Phe Val 485 490 495	1962
GCT GAT GTA TCA ATT AAA GTT GCA GAA GCA GTT GCT AAG AAA GCT CAA Ala Asp Val Ser Ile Lys Val Ala Glu Ala Val Ala Lys Lys Ala Gln 500 505 510 515	2010
GAA CAA GGT CTT ACT GAA TCT AAA GAA ACT GAT ATG GCT AAA GCA GTT Glu Gln Gly Leu Thr Glu Ser Lys Glu Thr Asp Met Ala Lys Ala Val 520 525 530	2058
CGT GAT CTT AAA TGG TAT CCA GAG TAC TAAGGGGAAT ATCTTAAATG Arg Asp Leu Lys Trp Tyr Pro Glu Tyr 535 540	2105
AAAAAACTTA AAGAAACGAA AATATCGGGA ATTAGTCTTC CCTTATATGC CTTTTTCGTA	2165
GCTGTCATCA TAGTTGTAAC ACTATTAGGA AAACCTCCAC TTGATATGGT AGGGTTAACT	2225
CTCCTACTTG TAACATTAGG CCACCTATTA TACTTCATAG GAGAAAAATT CCCTATTATG	2285
AATTCATACT TAGGTGGGGG ATCTGTTTTT ACTTTAATTG GTGCTACTCT ATTATCTTTC	2345
TTCCACATTG TTCCTTCAAA TGTTATTGGA GCAGTTTCCA ATTTTATGGG TGGAAAATTT	2405
GGATTTCTTG ATTTTATAT AGCTGCACTT ATCTGTGGAT CTATTTTAGG AATGAACAGA	2465
AATCTTTTGG TTAAAGCTTC CAAGAAATTT ATTCCGATTG CTTTAATCAC TATGGTTATT	2525
GGTTTCTTCT CAGTAGGTCT TGTAGGAATG CTTATTGGTA ATGGATTTGC TGATTCTGTA	2585
ATGTATGTTT CTATGCCAAT GATGTCAGGT GGTATGGGAG CCGGAATTAC TCACTCTCTC	2645
AAATCTATGC AGCCGGATTG GTCATGGAA ACCAAGCAG	2684



(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 540 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) DESCRIPTION OF THE SEQUENCE: SEQ ID NO: 2:

Met	Arg	Ala	His	Glu	Ile	Leu	Asn	Asn	Pro	Phe	Leu	Asn	Lys	Gly	Thr	1	5	10	15
Ala	Phe	Thr	Met	Lys	Asp	Arg	Gln	Glu	Leu	Gly	Leu	Ile	Gly	Leu	Leu	20	25	30	
Pro	Pro	Thr	Val	Gln	Thr	Ile	Glu	Glu	Gln	Ala	Glu	Gln	Thr	Tyr	Glu	35	40	45	
Gln	Tyr	Leu	Thr	Lys	Pro	Ser	Asp	Leu	Glu	Lys	Arg	His	Phe	Leu	Met	50	55	60	
Glu	Ile	Phe	Asn	Thr	Asn	Arg	Thr	Leu	Phe	Tyr	Tyr	Leu	Phe	Asn	Lys	65	70	75	80
His	Ile	Val	Glu	Phe	Asn	Pro	Val	Val	Tyr	Asp	Pro	Thr	Ile	Ala	Asp	85	90	95	
Thr	Ile	Glu	Asn	Tyr	Ser	His	Leu	Phe	Val	Asp	Pro	Gln	Tyr	Ala	Ala	100	105	110	
Tyr	Leu	Asp	Ile	Asn	His	Pro	Glu	Asn	Ile	Thr	Glu	Thr	Leu	Lys	Ser	115	120	125	
Ala	Ala	Gly	Asp	Arg	Glu	Ile	Arg	Pro	Ile	Val	Val	Thr	Asp	Ala	Glu	130	135	140	
Gly	Thr	Leu	Gly	Ile	Gly	Asp	Trp	Gly	Thr	Gln	Gly	Val	Asp	Ile	Ser	145	150	155	160
Val	Gly	Lys	Leu	Met	Ile	Tyr	Thr	Ala	Ala	Ala	Gly	Ile	Asp	Pro	Ala	165	170	175	
Ser	Val	Leu	Pro	Val	Val	Ile	Asp	Ala	Gly	Thr	Asn	Arg	Lys	Gly	Leu	180	185	190	
Leu	Glu	Asp	His	Leu	Tyr	Leu	Gly	Asn	His	Gln	Glu	Arg	Ile	Tyr	Gly	195	200	205	
Asp	Gln	Tyr	Tyr	Ser	Phe	Val	Asp	Gln	Phe	Val	Glu	Thr	Ala	Glu	Ser	210	215	220	
Ile	Phe	Pro	Lys	Leu	Tyr	Leu	His	Trp	Glu	Asp	Phe	Gly	Arg	Ser	Asn	225	230	235	240
Ala	Ala	Thr	Ile	Leu	Asn	Asn	Tyr	Lys	Thr	Lys	Ile	Pro	Thr	Phe	Asn	245	250	255	
Asp	Asp	Ile	Gln	Gly	Thr	Gly	Ile	Val	Val	Leu	Gly	Gly	Ile	Phe	Gly	260	265	270	
Ser	Leu	Asp	Ile	Thr	Gly	Glu	Lys	Leu	Thr	Asp	Gln	Val	Tyr	Leu	Cys	275	280	285	



Tyr Gly Gly Gly Ser Ala Gly Ala Gly Ile Ala Gly Arg Val His Ala
 290 295 300
 Glu Met Val Ser Glu Gly Leu Ser Glu Glu Glu Ala Tyr Lys His Phe
 305 310 315 320
 Phe Met Ile Asp Gln Gln Gly Leu Leu Phe Asp Asp Met Glu Asp Leu
 325 330 335
 Thr Pro Ala Gln Lys Pro Phe Ala Lys Lys Arg Ala Asp Tyr Lys Asp
 340 345 350
 Ala Gly Asp Met Thr Asp Leu Leu Asn Val Val Lys Thr Val Lys Pro
 355 360 365
 Thr Ile Leu Val Gly Thr Ser Thr Asn Pro Gly Ala Phe Thr Lys Glu
 370 375 380
 Val Val Glu Ala Met Cys Ala Asn Thr Glu Arg Pro Val Ile Phe Pro
 385 390 395 400
 Ile Ser Asn Pro Thr Lys Lys Met Glu Thr Thr Ala Glu Gln Val Ile
 405 410 415
 Glu Trp Ser Asp Gly Lys Ala Phe Val Ala Thr Gly Val Pro Ser Gly
 420 425 430
 Thr Ile Ser Tyr Lys Gly Val Asp Tyr Gln Ile Gly Gln Ala Asn Asn
 435 440 445
 Ser Leu Ile His Pro Gly Leu Gly Leu Gly Met Leu Ala Ser Glu Ala
 450 455 460
 Lys Leu Leu Thr Asp Glu Met Ile Gly Ala Ala Ala His Ser Leu Ser
 465 470 475 480
 Gly Leu Val Asp Pro Gly Lys Pro Gly Ala Pro Val Leu Pro Pro Phe
 485 490 495
 Glu Phe Val Ala Asp Val Ser Ile Lys Val Ala Glu Ala Val Ala Lys
 500 505 510
 Lys Ala Gln Glu Gln Gly Leu Thr Glu Ser Lys Glu Thr Asp Met Ala
 515 520 525
 Lys Ala Val Arg Asp Leu Lys Trp Tyr Pro Glu Tyr
 530 535 540



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated nucleic acid fragment, characterized in that it comprises a sequence of 1620 bp encoding the malolactic enzyme of *Lactococcus lactis*.
2. The nucleic acid fragment according to claim 1, characterized in that it comprises a sequence encoding the polypeptide SEQ. ID. NO 2.
3. The nucleic acid fragment according to claim 2, characterized in that it comprises the nucleotides 466 to 2085 of the sequence SEQ. ID. NO 1.
4. An expression cassette, characterized in that it comprises at least one fragment according to any one of claims 1 to 3, placed under the control of sequences for regulation of the expression of a gene.
5. The expression cassette according to claim 4, characterized in that the said regulatory sequences are active in yeast.
6. A recombinant vector characterized in that it comprises at least one fragment according to any one of claims 1 to 3.
7. A recombinant vector according to claim 6, characterized in that it comprises an expression cassette according to any one of claims 4 or 5.
8. A transformed eukaryotic or prokaryotic cell, characterized in that it contains at least one expression cassette according to any one of claims 4 or 5.
9. The transformed cell according to claim 8,



characterized in that it is a yeast cell.

10. A yeast cell according to claim 9, characterized in that the said yeast belongs to the genus *Saccharomyces*.

11. The yeast cell according to claim 9, characterized in that the said yeast belongs to the genus *Schizosaccharomyces*.

12. A process of malolactic fermentation comprising fermenting at least one yeast cell according to any one of claims 9 to 11 under suitable conditions.

DATED THIS 21ST DAY OF FEBRUARY 1997

INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA)

By Its Patent Attorneys:

GRIFFITH HACK

Fellows Institute of Patent
Attorneys of Australia



FIGURE 1

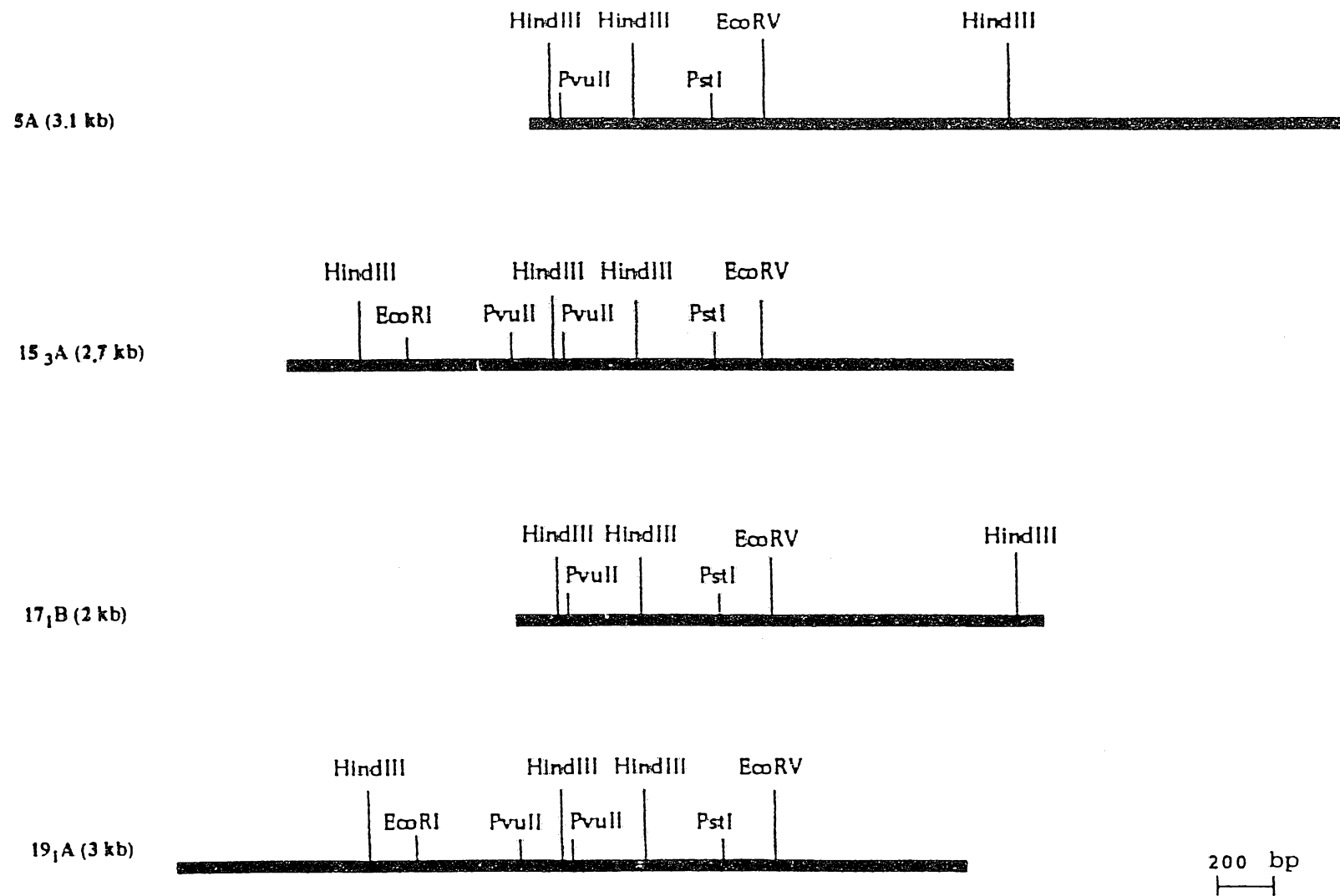


FIGURE 2

Seq L. lactis 5' ATGCGTGCACATGA 3'

primer 1 5' GGCTCGAGATGCGTGACATGA 3'

primer 12 5' TCGGATCCATGCGTGACATGA 3'
BamHI

Seq. Lactis 5' GAGTACTAA GGGGAATATCT 3'

primer 3 3' ATGATTCCCCTTATAGATCTGT 5'

XbaI

primer 13 3' CTCATGATTCCCCTCGAGTC 5'

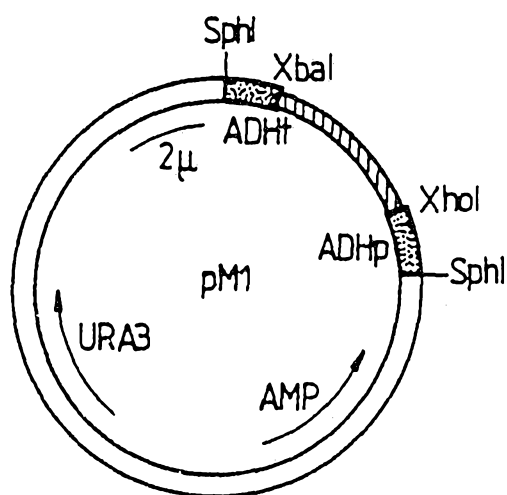


FIG. 3

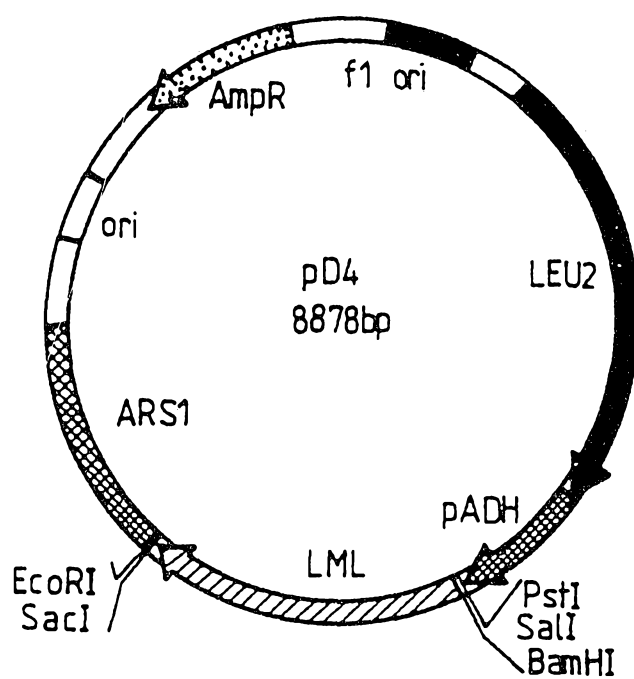


FIG.4

RAPPORT DE RECHERCHE INTERNATIONALE

Demande Internationale No.

PCT/FR 94/00589

A. CLASSEMENT DE L'OBJET DE LA DEMANDE

CIB 5 C12N9/04 C12N15/53 C12G1/02 C12N1/18

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 5 C12N

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
Y	EP,A,0 103 399 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 Mars 1984 voir le document en entier ---	1-12
Y	BIOTECHNOLOGY ABSTRACTS, London, GB, Ab.No. 92-03363 Renault P. 'Malolactic fermentation:...' cité dans la demande & GIM 90 (Pt.II, 905-16) 1990 ---	1-12
P,X	BIOSIS PREVIEWS DATABASE, Philadelphia, US Ab.No.97004447 Ansanay V. et al. 'Cloning, sequence and...' & FEBS Letters 332 (1-2).1993. 74-80. --- -/--	1-12

☒ Voir la suite du cadre C pour la fin de la liste des documents☒ Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités

- *A* document définissant l'état général de la technique, non considéré comme particulièrement pertinent
- *E* document antérieur, mais publié à la date de dépôt international ou après cette date
- *L* document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)
- *O* document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens
- *P* document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

- *T* document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention
- *X* document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément
- *Y* document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier
- *Z* document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

25 Août 1994

Date d'expédition du présent rapport de recherche internationale

27. 10. 94

Nom et adresse postale de l'administration chargée de la recherche internationale

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Fonctionnaire autorisé

Gurdjian, D

RAPPORT DE RECHERCHE INTERNATIONALE

Demande Internationale No.

PCT/FR 94/00589

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	BIOSIS PREVIEWS DATABASE, Philadelphia, US Ab.No. 83085283 RENAULT P P et al. 'SELECTION OF STREPTOCOCCUS...' & APPL ENVIRON MICROBIOL 53 (2). 1987. 320-324. -----	1-8

Renseignements relatifs aux membres de familles de brevets

PCT/FR 94/00589

Formulaire PCT/ISA/210 (nouveau families de brevets) (juillet 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR 94/00589

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N9/04 C12N15/53 C12G1/02 C12N1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 103 399 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 March 1984 see the whole document ---	1-12
Y	BIOTECHNOLOGY ABSTRACTS, London, GB, Ab.No. 92-03363 Renault P. 'Malolactic fermentation:....' cited in the application & GIM 90 (Pt.II, 905-16) 1990 ---	1-12
P, X	BIOSIS PREVIEWS DATABASE, Philadelphia, US Ab.No.97004447 Ansanay V. et al. 'Cloning, sequence and...' & FEBS Letters 332 (1-2).1993. 74-80. --- -/--	1-12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search

25 August 1994

Date of mailing of the international search report

27. 10. 94

Name and mailing address of the ISA

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Authorized officer

Gurdjian, D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR 94/00589

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOSIS PREVIEWS DATABASE, Philadelphia, US Ab.No.83085283 RENAULT P P et al. 'SELECTION OF STREPTOCOCCUS...' & APPL ENVIRON MICROBIOL 53 (2). 1987. 320-324. -----	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/FR 94/00589

Parent document cited in search report	Publication date	Patent family member(s)	Publication date
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EP-A-0103399

21-03-84

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18-09-84