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(54) Titre : BACTERIE RECOMBINANTE RESISTANT AUX BACTERIOPHAGES

(54) Title: BACTERIOPHAGE RESISTANT RECOMBINANT BACTERIA

(57) **Abrégé/Abstract:**

Recombinant bacteria containing phage-encoded resistance ("Per") and methods of making and using the same are disclosed. Such bacteria are made by (a) conducting a fermentation of a substrate in a medium containing a defined bacterial culture until bacteriophage are detected in the medium, the bacteriophage being specific to at least one bacteria in the medium; (b) isolating the bacteriophage; (c) digesting DNA of the bacteriophage to produce a library of DNA fragments; (d) transforming the bacteria susceptible to said bacteriophage with the library of DNA fragments to provide transformed bacteria; (e) selecting from among the transformed bacteria, a bacteriophage-resistant transformed bacteria; (f) adding bacteriophage resistant transformed bacteria to the medium; and (g) recommencing step (a). Also disclosed are bacterial cells which contain a first bacteriophage defense mechanism (Per), wherein Per comprises a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage. The bacterial cell is capable of being infected by a bacteriophage, the DNA of which, once injected into the bacterial cell, competes with Per for binding to DNA polymerase.

BACTERIOPHAGE RESISTANT RECOMBINANT BACTERIA

Abstract

Recombinant bacteria containing phage-encoded resistance ("Per") and methods of making and using the same are disclosed. Such bacteria are made by (a) conducting a fermentation of a substrate in a medium containing a defined bacterial culture until bacteriophage are detected in the medium, the bacteriophage being specific to at least one bacteria in the medium; (b) isolating the bacteriophage; (c) digesting DNA of the bacteriophage to produce a library of DNA fragments; (d) transforming the bacteria susceptible to said bacteriophage with the library of DNA fragments to provide transformed bacteria; (e) selecting from among the transformed bacteria, a bacteriophage-resistant transformed bacteria; (f) adding bacteriophage resistant transformed bacteria to the medium; and (g) recommencing step (a).

Also disclosed are bacterial cells which contain a first bacteriophage defense mechanism (Per), wherein Per comprises a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage. The bacterial cell is capable of being infected by a bacteriophage, the DNA of which, once injected into the bacterial cell, competes with Per for binding to DNA polymerase.

BACTERIOPHAGE RESISTANT RECOMBINANT BACTERIA

Field of the Invention

The present invention relates to bacteriophage resistant recombinant bacteria and their use in fermentation.

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Background of the Invention

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Production of cheese and cultured dairy products has long relied on the fermentation of milk by lactic acid-producing bacteria including species of *Lactococcus*. Since efficient fermentations are dependent on the growth and activity of these bacteria, great care is exercised to prepare starter cultures that are highly active and uncontaminated with undesirable microorganisms or bacteriophages. However, the fermentation process itself is nonaseptic, occurring in open vats with a nonsterile medium, e.g., pasteurized milk. It is, therefore, highly susceptible to contamination with bacteriophages. For the majority of strains of lactic acid bacteria employed in commercial dairy fermentations, lytic bacteriophages capable of halting growth and acid production can appear within one to two days after introducing the bacterial culture into the dairy plant.

Milk fermentations historically have relied on starter cultures composed of undefined mixtures of

lactic acid bacteria propagated without knowledge of,
or protection from, bacteriophages. Natural
bacteriophage contamination in these cultures
established an equilibrium of evolving bacteriophages
and bacteriophage-resistant variants. These cultures
were highly variable in day-to-day levels of acid
production, but remained moderately active and could be
used continuously in small fermentation factories.
Over the past 20 years, starter culture failures due to
bacteriophage infection have become prevalent
throughout the dairy industry. Increasing demand for
cultured milk products in recent years has necessitated
increases in both production capacity and process
efficiency such that larger volumes of milk are
processed, cheese vats are filled repeatedly within a
single day, and total processing time is shortened.
This modernization of the industry concurrently
increased the probability of bacteriophage
contamination and further dictated the use of defined
mixtures of lactic acid bacteria capable of uniform and
rapid rates of acid production. With the selection of
highly fermentative lactic acid bacteria and their
propagation under aseptic conditions, i.e., in the
absence of bacteriophages, the majority of cultures now
used by the industry are susceptible to bacteriophage
attack upon introduction into the cheese factory.

To cope with bacteriophage problems, a number
of methods have been developed to minimize
bacteriophage action during various fermentation
processes, particularly dairy fermentations.

Bacteriophage resistance in various lactic
acid bacteria is recognized generally as a plasmid-
encoded phenomenon. For example, U.S. 4,732,859 to
Hershberger et al. relates to a method of protecting
various genera of bacteria from naturally occurring
bacteriophage by providing host bacterial cells with a
restriction system that digests *Hha*II site-containing

foreign DNA, found in most naturally occurring phages. As bacteriophage DNA enters a host cell, the *HhaII* restriction endonuclease digests the DNA at *HhaII* sites and renders the bacteriophage non-functional and harmless. In more general terms, the invention involves transforming a bacterium with a recombinant DNA cloning vector which comprises a replicon that is functional in the bacterium, and a gene that expresses a functional protein (i.e., a restriction endonuclease which confers restriction activity to the bacterium).

U.S. 4,918,014 and 4,874,616, both to Vedamuthu, are directed to a method of imparting bacteriophage resistance to bacteriophage sensitive strands of *Streptococcus* group N, whereby a plasmid encoding the production of a mucoid substance is transferred via a plasmid into a bacteriophage sensitive strain. The lactic *Streptococci*, or "*Lactococci*," are said to be rendered bacteriophage resistant, and purportedly more stable for use in milk fermentation.

Bacteriophage resistance in *Lactococci* also has been found generally to be a plasmid-encoded phenomenon. Plasmids have been described which direct resistance via inhibition of adsorption, restriction and modification ("R/M"), and by aborting the bacteriophage infection ("Hsp"). Strategies that will be useful for the construction of bacteriophage-insensitive strains include the introduction of one or more resistance mechanisms within a single host, or introducing a single plasmid containing more than one resistance mechanism. For recent reviews, see Klaenhammer, T.R., *FEMS Microbiol. Rev.* 46:313-325 (1987) and Sanders, *Biochimie* 70: 411-422 (1988). A prerequisite for the success of these strategies is that the resistance mechanisms should work in combination to prevent bacteriophage proliferation. The insensitivity of any constructed, or natural,

isolate is probably a function of time, amount of use, and environmental conditions (Klaenhammer, 1984, Lawrence and Thomas, 1979). The presence of single plasmids encoding multiple resistances, or combinations of plasmids within a single strain, can also confer prolonged resistance phenotypes upon lactococcal strains. Inevitably, however, defense mechanisms used for long periods have succumbed to an evolving bacteriophage population. This has occurred, for example, in the case of pTR2030 transconjugants of the industrial strain of *L. lactis*, NCK202. pTR2030 is a conjugative plasmid which confers resistance to bacteriophage via Hsp and R/M. Bacteriophages recovered from the industry after prolonged use of NCK202 have overcome either one or both of these mechanisms.

The search for novel resistance determinants to add to the growing arsenal of available, independent genotypes has continued. In particular, bacteriophage defense mechanisms active against those that are insensitive to Hsp would provide a valuable adjunct to existing resistance genotypes in bacteria, particularly lactic acid bacteria. Most avenues of bacteriophage replication could be blocked to minimize the adaptation and proliferation of new bacteriophages, which in turn would increase the longevity of fermentation bacterial cultures.

Summary of the Invention

In accordance with the above, the present inventors have identified a heretofore unknown resistance mechanism of bacteria to bacteriophage, termed Phage encoded resistance, or "Per." Per is unique among bacterial genotypes since it is derived from the genome of the bacteriophage itself.

More specifically, a first aspect of the present invention comprises:

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(a) conducting a fermentation of a substrate in a medium containing a defined bacterial culture until bacteriophage are detected in the medium, the bacteriophage being specific to at least one bacteria in the medium;

(b) isolating the bacteriophage;

(c) digesting DNA of the bacteriophage to produce a library of DNA fragments;

(d) transforming the bacteria susceptible to said bacteriophage with the library of DNA fragments to provide transformed bacteria;

(e) selecting from among the transformed bacteria, a bacteriophage-resistant transformed bacteria;

(f) adding bacteriophage resistant transformed bacteria to the medium; and

(g) recommencing step (a).

A second aspect of the present invention comprises a bacterial cell which contains a first bacteriophage defense mechanism (Per), wherein Per comprises a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage. The bacterial cell is capable of being infected by a bacteriophage, the DNA of which, once injected into the bacterial cell, competes with Per for binding to DNA polymerase.

A third aspect of the present invention comprises a defined bacterial starter culture capable of fermenting a product, comprising a plurality of bacterial cells which contain a first bacteriophage defense mechanism (Per). Per comprises a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage. The bacterial cells are capable of being infected by a bacteriophage, the DNA of which, once injected into the bacterial cell, competes with Per for binding to DNA polymerase.

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A fourth aspect of the present invention comprises a method of conducting a fermentation of a product, which comprises providing to a fermentation medium, a defined bacterial starter culture comprising a plurality of
5 bacterial cells which contain a first bacteriophage defense mechanism (Per) comprising a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein the DNA of the bacteriophage, once injected into
10 the bacterial cell, competes with Per for binding to DNA polymerase.

A fifth aspect of the present invention involves a recombinant DNA vector comprising a bacteriophage origin of replication (ori) operatively associated with a DNA
15 sequence incapable of producing live bacteriophage.

In accordance with one embodiment of the present invention, there is provided a method of imparting bacteriophage resistance to lactococcal bacteria contained in a fermentative culture medium, comprising:

- 20 (a) conducting a fermentation of a milk substrate in a medium containing a defined bacterial culture including at least one lactococcal bacteria until bacteriophage are detected in the medium, said bacteriophage being specific to at least one
25 lactococcal bacteria in said medium;
- (b) isolating said bacteriophage;
- (c) digesting DNA of the bacteriophage to produce a library of DNA fragments;
- (d) transforming said one lactococcal bacteria
30 susceptible to said bacteriophage with said library of DNA fragments to provide transformed lactococcal bacteria;
- (e) selecting from among the transformed lactococcal bacteria a bacteriophage-resistant recombinant
35 lactococcal bacteria;

-6a-

(f) adding a predetermined amount of said bacteriophage-resistant recombinant lactococcal bacteria to the medium; and
(g) commencing fermentation of said substrate in said medium with said bacteriophage-resistant recombinant lactococcal bacteria.

In accordance with another embodiment of the present invention, there is provided a lactococcal bacterial cell containing a first bacteriophage defense mechanism, said first mechanism comprising a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein said lactococcal bacterial cell is capable of being infected by a bacteriophage, and wherein the DNA of said bacteriophage, once injected into said bacterial cell, competes with said first defense mechanism for binding to DNA polymerase.

In accordance with another embodiment of the present invention, there is provided a defined lactococcal bacterial starter culture capable of fermenting a predetermined product, comprising: a plurality of lactococcal bacterial cells, each containing a first bacteriophage defense mechanism, said bacteriophage defense mechanism comprising a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein said lactococcal bacterial cells are capable of being infected by a bacteriophage, and wherein the DNA of said bacteriophage, once injected into said bacterial cell, competes with said bacteriophage defense mechanism for binding to DNA polymerase.

In accordance with another embodiment of the present invention, there is provided a method of conducting a fermentation of a milk substrate to produce a dairy product, comprising:

providing to a fermentation medium, a defined

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lactococcal bacterial starter culture capable of fermenting a predetermined product, comprising: a plurality of lactococcal bacterial cells, each containing a first bacteriophage defense mechanism comprising a
5 bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein the DNA of said bacteriophage, once injected into said bacterial cell, competes with ori for binding to DNA polymerase; and
10 conducting the fermentation under conditions suitable to produce the product.

In accordance with another embodiment of the present invention, there is provided a recombinant DNA vector comprising a lactococcal bacteriophage origin of
15 replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, and wherein said vector is plasmid PTRK104.

In accordance with another embodiment of the present invention, there is provided a method of imparting
20 resistance to bacteriophage nck202.50 to lactococcal bacteria contained in a fermentative culture medium, comprising:

- (a) conducting a fermentation of a milk substrate in a medium containing a defined bacterial culture
25 including at least one lactococcal bacteria until bacteriophage nck202.50 is detected in the medium;
- (b) isolating said bacteriophage;
- (c) digesting DNA of the bacteriophage to produce a library of DNA fragments;
- 30 (d) transforming said one lactococcal bacteria susceptible to said bacteriophage with said library of DNA fragments to provide transformed lactococcal bacteria by ligating said DNA fragments to a replicating plasmid vector and transforming said one
35 bacteria susceptible to said bacteria with said vector;

-6c-

(e) selecting from among the transformed lactococcal bacteria a bacteriophage-resistant recombinant lactococcal bacteria resistant to bacteriophage nck202.50;

- 5 (f) adding a predetermined amount of said bacteriophage-resistant recombinant lactococcal bacteria to the medium; and
- (g) commencing fermentation of said substrate in said medium with said bacteriophage-resistant recombinant lactococcal bacteria.
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These aspects are more completely described hereinbelow. In addition, other aspects of the present invention not explicitly set forth herein will become apparent to those skilled in the art.

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Brief Description of the Drawings

Figures 1A and 1B show the restriction endonuclease maps of Phage nck202.50 (ϕ 50), wherein t.r. means terminal redundancy, and *pac* represents a packaging site.

20 The bacteriophage genome is represented as a circular molecule. Only two of the 13 HindIII sites are shown. The arrow denotes the direction of packaging. In **Fig. 1B**, the shaded box represents the 4.5-kb BamHI-HindIII bacteriophage fragment cloned in pTRK103 (Per+). **Figure**

25 **1C** shows the 1.4-kb EcoRV-HindIII fragment thereof.

Figure 2A depicts the physical map of the 1.4-kb insert in pTRK104. The positions of the ORF and direct repeats are indicated. **Figure 2B** shows the sequencing strategy for the pTRX104 insert. Only the repeated

30 sequence was determined from both strands.

Figure 3 shows the nucleotide sequence of the pTRK104 insert and deduced amino acid sequence of the open reading frame (ORF). The 236-bp direct repeat is indicated by the bold arrows. The 10-bp direct repeats are indicated by the thin arrows. The positions of both HindIII sites are overlined. M13 flanking sequences are boxed. These sequences have been deposited in GenBank under accession number M35639.

Figure 4 shows the large direct repeats having contained therein inverted repeats shown as hairpin loops. The positions of the inverted repeats are also indicated.

Figure 5A shows effect of lytic bacteriophage ϕ 50 on the growth of NCK215 (pSA3) (open boxes) and NCK214 (pTRK104) (filled boxes). Figure 5B shows the effect of lytic bacteriophages ϕ 31 and ϕ 50, together, on the growth of NCK214 (filled boxes), NCK204 (pTR2030) (open boxes), and NCK298 (pTR2030, pTRK104) (filled triangles).

Detailed Description of the Invention

Many bacteriophage genomes contain a unique origin of replication, hereinafter generically referred to as "ori." These regions typically contain special features such as binding sites for specific initiator proteins and adjacent stretches of AT-rich regions which presumably aid in the unwinding of DNA. See *The Bacteriophages*, (R. Calendar Ed. 1988) (Plenum Press), Volume 2 Chapter 3, pp. 146 et seq. The duplication of bacteriophage DNA begins at ori primed by RNA. The RNA primer is synthesized by either a host- or a bacteriophage-coded RNA polymerase or a primase enzyme, and is elongated into DNA by a host- or bacteriophage-coded DNA polymerase. *Id.* While the present inventors do not wish to be bound to any specific theory upon which the instantly disclosed invention operates, it appears that the presence of a given bacteriophage ori on a multi-copy factor such as a plasmid contained in a

bacterial cell interferes with the normal lytic process by titrating DNA polymerase and diverting bacteriophage replication towards the plasmid. In other words, *ori* presents a false target for DNA polymerase, which results in the uncontrolled replication of the plasmid DNA.

In accordance with the foregoing, a method for imparting bacteriophage resistance to fermentative bacteria capable of being infected by a bacteriophage has been devised. The first step in the method involves commencing a fermentation of a substrate in a medium containing a defined bacteria culture, and continuing fermentation until bacteriophage specific to at least one bacterial species in the medium are detected. By the term "specific," it is meant that the bacteriophage are capable of infecting the bacterial species, that is, the process whereby bacteriophage bind to the outer cell membrane of the bacterial cell and inject their genetic material into the bacteria, whereafter the bacteriophage genetic material duplicates, progeny bacteriophage produced, the bacterial cell lysed and the progeny bacteriophage released into the medium. Other bacterial cells are then infected by the progeny bacteriophage. Once the bacteriophage has been identified, it is isolated, and the genetic material, i.e., DNA, of the bacteriophage is digested and a library of DNA fragments is produced. The production of a DNA library using the genetic material of a given microorganism is a known technique, and is well documented in the prior art. See generally T. Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory 1982). Thereafter, the bacteria susceptible to the phage are transformed with the library of DNA fragments, also in accordance with known techniques *supra*, to provide recombinant bacteria.

When the library of DNA fragments is made, they must be joined to heterologous DNA which is incapable of coding for the phage from which the fragments are derived. In a preferred embodiment of the present invention, the DNA library is made by first inserting the fragments of the library into a plasmid vector, transforming the bacteria susceptible to the bacteriophage with the plasmid vector, and then propagating the transformed bacteria. However, other ways of cloning the DNA library are known in the art and will become apparent to those skilled therein without undue experimentation. For example, the library can be constructed in any host, such as *E. coli* or *Bacillus*, and transferred to the susceptible bacteria with an appropriate shuttle vector.

Bacteriophage resistant bacteria are selected from among the transformed bacteria by standard screening procedures, and then added to the fermentation medium. Fermentation is recommenced and continued until other bacteriophage specific to a given bacterial species are detected. This might occur, for example, if the culture medium contained two or more bacterial species, i.e., a mixed culture.

The art of fermentation is well known and the instant method is operable in a wide variety of fermentation processes. In a preferred embodiment according to the present invention, the instant method relates to bacteria capable of fermenting food substrates, and more particularly milk.

Bacteria capable of fermenting foods include those bacteria used in any type of food fermentation, including, but not limited to, the fermentation of milk, egg, meat, fruit, vegetables, and cereals. See generally Food Biotechnology, (D. Knorr Ed. 1987) (Marcel Dekker, Inc.); Fermented Foods (A. Rose Ed. 1982) (Academic Press); C. Pederson, Microbiology of Fermented Foods, (2d ed. 1979) (AVI Publishing Co.).

Bacteria used for the fermentation of meat (including beef, pork, and poultry) include, but are not limited to, lactic acid bacteria, *Pediococcus cerevisiae*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Micrococcus* species, *Leuconostoc citrovorum*, *Leuconostoc citrovorum*, and mixtures thereof. See Food Biotechnology, 538-39 (D. Knorr Ed. 1987); C. Pederson, Microbiology of Fermented Foods, 210-34 (2d ed. 1979); U.S. Patent No. 2,225,783 to Jensen and Paddock.

Bacteria used for the fermentation of vegetables (e.g., carrots, cucumbers, tomatoes, peppers, and cabbage) include, but are not limited to, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, and mixtures thereof. See Food Biotechnology, 540 (D. Knorr Ed. 1987); C. Pederson, Microbiology of Fermented Foods, 153-209 (2d ed. 1979); U.S. Patent No. 3,024,116 to Engelland; U.S. Patent No. 3,403,032 to Etchells et al.; U.S. Patent No. 3,932,674 to Etchells et al.; U.S. Patent No. 3,897,307 to Porubcan et al.

Bacteria used in the fermentation of dough formed from cereals (e.g., wheat, rye, rice, oats, barley, and corn) include yeasts such as *Saccharomyces cerevisiae* and *Candida utilis*; and lactic acid bacteria of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*, including, but not limited to *Lactobacillus delbrueckii*, *Lactobacillus debreuckii* subsp. *leichmanni*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus fermenti*, *Lactobacillus pastorianus*, *Lactobacillus buchneri*, and *Leuconostoc mesenteroides*. See generally Food Biotechnology, 235-70 (D. Knorr Ed. 1987); U.S. Patent No. 3,734,743 to Kline and Sugihara; U.S. Patent No. 3,681,083 to Everson; U.S. Patent No. 3,993,783 to Khoudokormoff and Langejan; U.S. Patent No. 3,843,800 to Langejan; U.S. Patent No. 3,410,692 to Wutzel.

Wine is produced by the fermentation of fruit juice, typically grape juice, with yeasts, such as *Saccharomyces cerevisiae* and *Saccharomyces ellipsoideus*, as well as with a broad variety of lactic acid bacteria including *Pediococcus pentosaceus*,
5 *Lactobacillus plantarum*, *Leuconostoc mesenteroides*,
Leuconostoc dextranicum, *Leuconostoc cremoris*,
Lactobacillus brevis, and *Lactobacillus fermenti*. Beer
is produced by the fermentation of malt with yeasts
10 such as *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*. See C. Pederson, Microbiology of Fermented Foods, 271-309 (2d ed. 1979).

Milk is fermented to produce products such as cheese, yoghurt, kefir, and acidophilus milk. Cheese
15 fermentation bacteria are discussed separately below. Otherwise, bacteria used for the fermentation of milk include, but are not limited to, *Lactobacillus debreuckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Streptococcus salivarius* subsp.
20 *thermophilus*, and mixtures thereof. See Food Biotechnology, 530 (D. Knorr Ed. 1987); C. Pederson, Microbiology of Fermented Foods, 105-35 (2d ed. 1979).

Bacteria used for the fermentation of milk to produce cheese include, but are not limited to,
25 *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, and mixtures thereof. See Food
30 Biotechnology, 530 (D. Knorr Ed. 1987); C. Pederson, Microbiology of Fermented Foods, 135-51 (2d ed. 1979).

Bacteria used for the fermentation of egg include *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and mixtures thereof. See Food
35 Biotechnology, 538-39 (D. Knorr Ed. 1987).

In a particularly preferred embodiment, the present invention is employed for the fermentation of

milk with the lactococci (previously classified as the group N Streptococci), such as *Lactococcus lactis* subsp. *lactis*, (also called *Lactococcus lactis*),
5 *Lactococcus lactis* subsp. *cremoris* (also called *Lactococcus cremoris*), and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (also called *Lactococcus diacetylactis*).

Starter cultures employed in practicing the present invention may be in any physical form,
10 including liquid cultures of the fermentation bacteria in a suitable growth medium, as well as lyophilized cultures and frozen cultures prepared therefrom.

Starter cultures employed in the present invention are preferably defined cultures, that is,
15 cultures of known bacterial content. Such defined cultures may be either single strain cultures, i.e., pure cultures, or multiple strain cultures, i.e., mixed cultures.

While the present invention is, in a preferred embodiment, directed to the fermentation of food, the invention may be practiced with any process involving fermentative bacteria susceptible to disruption by bacteriophage infection, including but not limited to processes for the production of
20 antibiotics, amino acids, and solvents. Products produced by fermentation which are known to have encountered bacteriophage infection, and the corresponding infected fermentation bacteria, include Cheddar and cottage cheese (*Lactococcus lactis*,
25 *Lactococcus cremoris*), Yogurt (*Lactobacillus bulgaricus*, *Streptococcus thermophilus*), Swiss cheese (*S. thermophilus*, *Lactobacillus lactis*, *Lactobacillus helveticus*), Blue cheese (*Leuconostoc cremoris*), Italian cheese (*L. bulgaricus*, *S. thermophilus*), Viili
30 (*Lactococcus cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc cremoris*), Yakult (*Lactobacillus casei*), casein (*Lactococcus cremoris*),
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Natto (*Bacillus subtilis* var. *natto*), Wine (*Leuconostoc oenos*), Sake (*Leuconostoc mesenteroides*), Polymyxin (*Bacillus polymyxa*), Colistin (*Bacillus colistrium*), Bacitracin (*Bacillus licheniformis*), L-Glutamic acid (*Brevibacterium lactofermentum*, *Microbacterium ammoniaphilum*), and acetone and butanol (*Colstridium acetobutylicum*, *Clostridium saccharoperbutylacetonicum*). See generally M. Sanders, Bacteriophages of Industrial Importance, in PHAGE ECOLOGY, 211-44 (S. Goyal, C. Berba and G. Bitton eds. 1987). Thus, the present invention may, for example, be employed in a fermentation process for producing any of the foregoing products with the foregoing bacteria in the manner described herein.

A second aspect of the present invention relates to a bacteriophage resistant, recombinant bacterial cell which contains phage encoded resistance (Per). The mechanism comprises a bacteriophage origin of replication (*ori*) operatively associated with a DNA sequence incapable of producing live bacteriophage. The bacteriophage *ori* incorporated into the bacterial cell ultimately competes with the bacteriophage *ori* contained on intact bacteriophage DNA genome injected into the bacteria upon infection for binding to DNA polymerase.

Once the bacteriophage that is capable of infecting a given bacterial cell is identified and isolated, the bacteriophage *ori* can be isolated and then cloned into the susceptible bacteria, thereby rendering the bacteria resistant to the bacteriophage. The bacteriophage *ori* can be identified and isolated in accordance with the method described above, or alternatively, from known bacteriophage DNA libraries prepared with plasmid or phage vectors.

Any bacterial species that is susceptible to infection by a given bacteriophage can be manipulated genetically in accordance with the instant invention to

produce bacteriophage resistant recombinant bacteria. Preferred bacteria include those that are capable of fermenting a food substrate, more preferably milk. In another preferred embodiment, the bacterial cell is a lactic acid bacterium; more preferred is a bacteria selected from the lactococci.

In another embodiment of the present invention, Per is carried by an extrachromosomal plasmid. The most preferred embodiment in this respect is *Lactococcus lactis* subsp. *lactis* NCK298, which has been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 USA, in accordance with the provisions of the Budapest Treaty, on August 10, 1990, and given ATCC Accession Number 68379. NCK298 contains plasmids pTRK104 and pTRK2030 (the latter conjugally introduced). pTRK104, the preparation of which is described in the Examples set forth below, is an *E. coli* plasmid containing a 1.4-kb region isolated from the genome of bacteriophage nck202.50 (ϕ 50). The genome map of ϕ 50 is shown in Fig. 1A. A physical map of the 1.4-kb insert, which contains Per, is illustrated in Figure 2A. Figure 2B illustrates the sequencing strategy for the pTRK104 insert. The 1.4-kb insert contains the ori of ϕ 50, which was found to comprise a 236-bp sequence illustrated in Fig. 3. The sequence and analysis of the insert are described more fully in Example 4 below.

In addition to encoding resistance to bacteriophage ϕ 50 from which it originated, Per is also active against another, genetically distinct bacteriophage (ϕ 48), which is also unaffected by Hsp. Both Per-sensitive bacteriophages were isolated on pTR2030-containing transconjugants and were found to be resistant to the Hsp mechanism encoded by pTR2030. In contrast, Hsp-sensitive bacteriophages, ϕ 31, ϕ 35, ϕ 36, were not affected by Per. Plaquing efficiencies and infective centers for these phages remained at 1.0 on

hosts bearing pTRK104. In the case of phages $\phi 48$ and $\phi 50$, adsorption was not affected, but the numbers of infective centers formed on pTRK104 bearing cells was reduced significantly.

5 The evidence set forth herein strongly
suggests that the Per region is the origin of
replication for bacteriophage $\phi 50$. First, since the
region involved in Per does not direct the expression
of a protein, it is probable that the resistance
10 encoded by Per reflects the titration of essential
bacteriophage regulatory signals by plasmids containing
this region. Second, the Per region can be shown to
have a negative impact on the replication of $\phi 50$ DNA,
whereas there is no effect on the replication of an
15 insensitive bacteriophage, $\phi 31$. Third, the presence of
the Per region on the plasmid pTRK139 leads to an
increase in its replication rate after infection with
 $\phi 50$, though not after infection with $\phi 31$. Finally, the
Per region itself bears the hallmarks associated with
20 bacteriophage replication origins, repeated and
inverted repeats and terminator-like structures. See
Bramhill, D. and Kornberg, A. Cell 54: 915-918 (1988);
Mosig, G., Am. Soc. Microbiol. A.S.M. (1983); Furth,
M.E. and Wickner, S.H., Lambda II, Cold Spring Harbor
25 Publications (1983).

As stated above, a successful bacteriophage
infection relies upon an interaction between a
bacteriophage or host-encoded DNA polymerase and the
bacteriophage ori. The presence of the ori on a
30 multicopy plasmid would be expected to interfere with
the normal lytic process by titrating DNA polymerase
and diverting replication towards the plasmid. This is
expected to result in the uncontrolled replication of
plasmid DNA. This theory is consistent with the
35 finding that Per of $\phi 50$ is effective only against
bacteriophage with which it shares homology ($\phi 48$) in
the region believed to encode the phage origin of

replication. Those bacteriophages unaffected by the specific Per sequence disclosed herein differ in that they must utilize an alternative *ori* sequence and are therefore not susceptible to any titration effects on the bacteriophage-directed DNA polymerase.

The Per phenotype is not a generalized feature of cloned bacteriophage DNA. A randomly selected bacteriophage fragment did not affect the proliferation of $\phi 50$, despite a larger region of homology (2.5-kb) to the bacteriophage than that possessed by pTRK104 (1.4-kb) or its derivatives. The possibility of homologous recombination and concomitant disruption of bacteriophage sequences as a cause of Per activity was accordingly ruled out.

Given the similarity of the Per and Hsp phenotypes when both are cloned in the vector pSA3, it appears that both affect similar points in the bacteriophage lytic cycle. However, it is obvious that they are genotypically unrelated. The Hsp essential region has been cloned and sequenced and shown to be a large single gene whereas the Per phenotype is the result of a non-coding region of repeated sequences. The Per region shares homology with both bacteriophages to which it encodes resistance, but the Hsp region does not. See Jarvis, A.W. and Klaenhammer, T.R., Appl. Environ. Microbiol. 51: 1272-1277 (1986). Finally, the Hsp mechanism is slightly heat sensitive in NCK203, whereas Per is not. Nonetheless, it is significant that the Hsp gene product has been shown to interfere with bacteriophage DNA replication. It may be that the Hsp gene product is also *ori*-specific, but only against the $\phi 31$ -type *ori*, and not that of bacteriophages $\phi 48$ and $\phi 50$.

L. lactis strain NCK298, which contains both pTR2030 and pTRK104, is insensitive to contaminating levels of bacteriophages representing both Per-resistant ($\phi 31$) and Hsp-resistant ($\phi 50$) groups. Thus,

Hsp+ and Per+ can work in concert to provide a more extensive range of insensitivity than either mechanism used in isolation.

5 Plasmid pTRK104 can be easily isolated from
L. lactis NCK298 by lysing the cells via the procedure
of Anderson, D.A., and McKay, L.L., Appl. Environ.
Microbiol. 46: 549-552 (1983). Plasmid pTRK104 can be
visualized among the other resident plasmids in NCK298
after electrophoretic migration of the DNA sample in
10 agarose gels; or isolated in pure form by
transformation into competent cells of *E. coli* and
selection for Cm^r transformants via standard protocols.
See Hill, c. et al., Appl. Environ. Microbiol. 55:
1684-1686.

15 A third aspect of the present invention
relates to a defined bacterial starter culture capable
of fermenting a predetermined product. The bacterial
starter culture comprises the bacteriophage resistant,
recombinant bacteria as described above. That is, the
20 recombinant bacteria contains at least a first
bacteriophage defense mechanism, Per, which comprises a
bacteriophage origin of replication, *ori*, operatively
associated with a DNA sequence incapable of producing
live bacteriophage. The use of the instant
25 bacteriophage resistant bacteria in a starting culture
increases the longevity of the culture, the end result
being a more economical fermentation process on an
industrial scale. Bacterial starter cultures
comprising the bacteriophage-resistant transformed
30 bacteria can be used in the fermentation of a variety
of products, as described above. A preferred bacterial
genus is the *lactococci*, and preferred species are *L.*
lactis subsp. *lactis* and *L. lactis* subsp. *cremoris*.

35 The bacterial starter culture may consist of
one bacterial strain, i.e., a pure culture. In this
case, substantially all, or at least a significant
portion of the bacterial starter culture would

generally comprise the transformed bacterial cells. In the alternative, the starter culture may comprise several bacterial strains, i.e., a defined mixed culture, wherein a portion of each bacterial strain is bacteriophage-resistant in accordance with the instant invention. The ideal situation in either case would be to provide starter cultures wherein substantially all the bacterial cells are rendered bacteriophage-resistant.

A fourth aspect of the present invention contemplates a method of conducting a fermentation of a product employing a bacterial starter culture of the instant invention. A fermentation process using the bacteriophage-resistant recombinant bacteria of the present invention may be conducted on a less interrupted basis since the problems of massive bacteriophage infection and subsequent lysis of a predominant portion of the initial bacterial starter culture are alleviated. For example, as explained in Example 6 below, bacteriophage surviving through a Per⁺ host are incapable of circumventing Per⁺ in subsequent infections.

The fermentation apparatus and conditions under which fermentation should be conducted may be selected and determined, respectively, by persons of ordinary skill in the art such as to produce the desired product while maintaining the viability of the bacteriophage resistant recombinant bacteria in the starter culture. Reference in this regard is made to the publications above.

A fifth aspect of the present invention relates to a recombinant DNA vector comprising a bacteriophage origin of replication, *ori*, operatively associated with a DNA sequence incapable of producing live bacteriophage. Any vector capable of transforming a given bacterial host cell can be used in accordance with the present invention. A preferred vector

according to the present invention is a plasmid, and more preferred is a plasmid isolated from *E. coli*. In addition, as described the examples below, shuttle vectors may be used since they are capable of replicating stably in two different hosts and thus can move DNA from one organism to another. In a more preferred embodiment of the present invention, the DNA sequence comprising *ori* is derived from a bacteriophage which is capable of infecting a fermentative bacterium. Bacteria that are capable of fermenting foods, particularly dairy products such as milk, are more preferred, i.e., lactic acid bacteria more particularly, those belonging to the *lactococci*.

In the most preferred embodiment, the recombinant vector is an *E. coli* plasmid containing the *ori* of $\phi 50$, designated pTRK104, the preparation and analysis of which are set forth in the Examples.

Isolation of bacteriophage resistance mechanisms from lytic bacteriophage genomes such as in the instant invention may provide a wealth of alternative defenses that can be employed to protect industrial strains against failure due to bacteriophage attack. The Per phenotype is not dependent on the production of repressors, nor on the transcription of antisense RNA. Because of this, no energy is expended by the resistant host, other than normal plasmid replication, on maintaining the Per response to bacteriophage attack. In addition, in at least one instance, the Per mechanism was effective against a genetically homologous bacteriophage ($\phi 48$) other than the "source bacteriophage" ($\phi 50$), which illustrates the potency of Per. Further, two distinct resistance mechanisms, such as Per⁺ and Hsp⁺ for example, can work in concert to provide a more extensive range of bacteriophage resistance than any sole resistance mechanism.

The examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLES

The bacterial strains used in the examples below are presented in Table 1. *L. lactis* strains were propagated at 30°C in M17 broth as described in Terzhagi, B.E. et al., *Appl. Environ. Microbiol.* 29:807-813 (1975), or in M17 broth with 0.5% glucose substituted for lactose (M17G) when appropriate. Bacteriophages were propagated and titrated as described in Klaenhammer, T.R. et al., *J. Gen. Microbiol.* 131:1531-1541 (1985). *Escherichia coli* strains were grown in LB broth at 37°C with shaking. See Silhavy, T.J. et al., "Experiments With Gene Fusions", Cold Spring Harbor, New York (1984). When required for selection, the following antibiotics were added at the indicated concentrations in micrograms per milliliter: for *E. coli*, ampicillin, 50; chloramphenicol, 20; for *lactococci*, erythromycin, 5. Solid surface conjugal matings and selection of Lac⁺ transconjugants were performed as described in Klaenhammer, T.R. et al., *J. Gen. Microbiol.* 131:1531-1541 (1985) and McKay, L.L. et al., *Appl. Environ. Microbiol.* 40:68-74 (1980). Protoplasts of *L. lactis* NCK203 were transformed as described in Kondo, J.K. et al., *Appl. Environ. Microbiol.* 43:1213-1215 (1982), with some modifications. See Hill, C.D. et al., *Appl. Environ. Microbiol.* 55:1684-1689 (1989). Transformants were selected on 1.5 ug erythromycin per ml.

Bacteriophage adsorption assays were conducted as described in Sanders, M.E. et al., *Appl. Environ. Microbiol.* 46:1125-1133 (1983). Center of Infection (COI) data were calculated as described in Klaenhammer, T.R. et al., *J. Gen. Microbiol.*, 131:1531-

1541 (1985). Growth curves were performed as follows; 10 ml of M17G, 50 μ l of 1 M CaCl_2 , and 200 μ l of an overnight culture, were added to a sterile cuvette and the OD_{600} was monitored with time. After 1 hr,
5 bacteriophage were added to a final level of 10^6 plaque forming unites per ml.

Plasmid isolation, restriction, ligation and transformation in *E. coli* DH1 was performed as described in Maniatis, T.E. et al., Molecular Cloning,
10 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Lactococcal bacteriophage DNA was isolated as previously described. See Jarvis, A.W. and T.R. Klaenhammer, Appl. Environ. Microbiol. 51: 1272-1277 (1986).

15 Nucleotide sequences of both strands were determined by the dideoxy chain termination method set forth in Sanger, F.S. et al., *Pro. Nat'l. Acad. Sci. (USA)* 74:5463-5467 (1977) and the Sequenase enzyme (Stratagene) using either the recombinant M13 single
20 stranded templates or pBluescript clones. Synthetic oligonucleotide primers (17-mers) were synthesized in those instances when a subclone was too large to be fully sequenced from commercially available primers.

Bacteriophage ϕ 50 replication was analyzed as
25 follows. Sensitive or resistant cells were freshly grown to an optical density of 0.5 at 600nm. CaCl_2 to a final concentration of 10mM, was added, followed by the addition of the test bacteriophage strain at a multiplicity of infection (MOI) greater than 1.0.
30 Infected cells were incubated at 30°C. At various time points, 1 ml of cells was harvested in an eppendorf centrifuge, and the pellet was frozen rapidly by immersion in ultra-cold ethanol (-70°C). After thawing on ice, the pellet was resuspended in 400 μ l of ice-
35 cold lysis solution (6.7% sucrose, 50 mM Tris, 1mM EDTA, pH 8.0). Lysozyme (20 μ l of a 10 mg/ml stock solution) was added and incubation on ice was continued

for 20 min. Sodium dodecyl sulfate (50 μ l of a 10% stock) was added. Proteinase K (20 μ l of a 20 mg/ml stock) was then added and the lysate incubated at 65°C for 20 min. The resulting clear suspension was
5 extracted once with TE-saturated phenol, followed by a chloroform:isoamyl-alcohol (24:1) extraction. One-tenth volume of 3M sodium acetate and two volumes of cold 95% ethanol were added to precipitate the total DNA. The DNA pellet was finally resuspended in 100 μ l
10 dH₂O. 5 μ l were generally sufficient for restriction analysis.

Unless indicated otherwise, all percents are by weight.

TABLE 1
Bacterial Strains and Plasmids

	Strain or Plasmid	Relevant Characteristics	Source or derivation
5	<u>L. lactis</u>		
	NCK202	<u>str15</u> , host for $\phi 50$, $\phi 48$, $\phi 31$	Hill et al., 1989b
	NCK203	R ⁻ /M ⁻ derivative of NCK202	Hill et al., 1989b
	NCK204	NCK203, pTR2030, Hsp ⁺ , R ⁺ /M ⁺	Hill et al., 1989b
10	NCK213	NCK203, pTRK103, Per ⁺	Transformant, this study
	NCK214	NCK203, pTRK104, Per ⁺	Transformant, this study
	NCK215	NCK203, pSA3	Transformant, this study
	NCK216	NCK203, pTRK106	Transformant, this study
15	NCK298	NCK203, pTR2030, pTRK104, pTR140, Lac ⁺ , Hsp ⁺ , R ⁺ /M ⁺ , Per ⁺	Transconjugant, this study
	NCK294	NCK203, pTRK136, Per ⁺	Transformant, this study
	NCK295	NCK203, pTRK137, Per ⁺	Transformant, this study
	NCK296	NCK203, pTRK138, Per ⁺	Transformant, this study

		Strain or Plasmid	Relevant Characteristics	Source or derivation
		<i>E. coli</i> DH1	Transformation host	Hanahan, 1983
5	Plasmids			
		pBluescript	Cloning vector, Ap ^r	Stratagene, La Jolla, CA.
		pSA3	Shuttle vector, Cm ^r , Em ^r , Tc ^r	Dao and Ferretti, 1985
		pSA34	<i>ori</i> selection vector, Cm ^r , Em ^r , Tc ^r	Sanders and Schultz, 1989
10		pTRK102	Ap ^r	pBluescript::4.5-kb ϕ 50 fragment
		pTRK103	Cm ^r , Em ^r , Per ⁺	pSA3::4.5-kb ϕ 50 fragment
15		pTRK104	Cm ^r , Em ^r , Per ⁺	<u>EcoRV</u> deletion of pTRK103
		pTRK105	Ap ^r	pBluescript::2.5-kb ϕ 50 fragment
		pTRK106	Cm ^r , Em ^r , Per ⁺	pSA3::2.5-kb ϕ 50 fragment
20		pTRK136	Cm ^r , Em ^r , Per ⁺	pTRK104, opposite orientation
		pTRK137	Cm ^r , Em ^r , Per ⁺	pTRK104, <u>Asp</u> 718 deletion

Strain or Plasmid	Relevant Characteristics	Source or derivation
pTRK138	Cm ^r , Em ^r , Per ⁺	pTRK104, <u>Sall</u> - <u>Xho</u> I deletion
pTRK141	Cm ^r , Em ^r	pSA34::4.5-kb ϕ 50 fragment
pTR2030	Hsp ⁺ , Tra ⁺ , R ⁺ /M ⁺	Klaenhammer and Sanozky, 1985

EXAMPLE 1

Cloning of per gene from Phage nck202.50

This example describes the isolation and characterization of a gene encoding bacteriophage encoded resistance. Phage nck202.50, designated ϕ 50, is a 29.8-kb virulent small isometric-headed bacteriophage. It possesses a unique *Bam*H1 site in addition to at least 13 *Hind*III sites which have not been mapped on the bacteriophage genome, as shown in Fig. 1A. To isolate *Per*, bacteriophage ϕ 50 DNA was digested with *Bam*H1 and *Hind*III and shotgun cloned into similarly digested pBluescript. Recombinant plasmids containing both possible *Bam*H1-*Hind*III inserts were recovered from transformants. The 4.5-kb insert from one of the plasmids, pTRK102, was subcloned as a *Bam*H1-*Sal*I fragment in the streptococcal-*E. coli* shuttle plasmid pSA3 in accordance with the method described in Dao, M.L. et al., *Appl. Environ. Microbiol.* 49:115-119 (1985), to create pTRK103, illustrated in Fig. 1B. pTRK103 was introduced to *L. lactis* NCK203 via protoplast transformation. NCK203 is a derivative of NCK202 which has been cured of a native R/M plasmid pTRK68. See Hill, C.K. et al., *Appl. Environ. Microbiol.*, 55:2416-2419 (1989). One NCK203

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transformant, NCK213, was verified as possessing intact pTRK103 by plasmid profile analysis. The plasmid was isolated from NCK213 and retransformed to *E. coli* DH1 and subjected to restriction enzyme analysis. No rearrangements in pTRK103 were detected (data not shown).

EXAMPLE 2

Identification of pTRK103 as Per⁺

The effect of pTRK103 on ϕ 50 was investigated using standard plaque assays. When present in NCK203, pTRK103 strongly inhibited the ability of ϕ 50 to form plaques. Plaques were pinpoint in size and difficult to enumerate; the EOP was estimated at 0.35. See Table 2. When present on NCK203 harboring the shuttle vector pSA3 without an insert, ϕ 50 gave normal plaques of 1.6 mm in diameter and an EOP of 1.0 (Table 2). NCK203 (pTRK103) also inhibited plaque formation by the small isometric headed bacteriophage ϕ 48. The plaque size was reduced and an EOP of 0.5 was estimated for this bacteriophage (Table 2). However, NCK203 (pTRK103) did not inhibit plaque formation by the small isometric-headed bacteriophages ϕ 31, ϕ 35 or ϕ 36 (data for ϕ 31 in Table 2, others not shown). pTRK103 was introduced into *L. lactis* MG1363 by protoplast transformation and one transformant, NCK219, was challenged with three bacteriophages lytic for that host. The presence of pTRK103 did not affect the plaque size or EOP of the prolate bacteriophage ϕ c2, or the small isometric bacteriophages ϕ p2 or ϕ sk1. Conversely, the Hsp⁺ plasmid pTK6 retarded normal plaque formation for ϕ c2, ϕ p2 and ϕ sk1 in MG1363. See Hill, C. et al. *Appl. Environ. Microbiol.*, 55:1684-1689 (1989). pTK6 also retarded normal plaque formation for ϕ 31, ϕ 35 and ϕ 36 in NCK203. See, Hill, C. et al., *Appl. Environ. Microbiol.* 55:2416-2419 (1989). The Hsp⁺

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activity directed by pTK6 did not inhibit either ϕ 48 or ϕ 50 in the NCK203 background.

5 A second clone was constructed to evaluate the possibility that the resistance encoded by pTRK103 is a general phenomenon of bacteriophage fragments distributed around the ϕ 50 genome. Bacteriophage ϕ 50 DNA was digested with *Hind*III and shotgun cloned into *Hind*III-digested pBluescript. A bank of clones with inserts representing the bacteriophage genome was
10 obtained. One recombinant plasmid, pTRK105, which contained an insert of approximately 2.5-kb was randomly selected, and the insert was subcloned in pSA3 as a *Bam*HI-*Xba*I fragment. The resultant recombinant plasmid, pTRK106, was introduced to NCK203 by
15 protoplast transformation. The pTRK106 transformant was challenged with ϕ 48 and ϕ 50. This randomly selected bacteriophage fragment had no effect on the plaque size or the efficiency of plating of either bacteriophage. See Table 2.

TABLE 2

Bacteriophage nck202.50 (ϕ 50), nck202.48 (ϕ 48), and nck202.31 (ϕ 31) reactions on L. lactis NCK203 and derivatives

5	PHAGE	HOST (PLASMID)	EOP	PLAQUE SIZE/mm
	ϕ 50	NCK203	1.0	1.6
	ϕ 50	NCK215 (pSA3)	1.0	1.6
	ϕ 50	NCK213 (pTRK103)	0.35	0.2
10	ϕ 48	NCK203	1.0	1.5
	ϕ 48	NCK213 (pTRK103)	0.5	0.2
	ϕ 31	NCK203	1.0	1.5
	ϕ 31	NCK213 (pTRK103)	1.0	1.5
	ϕ 50	NCK216 (pTRK106)	1.0	1.6
15	ϕ 48	NCK216 (pTRK106)	1.0	1.5
	ϕ 50	NCK214 (pTRK104)	0.42	0.2
	ϕ 48	NCK214 (pTRK104)	0.5	0.2
	ϕ 50	NCK298 (pTR2030, pTRK104)	0.44	0.2
20	ϕ 31	NCK298 (pTR2030, pTRK104)	$<10^{-8}$	no plaques

EXAMPLE 3

Subcloning the Per Region

pTRK103 contains four *EcoRV* sites, three of which are internal to the $\phi 50$ insert. See Fig. 1B.

5 pTRK103 was digested with *EcoRV*, religated and transformed to *E. coli* DH1. Cm^r -transformants were recovered containing plasmids which had been deleted of one or more of the *EcoRV* fragments. These plasmids were transformed to NCK203 and examined for their
10 effect on $\phi 48$ and $\phi 50$. All derivatives directed the same Per^+ resistance phenotype as did pTRK103. This localized the resistance determinant to a 1.4-kb *EcoRV*-*HindIII* fragment which remained undisturbed in all the in vitro deletions. See Fig. 1C. This fragment was
15 present in all deletion derivatives because there is no *EcoRV* site to the right-hand side of this region. The Per^+ phenotype against $\phi 48$ and $\phi 50$ (Table 2) is encoded by a plasmid containing only this 1.4-kb region, pTRK104, illustrated in Fig. 2A. The 1.4-kb insert in
20 pTRK104 was inverted by digesting the plasmid with *EcoRV* and *NruI*, which cut on either side of the insert (Fig. 2A). After religation, a recombinant plasmid was recovered, pTRK136, in which the insert was present in the opposite orientation. pTRK136 directed Per^+ at an
25 identical level to pTRK104, indicating that any information necessary for *Per* activity is located within the fragment.

EXAMPLE 4

Sequence and Analysis of Per

30 The 1.4-kb insert in pTRK104 was subcloned in M13mp18 and mp19 as an *EcoRV*-*ClaI* fragment and sequenced by the strategy illustrated in Figs. 2A and 2B. Sequence analysis revealed a single open reading frame (ORF) of 701-bp. See Fig. 3. An unexpected
35 result was the presence of a second *HindIII* site 69-bp

from the pSA3-derived *Hind*III site used in the original cloning protocol. This small fragment was either inserted fortuitously during the ligation reaction, or is a contiguous ϕ 50 fragment which was included as a result of a partial digestion. This region includes the potential start codon for the 701-bp ORF contained within the fragment. The 3' end of the insert proved difficult to sequence, and was resolved finally by conducting the polymerase reactions at 0°C, a recommended procedure for regions rich in secondary structure. The resulting unambiguous sequence revealed a direct repeat of 236-bp separated by 28-bp (Fig. 3). This extremely large direct repeat was almost 99% identical, with only 3 mismatches in the 236-bp repeat. Detected within the direct repeat were a number of inverted repeats capable of forming stable stem-loop structures. See Figs. 4A and 4B. These stable structures are typical of sequences associated with rho-independent termination. The 10-bp sequence, GGTACCGAGG, was also repeated four times within each larger direct repeat (Fig. 3). In addition, the palindromic sequence GGATCC was repeated 14 times overall in this 500-bp region.

EXAMPLE 5

Recognition of Repeated Sequences Responsible for Per Activity

pTRK104 was digested with *S*alI and *X*hoI and religated. The resulting construct, pTRK138, no longer possessed the putative start codon for the ORF. pTRK138 was reintroduced into *L. lactis* NCK203 and challenged with ϕ 50 and ϕ 48. The Per⁺ phenotype was observed (data not shown), indicating that the ORF is not involved in the bacteriophage resistance phenotype. A second derivative was constructed to assess the effect of the repeated sequence on the Per phenotype. The palindromic repeat GGATCC, present 14 times within

the repeated regions, is the recognition site for the type II restriction endonuclease Asp718 and its isoschizomer Kpn1. pTRK104 was digested with Asp718 and religated to form pTRK137. This plasmid lost approximately 400-bp, including most of the large direct and inverted repeats present in pTRK104. When introduced into NCK203, pTRK137 did not direct any resistance against either bacteriophage ϕ 48 or ϕ 50, from which it was concluded that the repeated sequence is essential for the Per⁺ phenotype.

EXAMPLE 6

Effect of pTRK104 on ϕ 50

The growth of NCK203 containing pTRK104, i.e., NCK214, at 30°C in the presence and absence of ϕ 50 was examined. See Fig. 5A. NCK214 grew normally in the presence of ϕ 50 at an initial bacteriophage level of 1×10^4 per ml. In comparison, the control strain NCK215, containing the cloning vector pSA3, was rapidly lysed under the same conditions. The mechanism of action of Per was examined further by conducting bacteriophage adsorption and center of infection (COI) studies on NCK214. Adsorption studies were performed at 30°C on NCK203 derivatives harboring either pSA3 (NCK215) or pTRK104 (NCK214). NCK214 adsorbed ϕ 50 at 87%, compared with 84% for the sensitive host, NCK215. However, the number of infective centers formed on NCK214 was 43% of that formed on NCK215. Standard plaque assays conducted at high temperature (39°C) revealed that Per is not heat sensitive. Similar plaque sizes and EOP were obtained at both 39°C and at 30°C (data not shown). Bacteriophage were inhibited to the same extent by pTRK104 regardless of whether or not the bacteriophage was first propagated on a host bearing pTRK104. Therefore bacteriophage surviving through a Per⁺ host are incapable of circumventing Per in subsequent infections.

EXAMPLE 7

Per⁺ Affects Bacteriophage DNA Replication

The internal replication of ϕ 50 DNA was followed using a rapid DNA isolation procedure in which bacteriophage-infected cells were harvested at various times and examined for bacteriophage DNA. Forty minutes after bacteriophage infection, the appearance of ϕ 50 DNA could be clearly detected in the propagating host, NCK203. Bacteriophage directed replication of the ϕ 50 genome prior to cell lysis was reflected in the accumulation of ϕ 50-specific DNA fragments. With increasing time after infection, bacteriophage DNA became more evident, suggesting continued replication. In the NCK203 derivative containing pTRK138, NCK296 (Per⁺), bacteriophage DNA replication over the identical time course was less pronounced suggesting that Per interferes with bacteriophage DNA replication. It is significant that the plasmid bearing Per, pTRK138, increased in copy number after infection. An increase in the copy number of pTRK138 was not apparent during ϕ 31 infection of NCK296, nor was there any decrease in ϕ 31 DNA replication. Thus, a plasmid bearing the bacteriophage replication ori should increase in copy number as a consequence of bacteriophage infection. It is noteworthy that the increased amount of pTRK138 evident in the ϕ 50-infected NCK296 was not present in a covalently closed circular (CCC) form. Uncut DNA preparations did not show increased amounts of the CCC plasmid, suggesting that pTRK138 is amplified as a concatemeric form during bacteriophage infection (data not shown). These data indicate that the Per region is the bacteriophage origin, or one of the bacteriophage origins of replication (ori).

The pSA3 cloning vector, contained in NCK215, was not amplified after bacteriophage ϕ 50 infection. Therefore, the presence of the repeated ϕ 50 DNA

sequence is required for amplification of pTRK138. The 4.5-kb insert from ϕ 50, contained in pTRK103, was subcloned to the pSA3 derivative pSA34, a derivative of pSA3 which has been deleted of the gram-positive origin of replication. See Sanders, M.E. et al., *Eur. Pat. App.* 88/118362.8. *L. lactis* NCK203 could not be transformed with the resulting construct, pTRK141. Thus, it was concluded that the putative bacteriophage ϕ 50 ori is incapable of supporting viable plasmid replication in the absence of a concurrent bacteriophage infection.

EXAMPLE 8

Combining Hsp⁺ and Per⁺ in NCK203

pTR2030 was introduced to NCK214 (pTRK104) by solid surface conjugation with a donor strain NCK1. Transconjugants were selected initially for acquisition of the Lac⁺ phenotype encoded by the non-conjugative plasmid pTR1040. Lac⁺ (pTR1040) Em^r (pTRK104) transconjugants were subsequently screened for the presence of pTR2030. One transconjugant, NCK298, containing both pTR2030 and pTRK104, was selected and examined for combined resistance to bacteriophages ϕ 50 and ϕ 31, insensitive to Hsp⁺ and Per⁺, respectively. NCK298 is completely resistant to ϕ 31 in standard plaque assays, a typical reaction for this bacteriophage on pTR2030-bearing hosts. The host bearing both plasmids also displayed the typical Per⁺ response against ϕ 50 (Table 2). Growth of NCK298 was not affected when both ϕ 50 and ϕ 31 were present at initial levels of 10⁶ pfu/ml. See Fig. 5B. Strains containing either pTR2030 or pTRK104 were inhibited by the presence of both types of bacteriophage. These data indicate that both resistance mechanisms can operate simultaneously in the NCK203 background and expand the bacteriophage resistance of this strain.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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WE CLAIM:

1. A method of imparting bacteriophage resistance to lactococcal bacteria contained in a fermentative culture medium, comprising:

- (a) conducting a fermentation of a milk substrate in a medium containing a defined bacterial culture including at least one lactococcal bacteria until bacteriophage are detected in the medium, said bacteriophage being specific to at least one lactococcal bacteria in said medium;
- (b) isolating said bacteriophage;
- (c) digesting DNA of the bacteriophage to produce a library of DNA fragments;
- (d) transforming said one lactococcal bacteria susceptible to said bacteriophage with said library of DNA fragments to provide transformed lactococcal bacteria;
- (e) selecting from among the transformed lactococcal bacteria a bacteriophage-resistant recombinant lactococcal bacteria;
- (f) adding a predetermined amount of said bacteriophage-resistant recombinant lactococcal bacteria to the medium; and
- (g) commencing fermentation of said substrate in said medium with said bacteriophage-resistant recombinant lactococcal bacteria.

2. The method of claim 1, wherein the defined bacterial culture is a pure culture.

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3. The method of claim 1, wherein the defined bacterial culture is a mixed culture.

4. The method of claim 1, wherein said step of transforming said one lactococcal bacteria susceptible to said bacteriophage is carried out by:

(a) inserting the fragments of the library into a plasmid vector; then

(b) transforming said bacteria susceptible to said bacteriophage with said plasmid vector; and then

(c) propagating the transformed bacteria.

5. A lactococcal bacterial cell containing a first bacteriophage defense mechanism, said first mechanism comprising a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein said lactococcal bacterial cell is capable of being infected by a bacteriophage, and wherein the DNA of said bacteriophage, once injected into said bacterial cell, competes with said first defense mechanism for binding to DNA polymerase.

6. A bacterial cell of claim 5, capable of fermenting a food substrate.

7. A bacterial cell of claim 6, wherein said food substrate is a dairy substrate.

8. A bacterial cell of claim 7, wherein said dairy substrate comprises milk.

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9. A bacterial cell of claim 5, wherein said ori and said operatively associated DNA sequence are carried by an extrachromosomal plasmid.

10. A bacterial cell of claim 9, wherein said extrachromosomal plasmid is pTRK104.

11. A defined lactococcal bacterial starter culture capable of fermenting a predetermined product, comprising: a plurality of lactococcal bacterial cells, each containing a first bacteriophage defense mechanism, said bacteriophage defense mechanism comprising a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein said lactococcal bacterial cells are capable of being infected by a bacteriophage, and wherein the DNA of said bacteriophage, once injected into said bacterial cell, competes with said bacteriophage defense mechanism for binding to DNA polymerase.

12. A defined bacterial starter culture of claim 11, which is a pure culture.

13. A defined bacterial starter culture of claim 11 or 12, which is capable of fermenting a food substrate.

14. A defined bacterial starter culture of claim 11, which is capable of fermenting milk.

15. A method of conducting a fermentation of a milk substrate to produce a dairy product, comprising:

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providing to a fermentation medium, a defined lactococcal bacterial starter culture capable of fermenting a predetermined product, comprising: a plurality of lactococcal bacterial cells, each containing a first bacteriophage defense mechanism comprising a bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, wherein the DNA of said bacteriophage, once injected into said bacterial cell, competes with ori for binding to DNA polymerase; and

conducting the fermentation under conditions suitable to produce the product.

16. A method of claim 15, wherein said defined bacterial starter culture is a pure culture.

17. A method of claim 15 or 16, wherein the product is a food product.

18. A method of claim 15 or 16, wherein the product is milk.

19. A recombinant DNA vector comprising a lactococcal bacteriophage origin of replication (ori) operatively associated with a DNA sequence incapable of producing live bacteriophage, and wherein said vector is plasmid PTRK104.

20. A vector of claim 19, wherein said bacteriophage is specific to a bacterium capable of fermenting a food product.

21. A vector of claim 20, wherein said food product is milk.

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22. A method of imparting resistance to bacteriophage nck202.50 to lactococcal bacteria contained in a fermentative culture medium, comprising:

- (a) conducting a fermentation of a milk substrate in a medium containing a defined bacterial culture including at least one lactococcal bacteria until bacteriophage nck202.50 is detected in the medium;
- (b) isolating said bacteriophage;
- (c) digesting DNA of the bacteriophage to produce a library of DNA fragments;
- (d) transforming said one lactococcal bacteria susceptible to said bacteriophage with said library of DNA fragments to provide transformed lactococcal bacteria by ligating said DNA fragments to a replicating plasmid vector and transforming said one bacteria susceptible to said bacteria with said vector;
- (e) selecting from among the transformed lactococcal bacteria a bacteriophage-resistant recombinant lactococcal bacteria resistant to bacteriophage nck202.50;
- (f) adding a predetermined amount of said bacteriophage-resistant recombinant lactococcal bacteria to the medium; and
- (g) commencing fermentation of said substrate in said medium with said bacteriophage-resistant recombinant lactococcal bacteria.

23. A method according to claim 22, wherein said lactococcal bacteria is *Lactococcus lactis*.

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24. A method according to claim 23, wherein said selecting step comprises selecting from among the transformed bacteria a bacteriophage-resistant recombinant bacteria containing plasmid pTRK104.

Figure 1A

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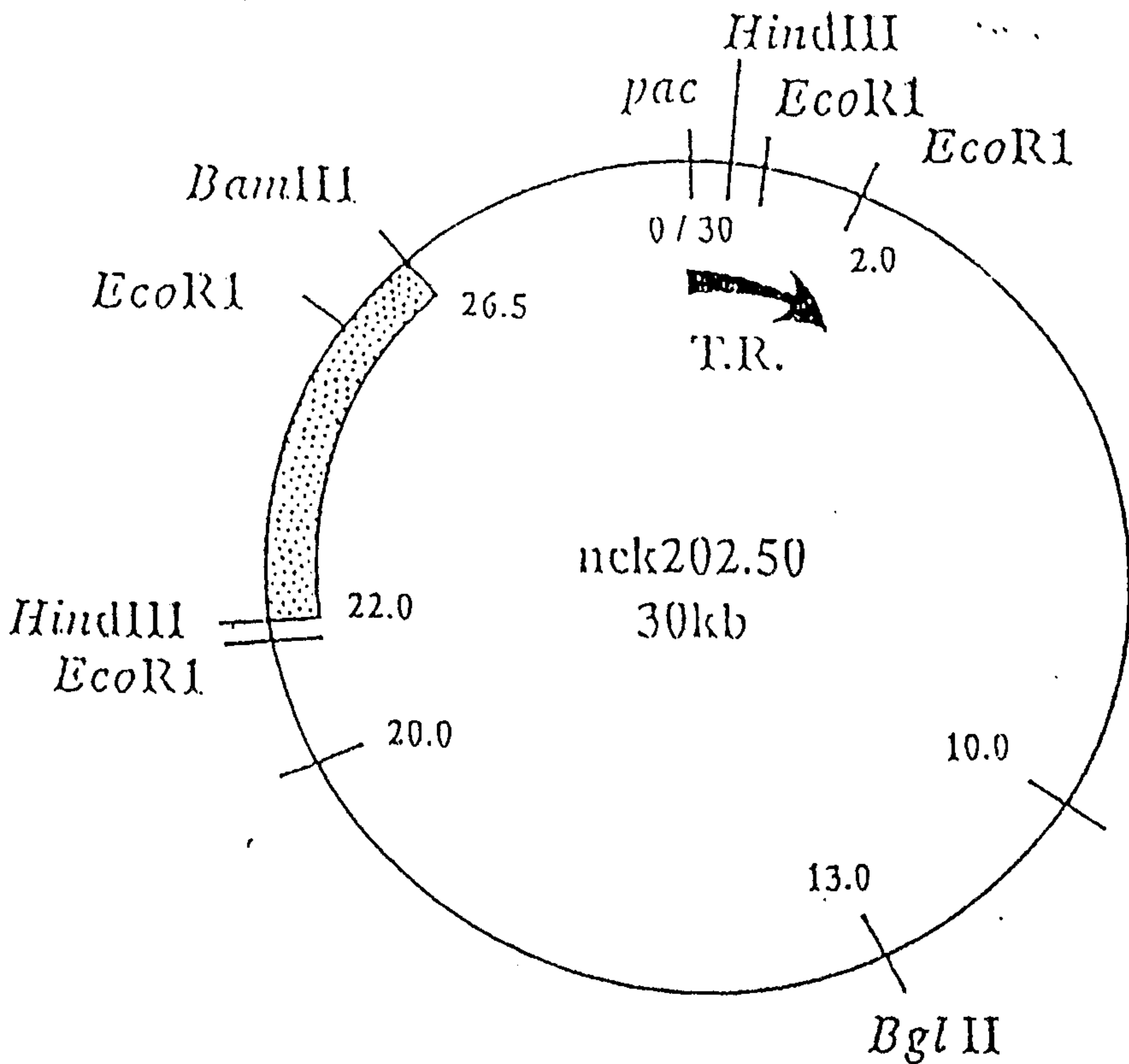


Figure 1B

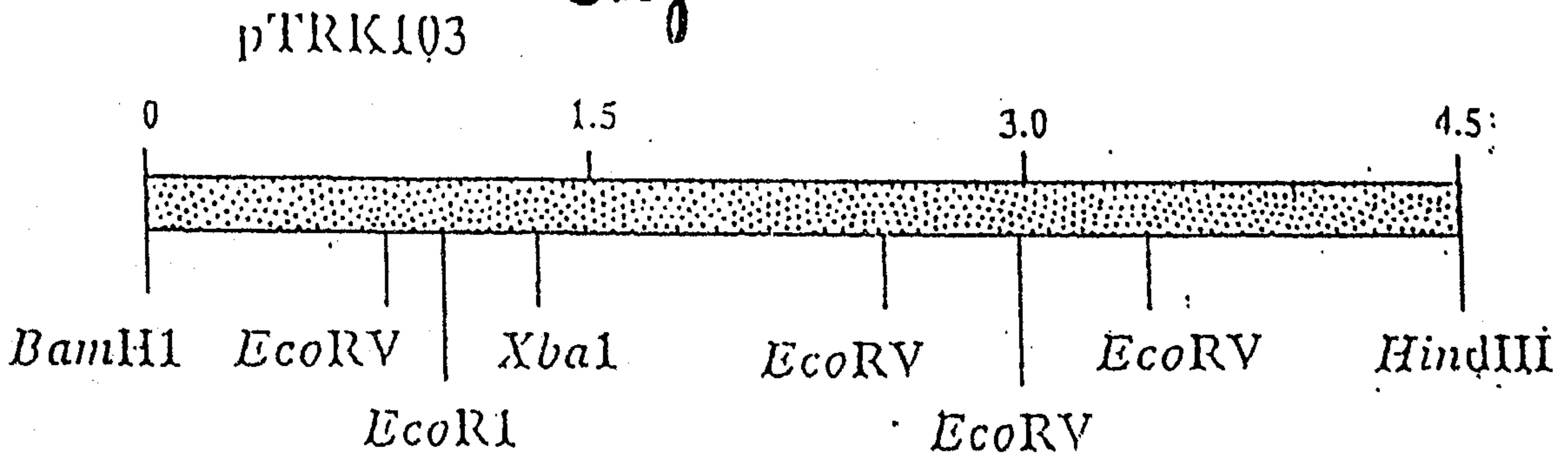
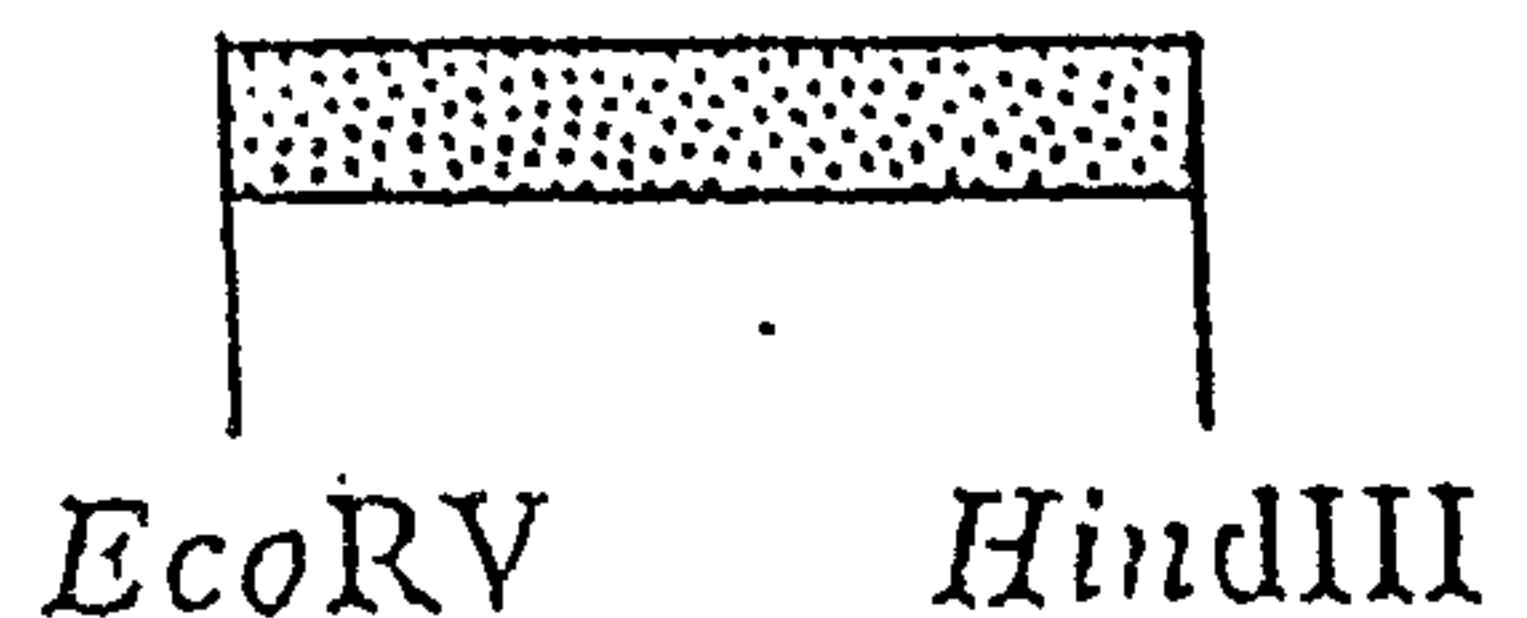


Figure 1C

pTRK104



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Figure 2A

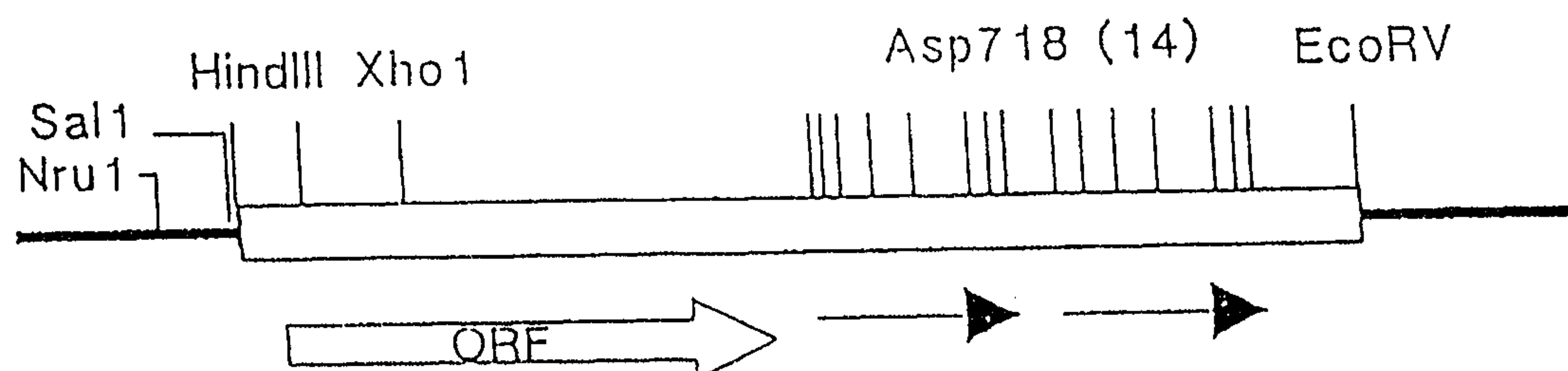
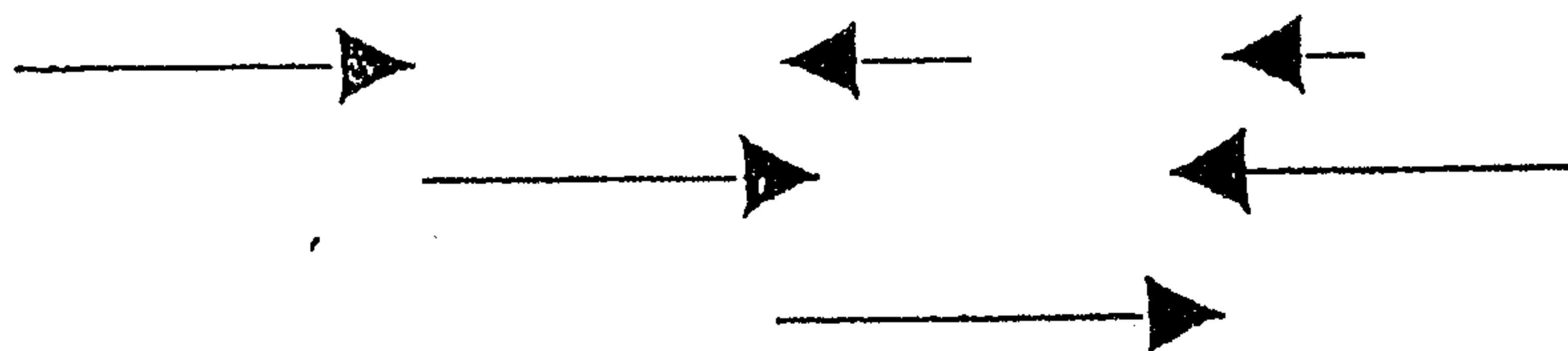


Figure 2B



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 MetAlaLeuAlaThrGluGlnGlyAspIleGlnGlyGluSerThrGluArgIleLeuLys
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 241 AATCTGACCAAGTGTGTTATGCGTTGATACAAATCGAACTGTTGCGCTCTCTGATGAAATG
 AsnLeuThrSerValLeuCysValAspThrAsnArgThrValAlaLeuSerAspGluMet
 301 GACGGCCGAAAAGTGTTAATTCAAATTTAAAGATCGTCCTAAAAATGAAACAGATATTCAA
 AspGlyArgLysValLeuIleGlnPheLysAspArgProLysAsnGluThrAspIleGln
 361 CGAGAGCGGATTTTCAAAAAATATTGGGATACTTTTACAAACAAAAGATAAAATTCCAGAT
 ArgGluArgIlePheLysLysTyrTrpAspThrPheThrThrLysAspLysIleProAsp
 421 ATTTCAGGATGTATAGGTTTCTTACTAAATTCGTTGGATTACTTTAACGAACTTGCGAAA
 IleSerGlyCysIleGlyPheLeuLeuAsnSerLeuAspTyrPheAsnGluLeuAlaLys
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 1141 GGTACCGAGGTTCTTACCATATATCAAAAGACTAATTAAATGTTTAAAGATATATATTATAT
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 1261 ATATGGCTTCGTGGAGGTTCCGAGGTGAGCAAAACAGCACTCTCATTTTTTATCAAAGGC
 1321 TATCAAGTCATTCCGTTGAACAGAAAAAAGTGGCACACCTATCACTAAATTTAAAGACATT
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Figure 4A

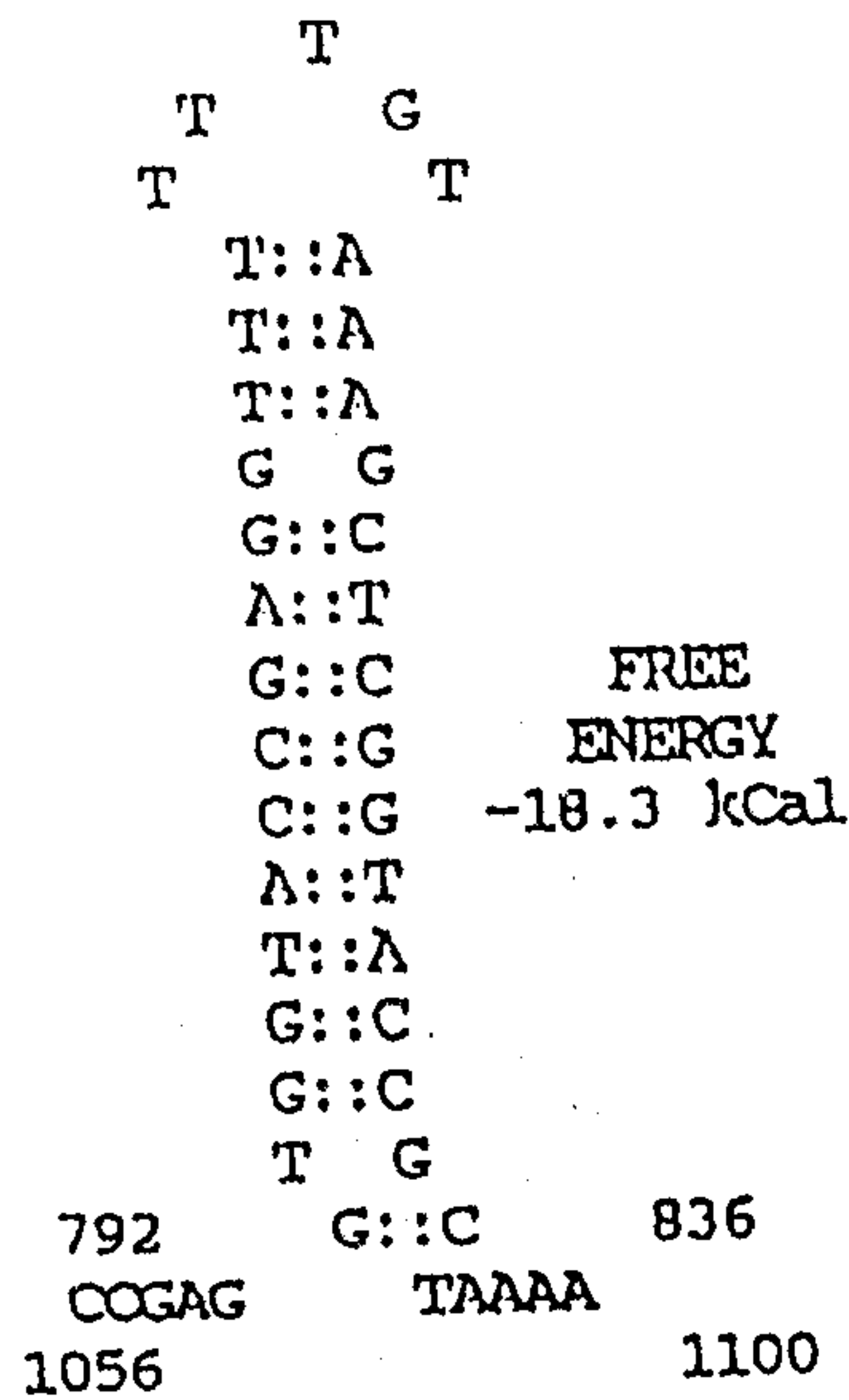
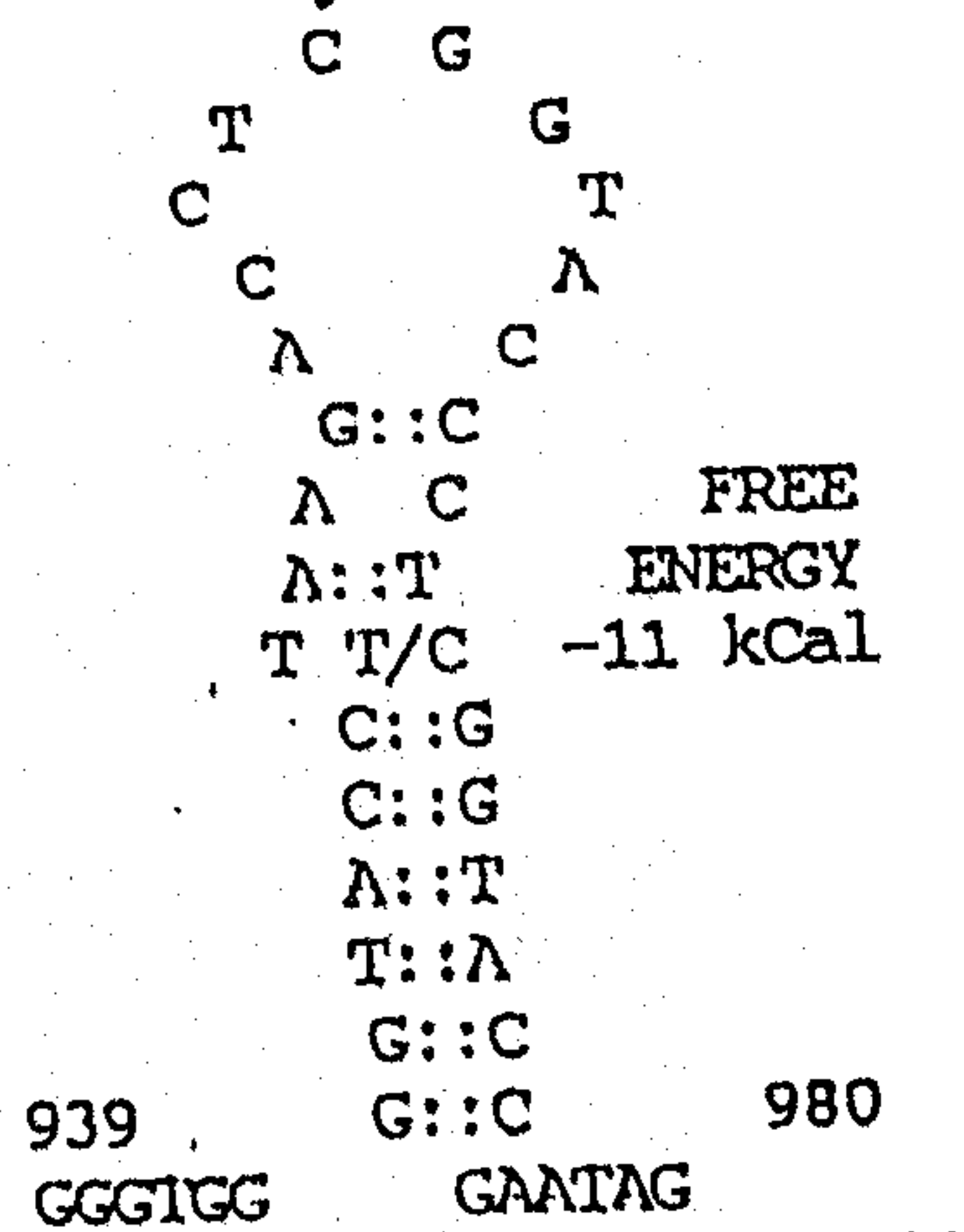


Figure 4B



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Figure 5A

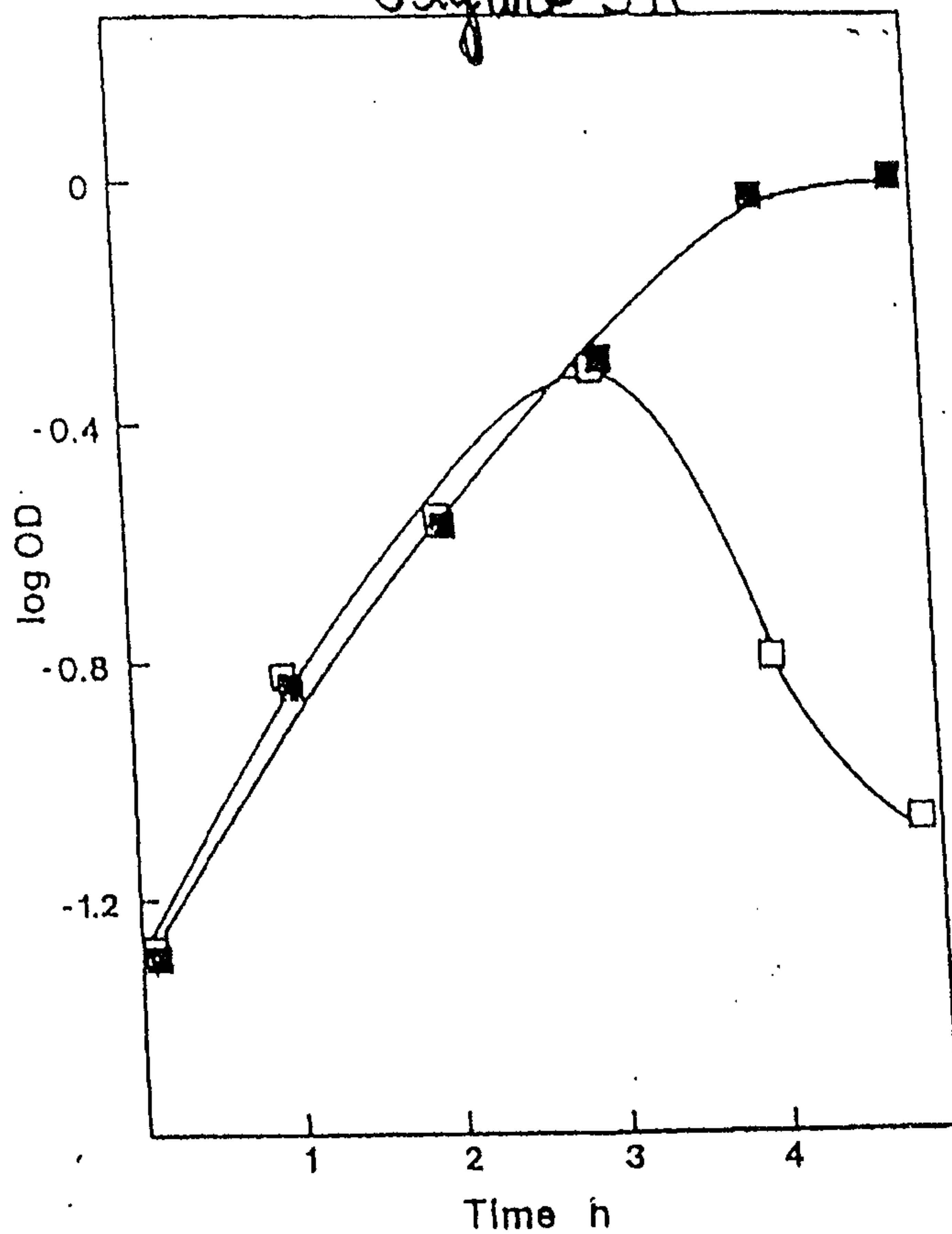
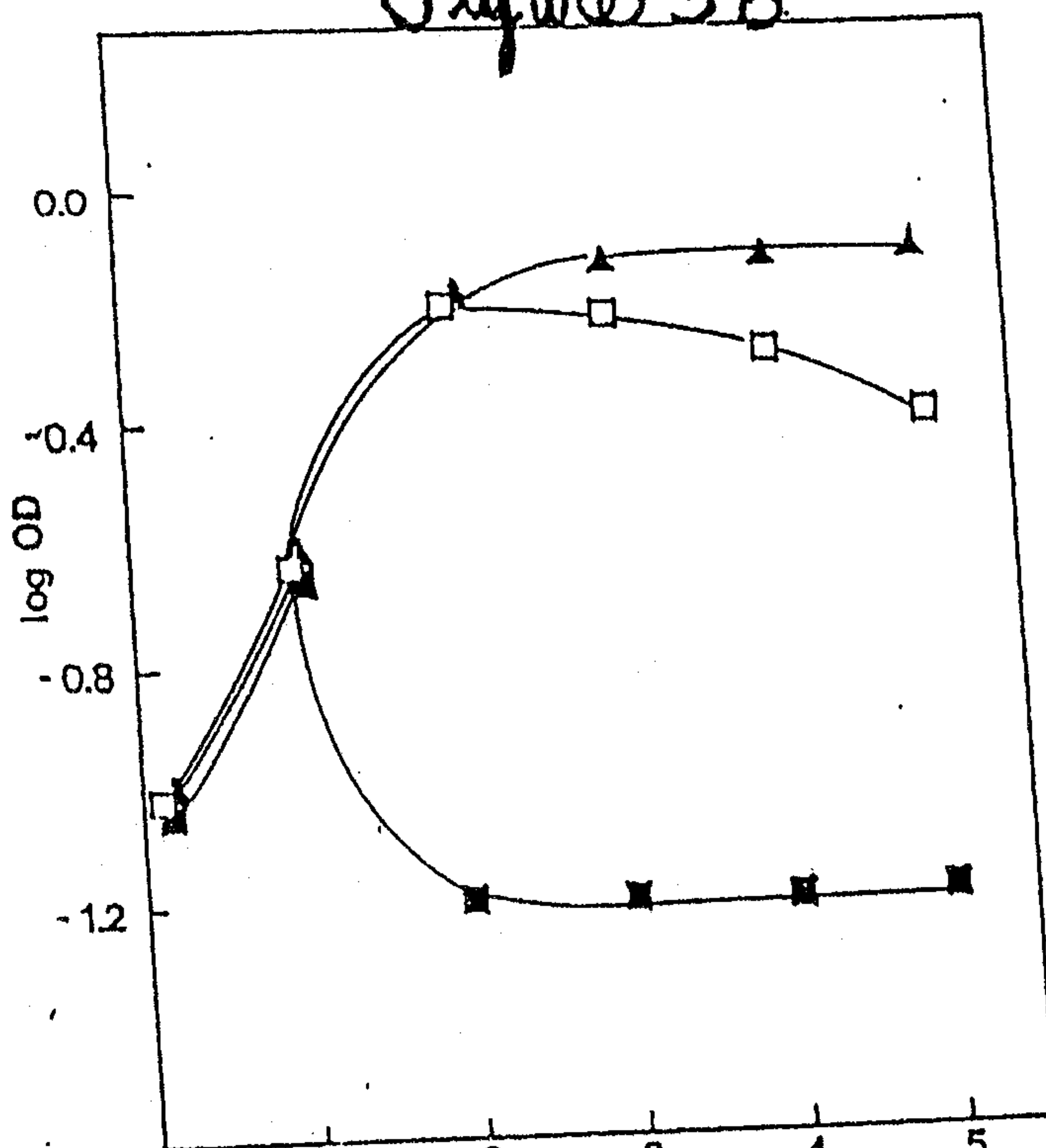


Figure 5B



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