BESCHWERDEKAMMERN DES EUROPÄISCHEN PATENTAMTS

BOARDS OF APPEAL OF THE EUROPEAN PATENT OFFICE

CHAMBRES DE RECOURS DE L'OFFICE EUROPEEN DES BREVETS

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File Number: T 609/90 - 3.3.2

Application No.: 83 305 058.6

Publication No.: 0 105 608

Title of invention: Method of protecting bacteria

Classification: C12N 15/00

D E C I S I O N
of 6 October 1992

Applicant: Eli Lilly and Company

Headword: Protecting bacteria/ELI LILLY

EPC Article 56

Keyword: "Inventive step - (yes)"

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Beschwerdekammern | Boards of Appeal | Chambres de recours

Case Number : T 609/90 - 3.3.2

D E C I S I O N
of the Technical Board of Appeal 3.3.2
of 6 October 1992

Appellant : Eli Lilly and Company
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Decision under appeal : Decision of Examining Division of the European Patent Office dated 2 March 1990 refusing European patent application No. 83 305 058.6 pursuant to Article 97(1) EPC.

Composition of the Board :

Chairman : P.A.M. Lançon
Members : U.M. Kinkeldey
E.M.C. Holtz

Summary of Facts and Submissions

I. European patent application No. 83 305 058.6 (publication No. 105 608) filed with 20 claims and relating to a method of protecting bacteria was rejected by the Examining Division. Claims 1 and 11 as refused read as follows:

"1. A recombinant DNA cloning vector that is useful for protecting a bacterium from bacteriophage freely living in the environment and containing double stranded DNA with a restriction site of specific activity, which comprises

1) a DNA segment comprising genes which coordinately and sequentially express in the bacterium
   a) a modification activity cognate to,
   b) a restriction activity of the same specificity as that of the restriction site,
2) a replicon that is functional in the bacterium, and
3) a gene that expresses a functional polypeptide comprising a human or non-human hormone or precursor or segment thereof.

11. A method for protecting a bacterium having an endogenous restriction-modification system from a bacteriophage which comprises transforming the bacterium with the recombinant DNA vector of any of Claims 1 to 10 and culturing the transformed bacterium under large scale fermentation conditions subject to the limitations

1) that the bacteriophage is free living in the environment and capable of infecting large scale bacterial fermentations and contains double stranded DNA with a restriction site of the same specificity as the restriction activity conferred to the bacterium, and

2) that the modification activity is expressed in the bacterium prior to the restriction activity."

II. The grounds given for rejection were that the application did not meet the requirement of Article 56 EPC.

In its decision the Examining Division took the view that the prior art already disclosed the problem of bacteriophage infection and its solution, i.e. the introduction of nucleotide sequences coding for restriction-modification activity and provided means to achieve this aim. The two prior art documents in question were

(1) PNAS, March 1981, Vol. 78(3), pages 1503 to 1507, and

(2) Gene, 1978, Vol. 3, pages 97-112.

On the other hand, nucleotide sequences coding for a hormone, their insertion into a plasmid and their subsequent expression were known before the priority date of the present application, for example from document

(3) EP-0 001 930.

Document (1) described plasmids containing nucleotide sequences coding for restriction-modification systems in order to protect bacteria against bacteriophage infection. Further protection had been obtained against various phages, as already disclosed in document (2) which described plasmid pDI10 which was used in the present patent application as a source for the nucleotide sequence coding for restriction-modification activity. It was therefore not surprising that the introduction of a heterologous restriction-modification system into a

bacterial strain containing an active restriction-modification system resulted in a better protection against phage infection than that conferred by the bacterium's own system. The fact that a bacterium was resistant to a given phage did not imply that said bacterium was resistant to other phages. Despite its own restriction-modification system, the bacterium could be sensitive to a given phage. This implied that such a bacterium containing an active restriction-modification system could be rendered resistant to said given phage by introduction of a heterologous restriction-modification system.

III. The Appellants appealed against the decision of the Examining Division and paid the appeal fee. Further, they filed a written statement setting out the grounds for appeal.

Together with their grounds for appeal a new set of 16 claims was filed. Claims 1 and 11 read as follows:

"1. A recombinant DNA cloning vector that is useful for protecting a bacterium from bacteriophage **freely living in the environment** and containing double stranded DNA with a restriction site of specific activity, which comprises

1) a DNA segment comprising genes which co-ordinately and sequentially express in the bacterium
   a) a modification activity cognate to,
   b) a restriction activity of the same specificity as that of the restriction site,
2) a replicon that is functional in the bacterium, and
3) a gene that expresses a functional polypeptide **comprising a human or non-human hormone or precursor or segment thereof.**

11. A method for protecting a bacterium from a bacteriophage which comprises transforming the bacterium with the recombinant DNA vector of any of Claims 1 to 10 and culturing the transformed bacterium under large scale fermentation conditions subject to the limitations

1) that the bacteriophage is free living in the environment and capable of infecting large scale bacterial fermentations and contains double stranded DNA with a restriction site of the same specificity as the restriction activity conferred to the bacterium, and
2) that the modification activity is expressed in the bacterium prior to the restriction activity."

(Emphasis of amendments compared to originally filed Claims 1 and 12 by the Board.)

Claims 2 to 10 and 12 to 16 remained unamended compared to the refused claims.

Oral proceedings took place on 6 October 1992.

The Appellant's arguments may be summarised as follows:

The Appellants were the first to encounter the problem of bacteriophage infection of large-scale fermentation cultures in the development of the production of insulin by recombinant DNA technology, the first product so to be made. The problem resulted in considerable losses, and the inventors developed a remarkably successful and practical solution to it, which was in no way prefigured by the prior art relating to laboratory techniques. The only area in large-scale fermentation of micro-organisms where this phenomenon of phage infection had already been observed was the cheese production. Although phage infection in

this field constituted a permanent problem a solution to it was not provided.

An uncertainty surrounding large-scale fermentation at the date of the invention had been that the nature of the infecting bacteriophage was not known. Five categories of bacteriophage had been recognised but it was not known which ones were responsible for the infections.

The skilled person faced with the unexpected problem of bacteriophage infection in large-scale fermentation of bacterial cultures producing heterologous proteins would have had the choice among several other possibilities to eliminate the bacteriophage at the time the invention was made: Thorough cleaning of the equipment and hyperfiltering, measures which would not influence the bacterial culture or the phage as such; choice of host range-mutants of the respective bacterium which would then be resistant against a particular phage; production of mutants which contain a mutation in their surface protein pattern with the effect that the sites normally recognisable by a phage are altered so that the phage does not inject its DNA into the bacterium whereby again phage resistance was achieved, or a reverse restriction mutant of the bacterium which would recognise phages which had been modified before infecting the bacterial culture whereby the DNA of the bacteriophage would be destroyed completely; finally, change of the cation concentration of the culture medium by chelating compounds whereby the phage infection would not occur.

Each of these solutions to reduce bacteriophage infection of the fermentation growth bore advantages and disadvantages. It was, therefore, not at all an obvious step to choose the introduction of a new restriction-modification system into the bacterial cells bound to

produce heterologous protein in a large-scale production system.

These submissions were supported by three affidavits of Drs. Muth, Seno and Hershberger.

The Appellants request that the decision under appeal be set aside and that a patent be granted on the basis of Claims 1 to 16 submitted on 9 July 1990 in Appendix 1 to the grounds of appeal or in accordance with three auxiliary requests submitted on the same date.

Reasons for the Decision

1. The appeal is admissible.

2. Amendments (Article 123(2) EPC

The originally filed set of claims has been amended during the proceedings. The independent product and method Claims 1 and 11 now on file correspond with one exception to be discussed infra, to those refused by the Examining Division, which did not object to the claims on the basis of Article 123(2) EPC. The Board agrees to this position because they find their support in the originally filed specification as follows:

Claim 1:
New Claim 1 is the counterpart of originally filed Claims 1 and 12, because originally filed Claim 12 related to a recombinant DNA cloning vector as defined in any of Claims 1 to 6. Originally filed Claims 1 to 6 in turn relate to a method for protecting a bacterium from a naturally occurring bacteriophage comprising transforming said bacterium with the recombinant DNA cloning vector

having certain characteristics. Further, the wording "naturally occurring bacteriophage" is changed into the expression "that the bacteriophage is freely living in the environment". This definition is not used literally in the originally specification. Instead the bacteriophages from which the bacterial culture has to be protected are defined as "naturally occurring", for example in originally filed Claim 1. In the Board's opinion, both expressions are interchangeable and have the same meaning so that this amendment is admissible.

Claim 11:
This process claim differs from the originally filed Claim 1 firstly in that the transformed bacterium is cultured "under large-scale fermentation conditions". This feature is the subject-matter of the whole disclosure and in particular described on page 2, lines 3 to 11 of the originally filed description.

Secondly, this claim contains the expression "that the bacteriophage is freely living in the environment" which here, like in Claim 1, is equally allowable.

In contrast to the refused Claim 11, Claim 11 now on file does not contain the feature that the transformed bacterium carries its own restriction-modification system. Support for this is provided in the original disclosure in Example 1, mentioning E. coli K12 C600, $R_K^-M_K^-$ as host cell.

Claims 2 to 10 and 12 to 16 are the same as refused by the Examining Division. Also, these claims were held to be allowable according to Article 123(2) by the Examining Division and the Board agrees with this opinion.

Consequently, there are no objections based on Article 123(2) EPC.

3. Novelty (Article 54 EPC)

The Examining Division did not object to novelty of any claims and the Board agrees to this position. Because the amended claims do not alter this situation, novelty is not at issue.

4. Inventive step (Article 56 EPC)

4.1 Unlike the Examining Division, the Board considers document (3) to represent the closest prior art document.

4.2 In document (3) a method for polypeptide production involving expression of a heterologous gene, a recombinant microbial cloning vehicle containing said gene and a bacterial culture transformed by said cloning vehicle are described. In particular the expression of the insulin AB chains on a small scale is disclosed.

To produce a reasonable amount of pharmaceutically or otherwise interesting proteins by the method disclosed in document (3) it is desired to use large-scale fermentation. As stated in the specification of the present patent application, supported by the three affidavits mentioned above (see paragraph III), the skilled circles were faced with the difficulty that the large-scale fermentation cultures were destroyed by bacteriophage infection.

4.3 In the light of this prior art the problem to be solved is to protect bacterial cultures producing a heterologous protein in large-scale fermentation from bacteriophage infection.

A solution to this problem is represented by the subject-matter of Claims 1 and 11. The whole specification and the affidavits provide sufficient information to make it plausible that the problem is actually solved.

4.4 Since document (3) relates to the basic technique of expressing a heterologous protein in a host-micro-organism only and does not yet provide a disclosure of large-scale production of this protein, this document, consequently, does not provide any information how to solve the problem of protecting the bacteria containing a heterologous gene for the expression of a heterologous protein from being destroyed by a bacteriophage.

The question is thus whether or not the skilled person was aware from his common general knowledge or other prior art documents of the possibility to protect the bacterial cultures by introducing a restriction-modification system into the bacteria, and whether or not he would have done so.

4.5 The skilled persons, that is the research workers interested in systems to produce in high amounts restriction enzymes, when looking for a solution to the aforementioned problem, would have come across documents (1) and (2). Both these documents, which at the time of their publication were of interest for the newly developing scientific field of genetic engineering, relate to scientific research work in the field of investigation of restriction enzymes.

4.6 Document (1) relates to the cloning and expression of the Pst I restriction-modification system in Escherichia coli. Transformants of Escherichia coli carrying the Pst I gene system inserted into the cloning vector pBR322 were

selected on the basis of acquired resistance to bacteriophage infection. This research work resulted in the observation that such transformant bacteria produced approximately ten times more Pst I endonuclease activity than did the native strain. The aim of the study was thus achieved, i.e. to produce higher amounts of restriction enzymes which had become essential tools for the manipulation and characterisation of DNA molecules. The effective cloning of the restriction-modification system was tested by the efficiency of plating of a phage.

4.7 Document (2) relates to the cloning of restriction and modification genes in Escherichia coli, namely the HhaII system. Again, the purpose and context of this research work was to investigate restriction-modification systems by cloning the system and introducing it into a receptor bacterial cell. As stated on page 109 in the chapter "Discussion", second paragraph, the authors of this document considered that a selection from recombinant strains carrying a restriction-modification system could be based either on the detection of the new methylation, i.e. modification, or new restriction properties. The authors chose screening by phage restriction because the host cell had no restriction-modification system of its own and the pBR322 plasmid vector which was used as the transportation vehicle to introduce the cloned restriction-modification system into the host imparted no new restriction activity. Thus, the appearance of a restriction phenotype after introducing recombinant plasmids should be an indication of expression of restriction-modification system genes carried by the inserted DNA. Individual recombinants could, therefore, easily be selected for restriction on plates seeded with phage.

By introducing a new restriction-modification system into the bacterial culture a bacterial clone, previously having been deficient in restriction and modification, became effective in reduction of plating efficiency only with five different kinds of phages (see document (2), page 103, last paragraph, third and second to last sentence).

4.8 The technical teaching of both prior art documents (1) and (2) relates to the investigation and characterisation of restriction systems, in particular the respective restriction enzyme. In order to prove that the cloned restriction system had been transferred successfully, the host bacterium that is to be transformed by the cloned restriction-modification genes must necessarily be void of such restriction-modification systems because otherwise the successful transformation cannot be recognised.

4.9 As emphasised by the Appellants in their grounds for appeal and during oral proceedings and by the affidavits as well, the skilled person was aware of the fact that a working restriction-modification system can protect bacteria from being infected by phages and could have thought of the possibility to introduce this system into the large-scale fermentation culture bacterium, but they contended that he would not have done it for two major reasons, namely first, the disadvantages to be encountered when searching to introduce such a system into the bacterial cells in question, particularly in large-scale production, and second, the existence of more promising possibilities to protect the bacterial cultures in question.

4.10 The Board observes that in fact it is to be expected that when in a plasmid, carrying already a heterologous gene coding for a protein not belonging to those of the plasmid

or host cell themselves, an additional gene is cloned, the level of expression of the heterologous protein will be reduced. The production of additional proteins such as the enzymes responsible for a restriction-modification system could compete for the energy capability or the ribosome capacity of the cell. The cell has a limited capacity to synthesise proteins and once that capacity has been reached, the synthesis of one protein can only be increased at the expense of another. Furthermore, the gene coding for the heterologous protein could be interfered with by the modification capacity of the restriction-modification system. The consequences of introducing the restriction-modification system could not, therefore, be predicted. As the Appellants submitted during oral proceedings, actually the yield of the desired heterologous protein was reduced by about 10 to 20% in bacterial cultures bearing an additional restriction-modification system.

4.11 In addition to the expected reduction of yield of the heterologous protein when manipulating the cells such that an additional burden is put on the cells, there in another uncertainty of the result of the introduction of restriction-modification systems which would have to be thoroughly weighed, namely that the type of phages which destroyed the valuable fermentation cultures had to be considered as a "wild-type" phage of unknown characteristics. The restriction-modification systems described in documents (1) and (2) were, however, tested with the well-known and highly investigated laboratory type phage λ (documents (1) and (2) and five other laboratory strains O80, h424, P1, BF23 and T5 (document (2)). It was, therefore, not at all sure that a newly introduced restriction-modification system would work efficiently on the DNA injected by wild-type phages of unknown history and characteristics.

4.12 A further uncertainty surrounding large-scale fermentation at the date of the invention was that the nature of the infecting bacteriophage was not known. Thus, there was only a marginal chance that the idea of introducing a restriction-modification system would work in practice. The introduction of an additional restriction-modification system would not have created any protection against an infecting phage, if for example the phage had one or more of the following characteristics:

- sites which can be modified,
- sites which had previously been chemically modified, rendering them resistant,
- single stranded DNA, or
- RNA.

Other, more promising means to protect bacterial cultures in large-scale fermentation would have been contemplated by the skilled person.

4.13 These are, for example, the use of hyperfilters in the fermentation system in connection with an extraordinarily thorough cleaning of the equipment. This alternative may sound trivial but it has undoubtedly the advantage that no manipulation of the living material itself would be necessary. In cases of already highly developed bacterial strains having been manipulated for the expression of heterologous protein, therefore, the solution of physical elimination of infecting phages from the equipment can be seen as a reasonable step.

4.14 Another solution which does not involve manipulation of the living material consists of changing the concentration of cations in the fermentation medium. It is known that phage infection does not occur if this concentration is

not convenient. Although the disadvantage of this method may lie in the negative influence to the growth of the bacterial cells themselves, this method remains promising.

4.15 A further solution, which is called the standard way to solve the problems in question in the affidavit by Dr. Hershberger, would have been to prepare typically phage-resistant strains. This is done by selection of so-called hostrang-mutants or mutants with changed surface proteins. These mutants are more easily obtainable than cells bearing newly introduced genes encoding restriction and modification enzymes because there is no need for the cumbersome genetic engineering work. The induction and selection can be carried out by exposing a large number of bacteria to the phage and screening for new mutant colonies that survive the infection. The disadvantage of mutants of this kind is that resistance is achieved against only one phage. This disadvantage, however, would not have had a negative influence on the skilled person's choice of this solution because the type of the infecting phage was not known.

4.16 Consequently, the skilled person was faced with a number of promising solutions to his problem.

4.17 When considering the disclosure of documents (1) and (2) in the light of the "could" versus "would" approach, (see decision T 2/83, OJ EPO 1984, 265), the Board comes to the conclusion that the mere fact that both documents disclose the cloning of a restriction-modification system and its use as a selection marker does not mean that they contain the necessary hint to render the invention obvious because of the severe disadvantages to be expected from the solution now claimed. Neither was the intention of both prior art documents (1) and (2) to "protect"

bacterial cultures in large-scale fermentations, nor did the receptor cells in these experiments contain a foreign gene coding for a heterologous protein.

If one argues that the disadvantage of a reduction in yield of the desired heterologous protein is a preferred situation compared to a complete destruction of the fermentation culture which would result in no yield at all, one has to take into account that the man skilled in the art was faced with alternative solutions, which would not necessarily reduce the yield of the valuable and desired heterologous protein.

4.18 Nevertheless, after having chosen this way despite the cumbersome work, the expectation of a reduction in the yield of the heterologous protein and the uncertainty with respect to the phage's nature an unexpected effective protection was observed. As emphasised by Dr. Muth in his affidavit, the chosen method was "extremely effective in preventing infection under large-scale fermentation conditions". He reported that the method was effective in all of 77 tests carried out, nine of which involved fermentation volumes of 1000 litres or more. In addition fermentations were challenged with at least 9 bacteriophage types isolated from infected fermentation tanks, and all of the trials were completely successful. In the Board's opinion this is a result which was surprising in view of what could have been expected.

Faced with the several possibilities stated above to protect the fermentation culture from infection by bacteriophages, the skilled man thus would have balanced advantages and disadvantages of the means known to him. Because of the foreseeable disadvantages of the introduction of a restriction-modification system into the bacteria culture, the Board is of the opinion that this

option would not have been seriously contemplated, if considered at all.

4.19 Consequently, the subject-matter of Claims 1 and 11, the one being the means for protecting the bacterial cultures, the other being the method, has to be considered as inventive. Claims 2 to 10 and 12 to 14 are directly or indirectly dependent on independent Claims 1 and 11 respectively and are, consequently, also allowable. Claims 15 and 16 relate to a bacterium transformed with a cloning vector of any of Claims 1 to 10. Since the latter is patentable, it follows that these claims are allowable as well.

5. Since the set of claims of the main request is patentable there is no need to discuss the claims of the auxiliary requests.

Order

For these reasons, it is decided that:

1. The decision under appeal is set aside.

2. The case is remitted to the first instance with the order to grant the patent on the basis of Claims 1 to 16 submitted on 9 July 1990 in appendix 1 to the grounds of appeal (main request), pages 1 to 31 and 33 to 46 of the description as published, page 32 of the description as amended by letter of 23 June 1986 and drawings as published.

The Registrar: The Chairman:

P. Martorana P. Lançon

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