

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 November 2000 (30.11.2000)

PCT

(10) International Publication Number
WO 00/71731 A1

(51) International Patent Classification⁷: **C12N 15/73**,
C07K 14/60, C12P 21/02, C12N 7/00, 1/06 // C12R 1/19

(21) International Application Number: PCT/US00/40020

(22) International Filing Date: 24 May 2000 (24.05.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/318,288 25 May 1999 (25.05.1999) US

(71) Applicant: **PHAGE BIOTECHNOLOGY CORPORATION** [US/US]; 18300 Von Karman Avenue, Suite 440, Irvine, CA 92612 (US).

(72) Inventors: **KORDYUM, Vitaliy, A.**; Artyoma St. 53, Apartment 33, 254053 Kiev-053 (UA). **CHERNYKH, Svetlana, I.**; Lomonosova St. 29, Apartment 6, 252127 Kiev-127 (UA). **SLAVCHENKO, Irina, Y.**; Revutskogo St. 44, Apartment 81, 253068 Kiev-068 (UA). **VOZIANOV, Oleksandr, F.**; Desyatinnaya St. 10, Apartment 6, 252025 Kiev-025 (UA).

(74) Agent: **SIMPSON, Andrew, H.**; Knobbe, Martens, Olson and Bear, LLP, 620 Newport Center Drive, 16th floor, Newport Beach, CA 92660 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PHAGE-DEPENDENT SUPER-PRODUCTION OF BIOLOGICALLY ACTIVE PROTEIN AND PEPTIDES

(57) Abstract: This invention relates to a method for enhancing the production of soluble and biologically active proteins and peptides in bacterial cells by infecting bacterial cells of the producer strain, which contain a plasmid with one or more targeted genes, with bacteriophage λ with or without the targeted gene(s). The phage increases synthesis of the targeted protein and induces lysis of the producer strain cells. Super-production is achieved by cultivating the producer strain cells under culture conditions that delay lytic development of the phage. The biologically active proteins and peptides subsequently accumulate in a soluble form in the culture medium as the cells of the producer strain are lysed by the phage.



WO 00/71731 A1

PHAGE-DEPENDENT SUPER-PRODUCTION OF BIOLOGICALLY ACTIVE PROTEIN AND PEPTIDESBackground of the InventionField of the Invention

5 This invention relates to recombinant DNA technology and more particularly to a new method for enhancing the production of heterologous proteins in bacterial host cells. The disclosed method involves infecting host cells, which contain plasmid encoding the gene of interest, with bacteriophage λ to induce lysis of the bacterial host cells. Super-production may be achieved in selected host cells either when the plasmid alone carries at least one copy of the heterologous DNA or when both plasmid and phage λ each carry at least one copy of the heterologous DNA.

Description of the Related Art

10 At present, genetic engineering methods allow creating microorganism strains capable of producing substantial amounts of various bioactive substances having important applications in medicine and industry. Typically, plasmid vectors into which a heterologous gene has been inserted are used to transform bacterial host cells. Different strains of *E. coli* are frequently used as recipient cells. Using such plasmid-dependent transformation methods, *E. coli*
15 cells have been engineered to produce a variety of valuable human peptides and proteins, including insulin, γ -interferon, a number of interleukins, superoxidedismutase, plasminogen activator, tumor necrosis factor, erythropoietin, etc. These substances are either already used in medical practice or undergoing different stages of clinical studies.

 However, the plasmid method has serious disadvantages. It is technologically complicated, since the desired product has to be extracted from bacterial cells after biosynthesis, which is a multi-stage process. For example,
20 interferon extraction involves disintegration of cells, buffer extraction, polyethylene-imine processing, clarification, precipitation by ammonium sulfate, dialysis, and centrifugation (Goeddel, EP 0043980). The necessity for such extraction and purification steps not only complicates production technology of the recombinant product, but also results in substantial losses, especially during large-scale industrial production.

 A further complicating factor is that at relatively high levels of expression of the cloned genes, the eukaryotic
25 proteins generated tend to accumulate in the cytoplasm of *E. coli* as insoluble aggregates, which are often associated with cell membranes. Consequently, the already difficult extraction and purification methods discussed above must be supplemented with additional technical procedures related to the extraction of the insoluble inclusion bodies. Usually, the insoluble proteins are solubilized using ionic detergents, such as SDS or laurylsarcosine, at increased temperatures or in the presence of denaturants, such as 8 M urea or 6-8 M guanidine-HCl.

30 Often, the final stage of purification involves renaturation and reoxidation of the solubilized polypeptides, which is required to restore functional activity. Disulfide bonds, which are necessary for proper folding of the protein in its native conformation, must be reformed. Renaturation procedures, such as disulfide interchange, may use expensive and relatively toxic reagents, like glutathione, and oxidized 2-mercaptoethanol or dithiothreitol. Further, the final yield of bioactive genetically-engineered proteins may be relatively low. Moreover, the presence of even trace
35 concentrations of the toxic reagents needed to solubilize and then re-establish secondary and tertiary protein structure

may prohibit subsequent clinical application of the proteins. Thus, the generation of targeted protein in the form of insoluble inclusion bodies within the bacterial host cells not only complicates the production of recombinant proteins and results in diminished yield, but may also render the final protein unsuitable for clinical use (Fisher, B., Sumner, I., Goodenough, P. *Biotech. and Bioeng.* 41:3-13, 1993).

5 The technological difficulties associated with the extraction of proteins produced by the expression of heterologous genes from plasmid-transformed bacterial host cells may be overcome by infecting the transformed bacterial host cells with bacteriophage, whose lytic pathway results in lysis of the bearer cell. Thus, the desired protein may be simply released into the culture medium (Breeze A.S. GB 2 143 238A). Accordingly, Breeze disclosed a method of increasing the yield of enzyme produced in *E. coli* by infecting the bacterial cells with phage λ carrying a
10 temperature-sensitive mutation in *cl* to provide controlled lysis. The *cl*-gene product is a repressor of early transcription and consequently blocks transcription of the late region of the phage DNA, which is required for head and tail assembly and cell lysis (Mantatis, T., Fritsch, E.F., Sambrook, J., *MOLECULAR CLONING: A LABORATORY MANUAL*, 1982, Cold Spring Harbor Laboratory Press). Bacteriophages carrying a temperature-sensitive mutation in *cl* are able to establish and maintain the lysogenic state as long as the cells are propagated at a temperature that allows
15 the *cl*-gene product to repress transcription of phage genes necessary for lytic growth. Accordingly, the transformed bacterial host cells may be cultivated at 30° C, wherein the *cl*-mediated suppression of phage DNA transcription continues and the phage remains in the lysogenic state, until the stage of maximum ferment production is reached. Subsequently, the culture temperature may be increased to 42° C for 30 minutes in order to inactivate the *cl* repressor and permit the phage to begin its lytic development. The host cells may then be incubated for 2-3 hours at 30° C to
20 allow complete lysis and release of the enzyme (Breeze A.S. GB 2 143 238A).

 Although Breeze teaches release of proteins from bacterial producer cells, it requires cultivating producers at temperatures not exceeding 30° C, which is not the optimum temperature for growth of *E. coli* cells. Synthesis at the optimum temperature (37° C) is not possible, since cells at temperatures exceeding 32° C undergo lysis before reaching the stage of maximum ferment accumulation due to the development of temperature-sensitive lytic prophage.
25 Furthermore, incubation of the bacterial host cells at 42° C for 30 min as disclosed by Breeze may activate proteases that destroy the targeted protein.

 Auerbach et al. (U.S. Patent No. 4,637,980) used a phage λ DNA fragment for inducing lytic release of recombinant products. In that method, like Breeze, the temperature-sensitive mutation in λ *cl*-gene product was used to provide temperature-dependent lysis of the bacterial host cells. The λ DNA fragment in Auerbach maintained
30 functional endolysin-encoding genes, N, Q, R and S, for producing lysozyme following inactivation of the *cl* repressor at 42° C. Most of the remaining phage genes were deleted; mutations in O and P genes prevented replication of the phage DNA. Consequently, the λ DNA was not a fully functional phage, capable of modulating expression of the targeted gene. Moreover, the λ DNA of Auerbach was not suitable for use as a vector for carrying targeted genes.

Further, as discussed above, incubation of the bacterial host cells at 42° to 44° C for 90-120 min as disclosed by Auerbach may activate proteases that destroy the targeted protein.

In addition to providing for the lytic release of intact protein from bacterial producer cells, bacteriophages have also been used as an alternative to bacterial plasmid-based vectors, for carrying heterologous DNA into host bacterial cells. (Murray, N.E. and Murray, K., *Nature* 251:476-481, 1974; Moir, A., Brammar, W.J., *Molec. gen. Genet.* 149:87-99, 1976). Typically, amplification of genes and their products is achieved during lytic growth of the phage, wherein the phage genome is integrated into the bacterial host DNA (Panasenکو, S.M., Cameron, J.R., Davis, R.V., Lehman, L.R., *Science* 196:188-189, 1977; Murray, N.E. and Kelley, W.S., *Molec. gen. Genet.* 175:77-87, 1979; Walter, F., Siegel, M., Malke, H., 1990, DD 276,694; Mory, Y., Revel, M., Chen, L., Sheldon, I.F., Yuti-Chernajovsky, 1983, GB 2,103,222A). Usually, either lysogenic cultures of recombinant phage λ are used for infecting the bacterial host cells, or "warmed up" bacterial cultures, already harboring recombinant lysogenic phage λ , are grown up to amplify expression of the heterologous genes.

Although there are examples of the successful use of λ vectors for expression of heterologous genes, λ vectors have been used primarily for gene cloning. Once cloned, the genes are transferred to plasmid vectors for more effective expression. For example, when *E. coli* is infected by phage λ Charon 4A C15, containing the human β -interferon gene, the quantity of interferon in cell lysate constituted 7.8×10^6 units/liter. After the DNA fragment bearing targeted gene was recloned from phage to plasmid, β -interferon yield increased to 1×10^8 units/liter (Moir, A., Brammar, W.J., *Molec. gen. Genet.* 149:87-99, 1976).

To increase the yield of heterologous protein generated in bacterial host cells by recombinant λ vectors, mutations in the phage genome have been introduced that cause phage λ to lose its ability to initiate bacterial cell lysis. Enhanced yield is thereby achieved by extending the period of time during which the heterologous gene is expressed by the bacterial host cells. Thus, for example, the yield of DNA ligase 1 in lysogenic cultures containing λ gt4ligS prophage, with amber-mutation in the S gene, was five times greater than the yield of DNA ligase 1 in lysogenic cultures containing λ gt4lig prophage without the amber-mutation (Panasenکو, S.M., Cameron, J.R., Davis, R.V., Lehman, L.R., *Science* 196:188-189, 1977). The phage λ S protein is required for lysis; therefore S⁻ mutants accumulate large numbers of intracellular progeny phage particles, as well as the targeted protein, without lysing the host cells (Mantiatis, T., Fritsch, E.F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press).

Similar increases in the yield of DNA polymerase 1 were reported for lysogenic cultures containing recombinant phage λ with amber-mutations in the S and Q genes, compared to recombinant phage λ without the amber-mutations (Murray, N.E. and Kelley, W.S., *Molec. gen. Genet.* 175:77-87, 1979). The phage λ Q protein is required for transcription of the late region of the phage DNA, which includes many genes involved in head and tail assembly and cell lysis. (Mantiatis, T., Fritsch, E.F., Sambrook, J., MOLECULAR CLONING: A LABORATORY MANUAL, 1982, Cold Spring Harbor Laboratory Press).

In U.S. Patent No. 4,710,463, Murray discloses lysogenizing a non-suppressing (Su^0) strain of *E. coli* with phage λ containing the temperature-sensitive mutation in *cl*, as well as mutations in λ S and E genes. Consequently, prolonged cultivation of the lysogenic *E. coli* at 37° C leads to high levels of production of the recombinant protein, which is retained within the cells, since these are not lysed by phage gene products in the normal way, and since the recombinant phage genome is not encapsidated, it remains available for transcription.

Despite the enhanced yield of heterologous proteins possible using λ -vectors with S and E mutations, the potential technical advantages of bacteriophage vectors related to the lytic release of targeted proteins, may be lost with these mutations, because the targeted proteins accumulate inside the bacterial cell. Thus, when a lysis-defective mutant λ -vector is used for production of heterologous protein, the extraction and purification steps, discussed above for bacterial cells transformed by plasmid vectors, along with the resultant losses, must be performed.

Summary of the Invention

The present invention relates to a method for producing a soluble and biologically active protein. The method comprises the steps of transforming an *E. coli* host cell with a plasmid having at least one copy of an expressible gene encoding the protein. The host cell is infected with a bacteriophage λ capable of growth without lysis at a permissive temperature and also capable of mediating lysis at a restrictive temperature. The host cells are cultivated at the permissive temperature until a level of production of the protein is at least 100 μ g/ml. The host cells are then lysed to release the soluble and biologically active protein by cultivating the host cell at the restrictive temperature.

The bacteriophage λ has a temperature-sensitive mutation, preferably, the temperature-sensitive mutation is *cl*₈₅₇. The restrictive temperature is greater than 32° C. The permissive temperature is less than about 32° C.

In one aspect of the present method, the bacteriophage λ has a mutation in at least one gene, the expression of which at the restrictive temperature mediates lysis of the host cell. The at least one gene may be selected from the group consisting of N, Q and R.

In one aspect of the present method, the *E. coli* host cell produces a suppressor for the repair of amber-mutations. Alternatively, in accordance with another aspect of the present method, the *E. coli* host cell lacks a suppressor for the repair of amber-mutations.

The infecting bacteriophage λ may be provided at a multiplicity of infection in a range of about 1 to about 100. More preferable, the infecting bacteriophage λ is provided at a multiplicity of infection in a range of about 10 to about 25, wherein lysis of the host cell at the restrictive temperature is delayed at higher multiplicities of infection relative to lower multiplicities of infection.

In a preferred mode of the present method, the bacteriophage λ contains at least one copy of the expressible gene encoding the protein.

The present invention is also related to an *E. coli* host cell, which is adapted to produce a soluble and biologically active protein at a concentration of at least 100 μ g/ml. The host cell has a plasmid with at least one copy

of an expressible heterologous gene encoding the protein. The host cell also contains a bacteriophage λ capable of growth without lysis at a permissive temperature and also capable of mediating lysis at a restrictive temperature.

The bacteriophage λ contained in the *E. coli* host cell of the present invention may have a temperature-sensitive mutation, preferably, the temperature-sensitive mutation is cl_{857} .

5 The bacteriophage λ contained in the *E. coli* host cell may also have a mutation in at least one gene, the expression of which at the restrictive temperature mediates lysis of the host cell. The at least one gene may be selected from the group consisting of N, Q and R.

In one preferred mode, the *E. coli* host cell contains a bacteriophage λ with cl_{857} , $Q_{am 117}$ and $R_{am 54}$ mutations.

10 In accordance with another preferred aspect of the *E. coli* host cell, the bacteriophage λ has at least one copy of an expressible heterologous gene encoding the protein.

In one mode, the *E. coli* host cell may lack a suppressor for repairing amber-mutations. In another mode, the *E. coli* host cell may be *recA* deficient.

In another preferred aspect of the present invention, the expressible heterologous gene encodes human alpha-2b interferon.

15

Detailed Description of the Preferred Embodiment

Bacteriophage λ is useful as a vector because more than 40% of the viral genome is not essential for lytic growth. This area of the λ genome, located in the central region of the λ DNA, between genes J and N, may be replaced by heterologous DNA encoding a desired product. That region is transcribed early during infection.

20 In order to maximize the expression of a targeted gene, whose synthesis information is recorded in the area of phage's early genes, special conditions for the phage's development must be provided to ensure proper replication. Further, transcription of the early area, containing the targeted gene, should be fostered, while transcription of the later genes, involved in cell lysis, should be decelerated. This slows down maturation of the λ particles and subsequent cell lysis. Consequently, the early phage products, including the targeted gene product, will accumulate in the bacterial cells. Deceleration of late transcription, thereby extending expression of the targeted gene, may be accomplished by: (1) mutations of phage genome that block expression of the later genes (2) increased multiplicity of infection, and/or (3) cultivation of the infected bacterial cells at a reduced temperature.

25

An important advantage of infecting producer cells with a bacteriophage is that the phage causes a profound rearrangement of all macromolecular synthesis in the bacterial host cells. By turning off transcription of bacterial genes, phages may increase the copying of the targeted gene, and consequently, increase the output of desired product.

30

In a preferred embodiment of the present super-production system, phage λ with amber-mutations that delay bacterial lysis (e.g., Q and R mutations) are provided in a strain of *E. coli*, designated Su^0 , which lacks the suppressor responsible for correcting amber-mutations in phage λ . In order to obtain a non-suppressing (Su^0) strain of *E. coli*, Su^0

clones are selected from the wild-type Su^+ population. Preferably, a selection marker is inserted into the phage DNA, e.g., tetracycline or ampicillin resistance.

Selection of Bacterial Strains

5 Selection of non-suppressing (Su^0) strains of *E. coli*, for example, *E. coli* K 802 was carried out with phage λ cl_{857} Nam7Nam53 bla tet (hereinafter λ bla N'). Strain *E. coli* C600 (λ bla N') served as source of the phage. This phage was obtained by insertion of plasmid pCV 11 (bla tet) at EcoRI site into single-site (EcoRI) vector carrying ts-mutation in repressor gene (cl_{857}). Then two amber-mutations were introduced into the phage N gene by recombination *in vivo*.

10 Clones were tested for non-lysogenicity with phage λ clear. In addition to phage λ bla N', phage λ cl_{857} $Q_{am117}R_{am54}$ was used to check for suppressor.

Media – Liquid nutrient media, LB and M9 as well as agar medium LB were used for bacterial culture growth (Miller J.H., 1972, Experiments in molecular genetics, Spring Cold Harbor, N.Y.).

15 Preparation of Phage Lysate – Lysogenic culture was grown in broth at 28° C under intense aeration to a density of 2×10^8 cells/ml followed by incubation at 43° C for 20 min. Then it was kept at 37° C under intense aeration. Cells were lysed in 60-80 min and phage was released into the cultural medium. Phage titer was estimated by a conventional two-layer technique. As a rule, 2×10^{10} PFU/ml of phage lysate were obtained.

20 As is known, phage λ N' mutant is not able to lyse the host cells and is present in cells in the form of extremely unstable plasmids. If the host cells contain suppressor, the amber-mutation is phenotypically corrected, the N protein is synthesized and the phage can develop lytically. This difference in the viability of Su^+ and Su^0 cells, infected by λ N', is used as a basis for selection of spontaneously appearing Su^0 revertants from the *E. coli* Su^+ cell population. Phage λ with an inserted plasmid that introduced the ampicillin and tetracycline resistance markers into cells was used to prevent the nonlysing Su^0 cells from masking the search for mutants. The phage also contains ts-mutation in the repressor gene that permits lytic development of such phage resulting in cell lysis.

25 If the medium supplemented with ampicillin and tetracycline is inoculated with Su^+ culture after its infection with phage λ bla N' with subsequent growth at 43° C, single suppressor-free cells containing phage λ bla N' in the form of plasmids must develop on plates. Su^0 derivatives of the parent cultures are obtained by curing the cells from the phage. The method can be subdivided into several stages.

1. Infection of Culture With Phage λ bla N'

30 The culture *E. coli* Su^+ was grown on the M9 medium with maltose at 37° C under intense agitation to a density of 1.2×10^8 cells/ml. The cells were infected with phage λ bla N' at a multiplicity of 5-10 particles per cell and incubated for 20 min at 20° C. Under given conditions, the infection efficiency is about 100%, in addition to the bulk of Su^+ cells, the phage also infects single Su^0 cells.

2. Selection of Suppressor-Free Cells Containing Marker Phage

After infection, cells were plated out on agar medium supplemented with 12 γ /ml tetracycline and 20 γ /ml ampicillin and grown at 43° C. In 24 h, single colonies developed, which were replated on agar medium with antibiotics and grown at 37° C.

3. Curing of the Selected Clones From Phage λ bla N'

Since phage λ N' in the *E. coli* Su^o cells is in the form of extremely unstable plasmids, in order to cure from the phage the selected clones were plated on selective agar medium without antibiotics and grown at 37° C. The number of cells that had lost the phage in the first passage on the medium without antibiotics amounted to 12-35%. The selection of such cells was carried out by monitoring the loss of antibiotic resistance and the acquisition of sensitivity to phage λ clear.

4. Testing of Cells for Repressor

The ability of phage λ with amber-mutations to form plaques on lawns of cured clones was checked. Isogenic suppressor-free derivatives of the parent *E. coli* Su⁺ strains are clones, on which phage λ bla N' did not form plaques, phage λ cl₈₅₇ Q_{am117} R_{am54} produced 1-3 x 10⁵ PFU/ml, and phage λ cl₈₅₇ without mutations in genes Q and R produced 1 x 10¹⁰ PFU/ml.

Using this method, Su^o revertants of *E. coli* K 802 Su⁺ were obtained. Based on the cell number at the moment of infection and the number of Su^o revertants among them, the frequency of occurrence of suppressor-free cells was about 3 x 10⁻⁷.

The use of suppressing (Su⁺) and non-suppressing (Su^o) host strains together with phage λ to achieve super-production of heterologous proteins and peptides may be more fully understood from the following working examples.

Example 1

Increased Synthesis of β -Lactamase in *E. coli* Transformed with pBR322 Carrying the β -Lactamase Gene (bla) following Infection by Phage λ

Bacterial cells, *E. coli* C-600 Su⁺, were transformed with plasmid pBR322-bla and cultivated in *Amino-peptid* medium (manufactured at the Leningrad meat processing and packing factory), diluted 1:1 in 0.15 M NaCl. The C-600 Su⁺/pBR322-bla transformants were then grown at 37° C to a density of 1 x 10⁸ cells/ml and divided into three portions. The control portion was left intact. The second portion was infected with phage λ having the temperature-sensitive mutation in cl, designated λ cl₈₅₇, and the third portion was infected with phage λ having the temperature-sensitive mutation in cl, as well as amber-mutations in the Q and R genes, designated λ cl₈₅₇ Q_{am 117} R_{am 54}. Phage mutations were accomplished by standard recombinant method *in vivo*. Phage multiplicity was approximately 10 phage bodies per 1 bacterial cell. The λ -treated cultures were incubated for 15 min at 37° C to inactivate the cl repressor, and then for 19 hr at 28° C. The control cultures were incubated at 37° C for the entire period. β -Lactamase activity was determined by iodometric assay as described by Chaykovakaya, S.M. and Venkina, T.G.

Antibiotics 7(5):453-456, 1962. A unit of activity is defined as the minimum quantity of ferment necessary to inactivate 1×10^{-7} M penicillin (60 units) in 1 hr at 37° C, pH 6.8-7.0.

Table 1

	Bacterial Cell Culture	β -Lactamase Activity (Units)
1	C-600 Su ⁺ /pBR322-bla	833
2	C-600 Su ⁺ /pBR322-bla + λ cl ₈₅₇	4400
3	C-600 Su ⁺ /pBR322-bla + λ cl ₈₅₇ Q _{am 117} R _{am 54}	8300

5 As shown in Table 1, β -lactamase synthesis in the C-600 Su⁺/pBR322-bla cells infected by phage λ with mutations in the later genes, Q and R, is almost 10 times greater than the synthesis in C-600 Su⁺/pBR322-bla control cells.

Example 2

Increased Output of β -Lactamase Encoded by bla-Gene

Contained in Both Plasmid and Phage

10 Cultures of *E. coli* W 3101 recA⁻13 Su^o with and without transformation by pBR322-bla were cultivated in *Aminopeptid* medium diluted 1:1 with 0.15 M NaCl, at 37° C to a density of 1×10^8 cells/ml. A recA⁻ strain was used because these cells have a reduced ability to conduct recombination in areas of extended homology in both plasmid and
 15 phage (e.g. bla-gene). Thus, recA⁻ cultures avoid exclusion of the homologous bla-gene. The cultures were divided into two portions. The first portion, which was not exposed to phage, was incubated at 37° C for 16 hr. The second portion was infected with phage λ cl₈₅₇ bla Q_{am 117} R_{am 54} at a multiplicity of about 10 phage bodies per 1 bacterial cell and cultivated for 2.5-3 hr at 37° C, and then for an additional 14 hr at 28° C. β -Lactamase activity was measured by the iodometric method. The results, shown in Table 2 (below), are expressed in units, as defined above for Table 1.

20 Phage λ cl₈₅₇ bla Q_{am 117} R_{am 54} was prepared from lysogenic cultures maintained at 28° C in *Aminopeptid* medium. When the bacterial cell density reached about 1×10^8 cells/ml, the cells were warmed for 20 minutes at 43° C in order to inactivate the cl repressor. Consequently, the prophage is excised from the bacterial genome and begins its lytic development. After 50 min, the cells underwent lysis, releasing 100-200 bodies each. At a density of 1×10^8 cells/ml, the cultures produced $1-2 \times 10^{10}$ phage bodies per ml. Thus, to infect bacterial cells with phage at a
 25 multiplicity of about 10 means that 1 ml of phage lysate (1×10^{10} phage bodies) was added to 10 ml of bacterial suspension (1×10^9 cells).

Table 2

	Bacterial Cell Culture	β -Lactamase Activity (Units)
1	W 3101 recA ⁻ 13 Su ^o /pBR322-bla	13,555

2	W 3101 recA ¹³ Su ^o + λ cl ₈₅₇ bla Q _{am 117} R _{am 54}	227,796
3	W 3101 recA ¹³ Su ^o /pBR322-bla + λ cl ₈₅₇ bla Q _{am 117} R _{am 54}	2,000,000

As shown in Table 2, bacterial cells which were transformed with both plasmid containing the targeted gene and phage carrying the same gene, produced about 10 times more recombinant protein (β -lactamase) than bacterial cells transformed with phage alone, and over 100 times more β -lactamase than bacterial cells transformed by plasmid alone.

Example 3

Super-Production of the β -Galactosidase Encoded by lac-Gene

Contained in Both Plasmid and Phage

Cultures of *E. coli* RLMI containing prophage λ cl₈₅₇ plac5 Q_{am 117} R_{am 54} (carrying a copy of the β -galactosidase gene, lac5) were grown in LB medium (Difco) at 30° C with intensive aeration to a density of approximately 1×10^8 cells/ml. The lysogenic culture was warmed to 43° C and incubated for 20 minutes to inactivate cl repressor. The temperature was then decreased to 37° C and the bacterial cells underwent lysis, with phages being formed at 1.2×10^{10} PFU/ml. Subsequently, 10 liters of phage lysate, containing about 1×10^{10} phage bodies (λ cl₈₅₇ plac5 Q_{am 117} R_{am 54}) per ml, were added to 40 liters of a suspension of *E. coli* Ca 77 Su^o transformed by plasmid pZ56 at a density of about 1×10^8 cells/ml in LB medium. Thus, the multiplicity of infection was 25, i.e., there were 25 phage bodies per bacterial cell.

After 7 hr at 37° C, recombinant β -galactosidase constituted 1.9 g per liter of culture medium. The activity of β -galactosidase was calculated by the method of Miller (Miller, J.H., EXPERIMENTS IN MOLECULAR GENETICS, 1972, Cold Spring Harbor Laboratory Press). A unit of activity was calculated as the minimum quantity of ferment required to hydrolyze 1 μ M ortho-nitrophenyl- β -D-galactoside to ortho-nitrophenol per min at 30° C, pH 7.0.

Example 4

Super-Expression of Human Interferon α -2b

Su⁺ and Su^o strains of *E. coli* K 802, transformed with a plasmid bearing a single copy of the gene encoding alpha-2 human interferon (pIF-2-trp), were grown in LB medium to a density of 1.5×10^8 cells/ml. These cells were then infected at a multiplicity of 15 with different phage λ lysates, as indicated in Table 3 (below). Cultivation continued with intensive aeration at 25° C for 13 hr. Control cultures, not infected with phage, were incubated at 37° C for the same period.

Table 3

	Bacterial Cell Culture	Phage	Interferon (Units/L)
1	K 802-pIF-2-trp Su ⁺		8.0×10^7

2	K 802-pIF-2-trp Su ⁺	λcl_{857}	77×10^7
3	K 802-pIF-2-trp Su ⁺	$\lambda cl_{857} Q_{am 117} R_{am 54}$	340×10^7
4	K 802-pIF-2-trp Su ⁺	$\lambda \cdot pIF-2-trp cl_{857} Q_{am 117} R_{am 54}$	1400×10^7
5	K 802-pIF-2-trp Su ^o	$\lambda \cdot pIF-2-trp cl_{857} Q_{am 117} R_{am 54}$	3000×10^7

As shown in Table 3, when bacterial cells which were transformed with plasmid containing the targeted gene were infected with phage λ containing only the temperature-sensitive mutation in *cl*, interferon expression increased by about 10-fold compared to control, non-infected cultures. Adding the amber-mutations in Q and R genes further increased expression by about 40-fold compared to control bacterial cells. Adding a copy of the interferon gene to phage λ with *cl*, Q and R mutations increased interferon synthesis by 175-fold over controls. Finally, when a non-suppressing, Su^o, strain of *E. coli*, transformed by a plasmid bearing a copy of the interferon gene, was infected with phage λ , also having a copy of the interferon gene, as well as *cl*, Q and R mutations, the bacterial host cells produced about 375 times more recombinant protein than the control cells transformed by plasmid alone.

Example 5

Enhanced Recovery of Biologically Active Recombinant Interferon by

Phage-Mediated Host Cell Lysis

Strain *E. Coli* SG 20050 was transformed by a plasmid bearing two copies of the human interferon alpha-2b gene (pIF-14) by standard methods. The transformant cells were grown up in 80 liters of LB medium at 37° C with intensive aeration to a density of 2×10^8 cells/ml. The culture was divided into two portions. The first was not infected with phage. The second was infected with phage λ lysate harvested from *E. coli* K 802/ $\lambda cl_{857} Q_{am 117} R_{am 54}$ at a multiplicity of 10 phage bodies per bacterial cell. The control cells were incubated for 19 hr at 37° C and the phage-infected cells were incubated for 19 hr at 21° C.

Interferon production in both control and phage-infected cultures was about 20% of the total cellular protein. However, the interferon in control cells was associated at least in part with insoluble inclusion bodies. Thus, it was not possible to determine its biological activity without solubilization, denaturation and renaturation. In contrast, the specific activity of the soluble interferon released into the medium following phage-mediated cell lysis, was readily determined by standard immunoenzyme assay. The interferon activity was 4×10^{10} IU/liter (200 mg/liter).

Pre-clinical toxicological studies of recombinant human alpha-2 β interferon produced by the phage super-production method of the present invention showed that the compound was practically non-toxic. Intra-abdominal and intra-muscular injections of the recombinant interferon in white mice and Wistar rats at 8.5×10^9 ME/kg (2.5×10^5 times the maximum human dose) and intravenous injections in mice and rabbits at 4.25×10^9 ME/kg (1.25×10^5 times the human therapeutic dose) produced no pronounced intoxication or death of the animals. Four months of injections in Wistar rats at 6×10^5 , 6×10^6 and 3×10^7 ME/kg (18, 180 and 900 times the human therapeutic dose, respectively)

showed no damage to the main organs and bodily systems of the experimental animals. Likewise, three months of intravenous injections in rabbits of 6×10^5 and 6×10^6 ME/kg, and two months of intra-muscular injections in dogs at 3×10^6 ME/kg showed no signs of damage to the organ systems.

5 During immunotoxic and allergenic analysis of recombinant interferon, the induction of cellular and humoral immunity reactions, as well as delayed and immediate hypersensitivity reactions were studied. The results indicated that no immunotoxic or allergenic influence was produced. The recombinant interferon also had no mutagenic or DNA-damaging effects in bacteria during metabolic activation *in vitro* or in bone marrow of mouse embryos *in vivo*.

10 Embryotoxic studies of recombinant interferon were conducted in pregnant hamadryad baboon females. Daily intra-muscular doses during organogenesis (20th to 50th days of pregnancy) caused defects in embryo development leading to miscarriage or stillbirth. Similar results were obtained for recombinant interferon analogs, and most probably could be explained by a powerful antiproliferative action of interferons. It is possible that the miscarriage may be attributed to a "cancellation" of immunologic tolerance of maternal organism towards the fetus, caused by immuno-modulating action of the protein.

15 Recombinant interferon was also studied in Ukrainian clinics. Based on these clinical studies, the recombinant interferon was shown to be useful in the treatment of a variety of human diseases and conditions. For example, recombinant interferon was effective in treating acute and chronic hepatitis B, acute viral, bacterial and mixed infections, acute and chronic septic diseases, herpetic infections, herpes zoster, papillomatosis of larynx, multiple sclerosis, and various cancers, including melanoma, renal cell carcinoma, bladder carcinoma, ovarian carcinoma, breast cancer, Kaposi's sarcoma and myeloma.

20 The contraindications in human clinical applications were prolonged (several months) use at high doses, allergy and pregnancy. The possible side effects noted were small and transitory "flu-like" symptoms and at prolonged regimens, leuko and trombocytopenia were marked.

25 While we have described a number of embodiments of this invention, it is apparent that our description of the invention can be altered to provide other embodiments that utilize the basic process of this invention. Therefore, it will be appreciated by those of skill in the art that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments that have been described in detail above by way of example.

WHAT IS CLAIMED IS:

1. A method for producing a soluble and biologically active protein, comprising:
transforming an *E. coli* host cell with a plasmid having at least one copy of an expressible gene
encoding said protein;
5 infecting the transformed host cell with a bacteriophage λ capable of growth without lysis at a
permissive temperature and also capable of mediating lysis at a restrictive temperature;
cultivating the host cell at the permissive temperature until a level of production of said protein is
at least 100 $\mu\text{g/ml}$; and
lysing the host cell to release the soluble and biologically active protein by cultivating the host cell
10 at the restrictive temperature.
2. The method of claim 1, wherein the bacteriophage λ has a temperature-sensitive mutation.
3. The method of claim 2, wherein the temperature-sensitive mutation is cl_{857} .
4. The method of claim 1, wherein said culture condition that induces lytic growth of the
bacteriophage λ is at a temperature of greater than 32° C.
- 15 5. The method of claim 1, wherein the permissive temperature is less than about 32° C.
6. The method of claim 1, wherein the bacteriophage λ has a mutation in at least one gene, the
expression of which at the restrictive temperature mediates lysis of the host cell.
7. The method of claim 6, wherein the at least one gene is selected from the group consisting of N, Q
and R.
- 20 8. The method of claim 1, wherein the *E. coli* host cell produces a suppressor for the repair of amber-
mutations.
9. The method of claim 1, wherein the *E. coli* host cell lacks a suppressor for the repair of amber-
mutations.
10. The method of claim 1, wherein the infecting bacteriophage λ is provided at a multiplicity of
25 infection in a range of about 1 to about 100.
11. The method of claim 1, wherein the infecting bacteriophage λ is provided at a multiplicity of
infection in a range of about 10 to about 25.
12. The method of claim 1, wherein lysis of the host cell at the restrictive temperature is delayed at
higher multiplicities of infection relative to lower multiplicities of infection.
- 30 13. The method of claim 1, wherein the bacteriophage λ contains at least one copy of an expressible
gene encoding said protein.
14. An *E. coli* host cell adapted to produce a soluble and biologically active protein at a concentration of
at least 100 $\mu\text{g/ml}$, said host cell having a plasmid with at least one copy of an expressible heterologous gene encoding

the protein, wherein said host cell contains a bacteriophage λ capable of growth without lysis at a permissive temperature and also capable of mediating lysis at a restrictive temperature.

15. The *E. coli* host cell of claim 14, wherein the bacteriophage λ has a temperature-sensitive mutation.

5 16. The *E. coli* host cell of claim 14, wherein the temperature-sensitive mutation is cl_{857} .

17. The *E. coli* host cell of claim 14, wherein the bacteriophage λ has a mutation in at least one gene, the expression of which at the restrictive temperature mediates lysis of the host cell.

18. The *E. coli* host cell of claim 17, wherein the at least one gene is selected from the group consisting of N, Q and R.

10 19. The *E. coli* host cell of claim 14, wherein the bacteriophage λ has cl_{857} , $Q_{am 117}$ and $R_{am 54}$ mutations.

20. The *E. coli* host cell of claim 14, wherein the bacteriophage λ has at least one copy of an expressible heterologous gene encoding the protein.

21. The host cell of claim 14, wherein the strain of *E. coli* lacks a suppressor for repairing amber-mutations.

15 22. The host cell of claim 14, wherein the strain of *E. coli* is *recA* deficient.

23. The host cell of claim 14, wherein the protein is human alpha-2b interferon.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/40020

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/73 C07K14/60 C12P21/02 C12N7/00 C12N1/06
//C12R1/19

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N C12P C12R

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

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

EP0-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 143 238 A (BREEZE DR ANTHONY STUART) 6 February 1985 (1985-02-06) cited in the application page 1, column 1 ---	1-5, 9-12, 14-16, 21,23
X	DATABASE WPI Section Ch, Week 198449 Derwent Publications Ltd., London, GB; Class D16, AN 1984-306248 XP002148368 & SU 1 089 119 A (AS UKR MOLECUL BIOL), 30 April 1984 (1984-04-30) abstract ---	1-22
A	--- -/--	23



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

26 September 2000

Date of mailing of the international search report

09/10/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

van Klompenburg, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/40020

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEN B -Y ET AL: "Temperature induction of bacteriophage lambda mutants in Escherichia coli" JOURNAL OF BIOTECHNOLOGY, NL, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 40, no. 2, 1 June 1995 (1995-06-01), pages 87-97, XP004036957 ISSN: 0168-1656 figures 1,3; table 1 ---	1-23
A	EP 0 140 864 A (SMITHKLINE BECKMAN CORP) 8 May 1985 (1985-05-08) page 6, line 5 -page 7, line 10 claims 1-46; examples 1-9 ---	1-23
A	EP 0 372 707 A (PESTKA SIDNEY) 13 June 1990 (1990-06-13) page 21, line 25 - line 32; claims 1-51 -----	23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/40020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2143238 A	06-02-1985	NONE	
SU 1089119 A	30-04-1984	NONE	
EP 0140864 A	08-05-1985	US 4637980 A AT 51024 T AU 583014 B AU 3163884 A CA 1219825 A DE 3481629 D DK 380684 A ES 534963 D ES 8602124 A ES 543207 D ES 8703524 A FI 89507 B GR 80053 A HU 35010 A, B IE 57797 B IL 72623 A JP 7087787 B JP 7115974 A JP 1970292 C JP 6098018 B JP 60062985 A JP 1993922 C JP 6121691 A JP 7014348 B NO 843183 A, B, NZ 209128 A NZ 225296 A PT 79045 A ZA 8406132 A ZW 12684 A	20-01-1987 15-03-1990 20-04-1989 14-02-1985 31-03-1987 19-04-1990 10-02-1985 01-11-1985 01-03-1986 16-02-1987 01-05-1987 30-06-1993 12-12-1984 28-05-1985 07-04-1993 29-04-1990 27-09-1995 09-05-1995 18-09-1995 07-12-1994 11-04-1985 22-11-1995 06-05-1994 22-02-1995 11-02-1985 26-06-1990 26-06-1990 01-09-1984 26-06-1985 21-11-1984
EP 0372707 A	13-06-1990	AT 135014 T CA 2001719 A DE 68925869 D DE 68925869 T ES 2087869 T GR 3020060 T IL 92124 A JP 2288900 A US 5986061 A	15-03-1996 28-04-1990 11-04-1996 14-11-1996 01-08-1996 31-08-1996 31-10-1996 28-11-1990 16-11-1999