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[54] **PROCESS FOR PRODUCING BIOLOGICALLY FUNCTIONAL MOLECULAR CHIMERAS**

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Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 959,288, Nov. 9, 1978, which is a continuation-in-part of Ser. No. 687,430, May 17, 1976, abandoned, which is a continuation-in-part of Ser. No. 520,691, Nov. 4, 1974.

[51] **Int. Cl.**[3] **C12P 21/00**

[52] **U.S. Cl.** .... **435/68;** 435/172; 435/231; 435/183; 435/317; 435/849; 435/820; 435/91; 435/207; 260/112.5 S; 260/27R; 435/212

[58] **Field of Search** .... 195/1, 28 N, 28 R, 112, 195/78, 79; 435/68, 172, 231, 183

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[57] **ABSTRACT**

Method and compositions are provided for replication and expression of exogenous genes in microorganisms. Plasmids or virus DNA are cleaved to provide linear DNA having ligatable termini to which is inserted a gene having complementary termini, to provide a biologically functional replicon with a desired phenotypical property. The replicon is inserted into a microorganism cell by transformation. Isolation of the transformants provides cells for replication and expression of the DNA molecules present in the modified plasmid. The method provides a convenient and efficient way to introduce genetic capability into microorganisms for the production of nucleic acids and proteins, such as medically or commercially useful enzymes, which may have direct usefulness, or may find expression in the production of drugs, such as hormones, antibiotics, or the like, fixation of nitrogen, fermentation, utilization of specific feedstocks, or the like.

14 Claims, No Drawings

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PROCESS FOR PRODUCING BIOLOGICALLY FUNCTIONAL MOLECULAR CHIMERAS

The invention was supported by generous grants of NIH, NSF and the American Cancer Society.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuatin-in-part of applicatin Ser. No. 959,288, filed Nov. 9, 1978, which is a continuation of application Ser. No. 687,430 filed May 17, 1976, now abandoned, which was a continuation-in-part of application Ser. No. 520,691, filed Nov. 4, 1974, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

Although transfer of plasmids among strains of *E. coli* and other Enterobacteriaceae has long been accomplished by conjugation and/or transduction, it has not been previously possible to selectively introduce particular species of plasmid DNA into these bacterial hosts or other microorganisms. Since microorganisms that have been transformed with plasmid DNA contain autonomously replicating extrachromosomal DNA species having the genetic and molecular characteristics of the parent plasmid, transformation has enabled the selective cloning and amplification of particular plasmid genes.

The ability of genes derived from totally different biological classes to replicate and be expressed in a particular microorganism permits the attainment of interspecies genetic recombination. Thus, it becomes practical to introduce into a particular microorganism, genes specifying such metabolic or synthetic functions as nitrogen fixation, photosynthesis, antibiotic production, hormone synthesis, protein synthesis, e.g. enzymes or antibodies, or the like—functions which are indigenous to other classes of organisms—by linking the foreign genes to a particular plasmid or viral replicon.

BRIEF DESCRIPTION OF THE PRIOR ART

References which relate to the subject invention are Cohen, et al., Proc. Nat. Acad, Sci., USA, 69, 2110 (1972); ibid, 70, 1293 (1973); ibid, 70, 3240 (1973); ibid, 71, 1030 (1974); Morrow, et al., Proc. Nat. Acad. Sci., 71, 1743 (1974); Novick, Bacteriological Rev., 33, 210 (1969); and Hershfeld, et al., Proc. Nat. Acad. Sci., in press; Jackson, et al., ibid, 69, 2904 (1972);

SUMMARY OF THE INVENTION

Methods and compositions are provided for genetically transforming microorganisms, particularly bacteria, to provide diverse genotypical capability and producing recombinant plasmids. A plasmid or viral DNA is modified to form a linear segment having ligatable termini which is joined to DNA having at least one intact gene and complementary ligatable termini. The termini are then bound together to form a "hybrid" plasmid molecule which is used to transform susceptible and compatible microorganisms. After transformation, the cells are grown and the transformants harvested. The newly functionalized microorganisms may then be employed to carry out their new function; for example, by producing proteins which are the desired end product, or metabolities of enzymic conversion, or be lysed and the desired nucleic acids or proteins recovered.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The process of this invention employs novel plasmids, which are formed by inserting DNAhaving one or more intact genes into a plasmid in such a location as to permit retention of an intact replicator locus and system (replicon) to provide a recombinant plasmid molecule. The recombinant plasmid molecule will be referred to as a "hybrid" plasmid or plasmid "chimera." The plasmid chimera contains genes that are capable of expressing at least one phenotypical property. The plasmmid chimera is used to transform a susceptible and competent microorganism under conditions where transformation occurs. The microorganism is then grown under conditions which allow for separation and harvesting of transformants that contain the plasmid chimera.

The process of this invention will be divided into the following stages:

I. preparation of the recombinant plasmid or plasmid chimera;

II. transformation or preparation of transformants; and

III. replication and transcription of the recombinant plasmid in transformed bacteria.

Preparation of Plasmid Chimera

In order to prepare the plasmid chimera, it is necessary to have a DNA vector, such as a plasmid or phage, which can be cleaved to provide an intact replicator locus and system (replicon), where the linear segment has ligatable termini or is capable of being modified to introduce ligatable termini. Of particular interest are those plasmids which have a phenotypical property, which allow for ready separation of transformants from the parent microorganism. The plasmid will be capable of replicating in a microorganism, particularly a bacterium which is susceptible to transformation. Various unicellular microorganisms can be transformed, such as bacteria, fungii and algae. That is, those unicellular organisms which are capable of being grown in cultures of fermentation. Since bacteria are for the most part the most convenient organisms to work with, bacteria will be hereinafter referred to as exemplary of the other unicellular organisms. Bacteria, which are susceptible to transformation, include members of the Enterobacteriaceae, such as strains of *Escherichia coli;* Salmonella; Bacillaceae, such as *Bacillus subtilis;* Pneumococcus; Streptococcus, and *Haemophilus influenzae.*

A wide variety of plasmids may be employed of greatly varying molecular weight. Normally, the plasmids employed will have molecular weights in the range of about 1×10^6 to 50×10^6d, more usually from about 1 to 20×10^6d, and preferably, from about 1 to 10×10^6d. The desirable plasmid size is determined by a number of factors. First, the plasmid must be able to accommodate a replicator locus and one or more genes that are capable of allowing replication of the plasmid. Secondly, the plasmid should be of a size which provides for a reasonable probability of recircularization with the foreign gene(s) to form the recombinant plasmid chimera. Desirably, a restriction enzyme should be available, which will cleave the plasmid without inactivating the replicator locus and system associated with the replicator locus. Also, means must be provided for providing ligatable termini for the plasmid, which are

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complementary to the termini of the foreign gene(s) to allow fusion of the two DNA segments.

Another consideration for the recombinant plasmid is that it be compatible with the bacterium to be transformed. Therefore, the original plasmid will usually be derived from a member of the family to which the bacterium belongs.

The original plasmid should desirably have a phenotypical property which allows for the separation of transformant bacteria from parent bacteria. Particularly useful is a gene, which provides for survival selection. Survival selection can be achieved by providing resistance to a growth inhibiting substance or providing a growth factor capability to a bacterium deficient in such capability.

Conveniently, genes are available, which provide for antibiotic or heavy metal resistance or polypeptide resistance, e.g. colicin. Therefore, by growing the bacteria on a medium containing a bacteriostatic or bacteriocidal substance, such as an antibiotic, only the transformants having the antibiotic resistance will survive. Illustrative antibiotics include tetracycline, streptomycin, sulfa drugs, such as sulfonamide, kanamycin, neomycin, penicillin, chloramphenicol, or the like.

Growth factors include the synthesis of amino acids, the isomerization of substrates to forms which can be metabolized or the like. By growing the bacteria on a medium which lacks the appropriate growth factor, only the bacteria which have been transformed and have the growth factor capability will clone.

One plasmid of interest derived from *E. coli* is referred to as pSC101 and is described in Cohen, et al., Proc. Nat. Acad. Sci., USA, 70, 1293 (1972), (referred to in that article as Tc6-5). Further description of this particular plasmid and its use is found in the other articles previously referred to.

The plasmid pSC101 has a molecular weight of about 5.8×10^6d and provides tetracycline resistance.

Another plasmid of interest is colicinogenic factor EI (ColE1), which has a molecular weight of 4.2×10^6d, and is also derived from *E. coli.* The plasmid has a single EcoRI substrate site and provides immunity to colicin E1.

In preparing the plasmid for joining with the exogenous gene, a wide variety of techniques can be provided, including the formation of or introduction of cohesive termini. Flush ends can be joined. Alternatively, the plasmid and gene may be cleaved in such a manner that the two chains are cleaved at different sites to leave extensions at each end which serve as cohesive termini. Cohesive termini may also be introduced by removing nucleic acids from the opposite ends of the two chains or alternatively, introducing nucleic acids at opposite ends of the two chains.

To illustrate, a plasmid can be cleaved with a restriction endonuclease or other DNA cleaving enzyme. The restriction enzyme can provide square ends, which are then modified to provide cohesive termini or can cleave in a staggered manner at different, but adjacent, sites on the two strands, so as to provide cohesive termini directly.

Where square ends are formed such as, for example, by HIN (Haemophilus influenzae RII) or pancreatic DNAse, one can ligate the square ends or alternatively one can modify the square ends by chewing back, adding particular nucleic acids, or a combination of the two. For example, one can employ appropriate transferases to add a nucleic acid to the 5' and 3' ends of the DNA. Alternatively, one can chew back with an enzyme, such as a λ-exonuclease, and it is found that there is a high probability that cohesive termini will be achieved in this manner.

An alternative way to achieve a linear segment of the plasmid with cohesive termini is to employ an endonuclease such as EcoRI. The endonuclease cleaves the two strands at different adjacent sites providing cohesive termini directly.

With flush ended molecules, a T_4 ligase may be employed for linking the termini. See, for example, Scaramella and Khorana, J. Mol. Biol. 72: 427–444 (1972) and Scaramella, DNAS 69: 3389 (1972), whose disclosure is incorporated herein by reference.

Another way to provide ligatable termini is to leave employing DNAse and Mn^{++} as reported by Lai and Nathans, J. Mol. Biol, 89: 179 (1975).

The plasmid, which has the replicator locus, and serves as the vehicle for introduction of a foreign gene into the bacterial cell, will hereafter be referred to as "the plasmid vehicle."

It is not necessary to use plasmid, but any molecule capable of replication in bacteria can be employed. Therefore, instead of plasmid, viruses may be employed, which will be treated in substantially the same manner as the plasmid, to provide the ligatable termini for joining to the foreign gene.

If production of cohesive termini is by restriction endonuclease cleavage, the DNA containing the foreign gene(s) to be bound to the plasmid vehicle will be cleaved in the same manner as the plasmid vehicle. If the cohesive termini are produced by a different technique, an analogous technique will normally be employed with the foreign gene. (By foreign gene is intended a gene derived from a source other than the transformant strain.) In this way, the foreign gene(s) will have ligatable termini, so as to be able to covalently bonded to the termini of the plasmid vehicle. One can carry out the cleavage or digest of the plasmids together in the same medium or separately, combine the plasmids and recircularize the plasmids to form the plasmid chimera in the absence of active restriction enzyme capable of cleaving the plasmids.

Descriptions of methods of cleavage with restriction enzymes may be found in the following articles: Greene, et al., ***Methods in Molecular Biology,*** Vol. 9, ed. Wickner, R. B., (Marcel Dekker, Inc., New York), "DNA Replication and Biosynthesis"; Mertz and Davis, 69, Proc. Nat. Acad. Sci., USA, 69, 3370 (1972);

The cleavage and non-covalent joining of the plasmid vehicle and the foreign DNA can be readily carried out with a restriction endonuclease, with the plasmid vehicle and foreign DNA in the same or different vessels. Depending on the number of fragments, which are obtained from the DNA endonuclease digestion, as well as the genetic properties of the various fragments, digestion of the foreign DNA may be carried out separately and the fragments separated by centrifugation in an appropriate gradient. Where the desired DNA fragment has a phenotypical property, which allows for the ready isolation of its transformant, a separation step can usually be avoided.

Endonuclease digestion will normally be carried out at moderate temperatures, normally in the range of 10° to 40° C. in an appropriately buffered aqueous medium, usually at a pH of about 6.5 to 8.5. Weight percent of total DNA in the reaction mixture will generally be about 1 to 20 weight percent. Time for the reaction will

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vary, generaly being from 0.1 to 2 hours. The amount of endonuclease employed is normally in excess of that required, normally being from about 1 to 5 units per 10 μg of DNA.

Where cleavage into a plurality of DNA fragments results, the course of the reaction can be readily followed by electrophoresis. Once the digestion has gone to the desired degree, the endonuclease is inactivated by heating above about 60° C. for five minutes. The digestion mixture may be worked up by dialysis, gradient separation, or the like, or used directly.

After preparation of the two double stranded DNA sequences, the foreign gene and vector are combined for annealing and/or ligation to provide for a functional recombinant DNA structure. With plasmids, the annealing involves the hydrogen bonding together of the cohesive ends of the vector and the foreign gene to form a circular plasmid which has cleavage sites. The cleavage sites are then normally ligated to form the complete closed and circularized plasmid.

The annealing, and as appropriate, recircularization can be performed in whole or in part in vitro or in vivo. Preferably, the annealing is performed in vitro. The annealing requires an appropriate buffered medium containing the DNA fragments. The temperature employed initially for annealing will be about 40° to 70° C., followed by a period at lower temperature, generaly from about 10° to 30° C. The molar ratio of the two segments will generally be in the range of about 1–5:5–1. The particular temperature for annealing will depend upon the binding strength of the cohesive termi. While 0.5 hr to 2 or more days may be employed for annealing, it is believed that a period of 0.5 to 6 hrs may be sufficient. The time employed for the annealing will vary with the temperature employed, the nature of the salt solution, as well as the nature of the cohesive termini.

The ligation, when in vitro, can be achieved in conventional ways employing DNA ligase. Ligation is conveniently carried out in an aqueous solution (pH 6–8) at temperatures in the range of about 5° to 40° C. The concentration of the DNA will generally be from about 10 to 100 g/ml. A sufficient amount of the DNA ligase or other ligating agent e.g. T_4 ligase, is employed to provide a convenient rate of reaction, generally ranging from about 5 to 50 U/ml. A small amount of a protein e.g. albumin, may be added at concentrations of about 10 to 200 g/ml. The ligation with DNA ligase is carried out in the presence of magnesium at about 1–10 mM.

At the completion of the annealing or ligation, the solution may be chilled and is ready for use in transformation.

It is not necessary to ligate the recircularized plasmid prior to transformation, since it is found that this function can be performed by the bacterial host. However, in some situations ligation prior to transformation may be desirable.

The foreign DNA can be derived from a wide variety of sources. The DNA may be derived from eukaryotic or prokaryotic cells, viruses, and bacteriophage. The fragments employed will generally have molecular weights in the range of about 0.5 to 20×10^6d, usually in the range of 1 to 10×10^6d. The DNA fragment may include one or more genes or one or more operons.

Desirably, if the plasmid vehicle does not have a phenotypical property which allows for isolation of the transformants, the foreign DNA fragment should have such property. Also, an intact promoter and base sequences coding for initiation and termination sites should be present for gene expression.

In accordance with the subject invention, plasmids may be prepared which have replicons and genes which could be present in bacteria as a result of normal mating of bacteria. However, the subject invention provides a technique, whereby a replicon and gene can coexist in a plasmid, which is capable of being introduced into a unicellular organism, which could not exist in nature. The first type of plasmid which cannot exist in nature is a plasmid which derives its replicon from one organism and the exogenous gene from another organism, where the two organisms do not exchange genetic information. In this situation, the two organisms will either be eukaryotic or prokaryotic. Those organisms which are able to exchange genetic information by mating are well known. Thus, prior to this invention, plasmids having a replicon and one or more genes from two sources which do not exchange genetic information would not have existed in nature. This is true, even in the event of mutations, and induced combinations of genes from different strains of the same species. For the natural formation of plasmids formed from a replicon and genes from different microorganisms it is necessary that the microorganisms be capable of mating and exchanging genetic information.

In the situation, where the replicon comes from a eukaryotic or prokaryotic cell, and at least one gene comes from the other type of cell, this plasmid heretofore could not have existed in nature. Thus, the subject invention provides new plasmids which cannot naturally occur and can be used for transformation of unicellular organisms to introduce genes from other unicellular organisms, where the replicon and gene could not previously naturally coexist in a plasmid.

Besides naturally occurring genes, it is feasible to provide synthetic genes, where fragments of DNA may be joined by various techniques known in the art. Thus, the exogenous gene may be obtained from natural sources or from synthetic sources.

The plasmid chimera contains a replicon which is compatible with a bacterium susceptible of transformation and at least one foreign gene which is directly or indirectly bonded through deoxynucleotides to the replicon to form the circularized plasmid structure. As indicated previously, the foreign gene normally provides a phenotypical property, which is absent in the parent bacterium. The foreign gene may come from another bacterial strain, species or family, or from a plant or animal cell. The original plasmid chimera will have been formed by in vitro covalent bonding between the replicon and foreign gene. Once the originally formed plasmid chimera has been used to prepare transformants, the plasmid chimera will be replicated by the bacterial cell and cloned in vivo by growing the bacteria in an appropriate growth medium. The bacterial cells may be lysed and the DNA isolated by conventional means or the bacteria continually reproduced and allowed to express the genotypical property of the foreign DNA.

Once a bacterium has been transformed, it is no longer necessary to repeat the in vitro preparation of the plasmid chimera or isolate the plasmid chimera from the transformant progeny. Bacterial cells can be repeatedly multiplied which will express the genotypical property of the foreign gene.

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One method of distinguishing between a plasmid which originates in vivo from a plasmid chimera which originates in vitro is the formation of homoduplexes between an in vitro prepared plasmid chimera and the plasmid formed in vivo. It will be an extremely rare event where a plasmid which originates in vivo will be the same as a plasmid chimera and will form homoduplexes with plasmid chimeras. For a discussion of homoduplexes, see Sharp, Cohen and Davidson, J. Mol. Biol., 75, 235 (1973), and Sharp, et al, ibid, 71, 471 (1972).

The plasmid derived from molecular cloning need not homoduplex with the in vitro plasmid originally employed for transformation of the bacterium. The bacterium may carry out modification processes, which will not affect the portion of the replicon introduced which is necessary for replication nor the portion of the exogenous DNA which contains the gene providing the genotypical trait. Thus, nucleotides may be introduced or excised and, in accordance with naturally occurring mating and transduction, additional genes may be introduced. In addition, for one or more reasons, the plasmids may be modified in vitro by techniques which are known in the art. However, the plasmids obtained by molecular cloning will homoduplex as to those parts which relate to the original replicon and the exogenous gene.

II. Transformation

After the recombinant plasmid or plasmid chimera has been prepared, it may then be used for the transformation of bacteria. It should be noted that the annealing and ligation process not only results in the formation of the recombinant plasmid, but also in the recircularization of the plasmid vehicle. Therefore, a mixture is obtained of the original plasmid, the recombinant plasmid, and the foreign DNA. Only the original plasmid and the DNA chimera consisting of the plasmid vehicle and linked foreign DNA will normally be capable of replication. When the mixture is employed for transformation of the bacteria, replication of both the plasmid vehicle genotype and the foreign genotype will occur with both genotypes being replicated in those cells having the recombinant plasmid.

Various techniques exist for transformation of a bacterial cell with plasmid DNA. A technique, which is particularly useful with *Escherichia coli*, is described in Cohen, et al., ibid, 69, 2110 (1972). The bacterial cells are grown in an appropriate medium to a predetermined optical density. For example, with *E. coli strain C600*, the optical density was 0.85 at 590 nm. The cells are concentrated by chilling, sedimentation and washing with a dilute salt solution. After centrifugation, the cells are resuspended in a calcium chloride solution at reduced temperatures (approx. 5°–15° C.), sedimented, resuspended in a smaller volume of a calcium chloride solution and the cells combined with the DNA in an appropriately buffered calcium chloride solution and incubated at reduced temperatures. The concentration of Ca^{++} will generally be about 0.01 to 0.1 M. After a sufficient incubation period, generally from about 0.5–3.0 hours, the bacteria are subjected to a heat pulse generally in the range of 35° to 45° C. for a short period of time; namely from about 0.5 to 5 minutes. The transformed cells are then chilled and may be transferred to a growth medium, whereby the transformed cells having the foreign genotype may be isolated.

An alternative transformation technique may be found in Lederberg and Cohen, I. Bacteriol., 119, 1072 (1974), whose disclosure is incorporated herein by reference.

III. Replication and Transcription of the Plasmid

The bacterial cells, which are employed, will be of such species as to allow replication of the plasmid vehicle. A number of different bacteria which can be employed, have been indicated previously. Strains which lack indigenous modification and restriction enzymes are particularly desirable for the cloning of DNA derived from foreign sources.

The transformation of the bacterial cells will result in a mixture of bacterial cells, the dominant proportion of which will not be transformed. Of the fraction of cells which are transformed, some significant proportion, but normally a minor proportion, will have been transformed by recombinant plasmid. Therefore, only a very small fraction of the total number of cells which are present will have the desired phenotypical characteristics.

In order to enhance the ability to separate the desired bacterial clones, the bacterial cells, which have beeen subjected to transformation, will first be grown in a solution medium, so as to amplify the absolute number of the desired cells. The bacterial cells may then be harvested and streaked on an appropriate agar medium. Where the recombinant plasmid has a phenotype, which allows for ready separation of the transformed cells from the parent cells, this will aid in the ready separation of the two types of cells. As previously indicated, where the genotype provides resistance to a growth inhibiting material, such as an antibiotic or heavy metal, the cells can be grown on an agar medium containing the growth inhibiting substance. Only available cells having the resistant genotype will survive. If the foreign gene does not provide a phenotypical property, which allows for distinction between the cells transformed by the plasmid vehicle and the cells transformed by the plasmid chimera, a further step is necessary to isolate the replicated plasmid chimera from the replicated plasmid vehicle. The steps include lysing of the cells and isolation and separation of the DNA by conventional means or random selection of transformed bacteria and characterization of DNA from such transformants to determine which cells contain molecular chimeras. This is accomplished by physically characterizing the DNA by electrophoresis, gradient centrifugation or electron microscopy.

Cells from various clones may be harvested and the plasmid DNA isolated from these transformants. The plasmid DNA may then be analyzed in a variety of ways. One way is to treat the plasmid with an appropriate restriction enzyme and analyze the resulting fragments for the presence of the foreign gene. Other techniques have been indicated above.

Once the recombinant plasmid has been replicated in a cell and isolated, the cells may be grown and multiplied and the recombinant plasmid employed for transformation of the same or different bacterial strain.

The subject process provides a technique for introducing into a bacterial strain a foreign capability which is genetically mediated. A wide variety of genes may be employed as the foreign genes from a wide variety of sources. Any intact gene may be employed which can be bonded to the plasmid vehicle. The source of the gene can be other bacterial cells, mammalian cells, plant

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