EVOLUTION OF WHOLE CELLS AND ORGANISMS BY RECURSIVE SEQUENCE RECOMBINATION

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in part of 09/116,188 The subject application claims priority to this prior application, which is also incorporated by reference in its entirety for all purposes

FIELD OF THE INVENTION

The invention applies the technical field of molecular genetics to evolve the genomes of cells and organisms to acquire new and improved properties.

BACKGROUND

WO 98/31837 (PCT/US98/00852) provides pioneering technology for evolving the genome of whole cells and organisms. One of skill will appreciate that the technology provided in WO 98/31837 is fundamental to the ability of one of skill rapidly to evolve cells and whole organisms. For example, the document teaches a variety of recursive methods of artificially recombining nucleic acids in vivo, including entire genomes, and ways of selecting resulting recombinant organisms

This ability to evolve genes artifically is of fundamental importance. For example, cells have a number of well-established uses in molecular biology, medicine and industrial processes. For example, cells are commonly used as hosts for manipulating DNA in processes such as transformation and recombination. Cells are used for expression of recombinant proteins encoded by DNA transformed/transfected or otherwise introduced into the cells. Some types of cells are used as progenitors for generation of transgenic animals and plants. Although all of these processes are now routine, prior to the technology provided by WO 98/31837, the genomes of the cells used in these processes had evolved little from the genomes of natural cells, and particularly not toward acquisition of new or improved properties for use in the above processes.

Additional methods of recursively recombining nucleic acids in vivo and selecting resulting recombinants would be of use. The present invention provides a number of new and valuable methods and compositions for whole and partial genome evolution.

09 JAN 2003

SUMMARY OF THE INVENTION

In one aspect, the invention provides methods of evolving a cell to acquire a desired function Such methods entail, e g., introducing a library of DNA fragments into a plurality of cells, whereby at least one of the fragments undergoes recombination with a segment in the genome or an episome of the cells to produce modified cells Optionally, these modified cells are bred to increase the diversity of the resulting recombined cellular population The modified cells, or the recombined cellular population are then screened for modified or recombined cells that have evolved toward acquisition of the desired function. DNA from the modified cells that have evolved toward the desired function is then optionally recombined with a further library of DNA fragments, at least one of which undergoes recombination with a segment in the genome or the episome of the modified cells to produce further modified cells. The further modified cells are then screened for further modified cells that have further evolved toward acquisition of the desired function. Steps of recombination and screening/selection are repeated as required until the further modified cells have acquired the desired function. In one preferred embodiment, modified cells are recursively recombined to increase diversity of the cells prior to performing any selection steps on any resulting cells.

In some methods, the library or further library of DNA fragments is coated with recA protein to stimulate recombination with the segment of the genome. The library of fragments is optionally denatured to produce single-stranded DNA, which are annealed to produce duplexes, some of which contain mismatches at points of variation in the fragments. Duplexes containing mismatches are optionally selected by affinity chromatography to immobilized MutS.

Optionally, the desired function is secretion of a protein, and the plurality of cells further comprises a construct encoding the protein. The protein is optionally inactive unless secreted, and further modified cells are optionally selected for protein function. Optionally, the protein is toxic to the plurality of cells, unless secreted. In this case, the modified or further modified cells which evolve toward acquisition of the desired function are screened by propagating the cells and recovering surviving cells.

In some methods, the desired function is enhanced recombination. In such methods, the library of fragments sometimes comprises a cluster of genes collectively conferring recombination capacity. Screening can be achieved using cells carrying a gene encoding a marker whose expression is prevented by a mutation removable by recombination. The cells are screened by their expression of the marker resulting from removal of the mutation by recombination.

In some methods, the plurality of cells are plant cells and the desired property is improved resistance to a chemical or microbe The modified or further modified cells (or whole plants) are exposed to the chemical or microbe and modified or further modified cells having evolved toward the acquisition of the desired function are selected by their capacity to survive the exposure

In some methods, the plurality of cells are embryonic cells of an animal, and the method further comprises propagating the transformed cells to transgenic animals.

The plurality of cells can be a plurality of industrial microorganisms that are enriched for microorganisms which are tolerant to desired process conditions (heat, light, radiation, selected pH, presence of detergents or other denaturants, presence of alcohols or other organic molecules, etc.)

The invention further provides methods for performing *in vivo* recombination. At least first and second segments from at least one gene are introduced into a cell, the segments differing from each other in at least two nucleotides, whereby the segments recombine to produce a library of chimeric genes. A chimeric gene is selected from the library having acquired a desired function.

The invention further provides methods of predicting efficacy of a drug in treating a viral infection. Such methods entail recombining a nucleic acid segment from a virus, whose infection is inhibited by a drug, with at least a second nucleic acid segment from the virus, the second nucleic acid segment differing from the first nucleic acid segment in at least two nucleotides, to produce a library of recombinant nucleic acid segments. Host cells are then contacted with a collection of viruses having genomes including the recombinant nucleic acid segments in a media containing the drug, and progeny viruses resulting from infection of the host cells are collected.

A recombinant DNA segment from a first progeny virus recombines with at least a recombinant DNA segment from a second progeny virus to produce a further library of recombinant nucleic acid segments. Host cells are contacted with a collection of viruses having genomes including the further library or recombinant nucleic acid segments, in media containing the drug, and further progeny viruses are produced by the host cells. The recombination and selection steps are repeated, as desired, until a further progeny virus has acquired a desired degree of resistance to the drug, whereby the degree of resistance acquired and the number of repetitions needed to acquire it provide a measure of the efficacy of the drug in treating the virus. Viruses are optionally adapted to grow on particular cell lines.

The invention further provides methods of predicting efficacy of a drug in treating an infection by a pathogenic microorganism These methods entail delivering a library of DNA fragments into a plurality of microorganism cells, at least some of which undergo recombination with segments in the genome of the cells to produce modified microorganism cells Modified microorganisms are propagated in a media containing the drug, and surviving microorganisms are recovered DNA from surviving microorganisms is recombined with a further library of DNA fragments at least some of which undergo recombination with cognate segments in the DNA from the surviving microorganisms to produce further modified microorganisms cells. Further modified microorganisms are propagated in media containing the drug, and further surviving microorganisms are collected. The recombination and selection steps are repeated as needed, until a further surviving microorganism has acquired a desired degree of resistance to the drug. The degree of resistance acquired and the number of repetitions needed to acquire it provide a measure of the efficacy of the drug in killing the pathogenic microorganism.

The invention further provides methods of evolving a cell to acquire a desired function These methods entail providing a populating of different cells. The cells are cultured under conditions whereby DNA is exchanged between cells, forming cells with hybrid genomes. The cells are then screened or selected for cells that have evolved toward acquisition of a desired property. The DNA exchange and screening/selecting steps are repeated, as needed, with the screened/selected cells from one cycle forming the population of different cells in the next cycle, until a cell has acquired the desired property.

Mechanisms of DNA exchange include conjugation, phage-mediated transduction, liposome delivery, protoplast fusion, and sexual recombination of the cells. Optionally, a library of DNA fragments can be transformed or electroporated into the cells

As noted, some methods of evolving a cell to acquire a desired property are effected by protoplast-mediated exchange of DNA between cells. Such methods entail forming protoplasts of a population of different cells. The protoplasts are then fused to form hybrid protoplasts, in which genomes from the protoplasts recombine to form hybrid genomes. The hybrid protoplasts are incubated under conditions promoting regeneration of cells. The regenerated cells can be recombined one or more times (i.e., via protoplasting or any other method than combines genomes of cells) to increase the diversity of any resulting cells. Preferably, regenerated cells are recombined several times, e.g., by protoplast fusion to generate a diverse population of cells.

The next step is to select or screen to isolate regenerated cells that have evolved toward acquisition of the desired property. DNA exchange and selection/screening steps are repeated, as needed, with regenerated cells in one cycle being used to form protoplasts in the next cycle until the regenerated cells have acquired the desired property Industrial microorganisms are a preferred class of organisms for conducting the above methods. Some methods further comprise a step of selecting or screening for fused protoplasts free from unfused protoplasts of parental cells. Some methods further comprise a step of selecting or screening for fused protoplasts with hybrid genomes free from cells with parental genomes. In some methods, protoplasts are provided by treating individual cells, mycelia or spores with an enzyme that degrades cell walls. In some methods, the strain is a mutant that is lacking capacity for intact cell wall synthesis, and protoplasts form spontaneously. In some methods, protoplasts are formed by treating growing cells with an inhibitor of cell wall formation to generate protoplasts.

In some methods, the desired property is expression and/or secretion of a protein or secondary metabolite, such as an industrial enzyme, a therapeutic protein, a primary metabolite such as lactic acid or ethanol, or a secondary metabolite such as erythromycin cyclosporin A or taxol. In other methods it is the ability of the cell to convert compounds provided to the cell to different compounds. In yet other methods, the desired property is capacity for meiosis. In some methods, the desired property is compatibility to form a heterokaryon with another strain.

The invention further provides methods of evolving a cell toward acquisition of a desired property. These methods entail providing a population of different cells. DNA is isolated from a first subpopulation of the different cells and encapsulated in liposomes. Protoplasts are formed from a second subpopulation of the different cells Liposomes are fused with the protoplasts, whereby DNA from the liposomes is taken up by the protoplasts and recombines with the genomes of the protoplasts. The protoplasts are incubated under regenerating conditions. Regenerating or regenerated cells are then selected or screened for evolution toward the desired property.

The invention further provides methods of evolving a cell toward acquisition of a desired property using artificial chromosomes. Such methods entail introducing a DNA fragment library cloned into an artificial chromosome into a population of cells. The cells are then cultured under conditions whereby sexual recombination occurs between the cells, and DNA fragments cloned into the artificial chromosome recombines by homologous

recombination with corresponding segments of endogenous chromosomes of the populations of cells, and endogenous chromosomes recombine with each other Cells can also be recombined via conjugation Any resulting cells can be recombined via any method noted herein, as many times as desired, to generate a desired level of diversity in the resulting recombinant cells. In any case, after generating a diverse library of cells, the cells that have evolved toward acquisition of the desired property are screened and/or selected for a desired property. The method is then repeated with cells that have evolved toward the desired property in one cycle forming the population of different cells in the next cycle. Here again, multiple cycles of in vivo recombination are optionally performed prior to any additional selection or screening steps.

The invention further provides methods of evolving a DNA segment cloned into an artificial chromosome for acquisition of a desired property. These methods entail providing a library of variants of the segment, each variant cloned into separate copies of an artificial chromosome The copies of the artificial chromosome are introduced into a population of cells. The cells are cultured under conditions whereby sexual recombination occurs between cells and homologous recombination occurs between copies of the artificial chromosome bearing the variants. Variants are then screened or selected for evolution toward acquisition of the desired property.

The invention further provides hyperrecombinogenic recA proteins. Examples of such proteins are from clones 2, 4, 5, 6 and 13 shown in Fig. 13.

The method also provides methods of reiterative pooling and breeding of higher organisms. In the methods, a library of diverse multicellular organisms are produced (e.g., plants, animals or the like). A pool of male gametes is provided along with a pool of female gametes. At least one of the male pool or the female pool comprises a plurality of different gametes derived from different strains of a species or different species. The male gametes are used to fertilize the female gametes. At least a portion of the resulting fertilized gametes grow into reproductively viable organisms. These reproductively viable organisms are crossed (e.g., by pairwise pooling and joining of the male and female gametes as before) to produce a library of diverse organisms. The library is then selected for a desired trait or property.

The library of diverse organisms can comprise a plurality of plants such as Gramineae, Fetucoideae, Poacoideae, Agrostis, Phleum, Dac ylis, Sorgum, Setaria, Zea, Oryza, Triticum, Secale, Avena, Hordeum, Saccharum, Poa, Festuca, Stenotaphrum,

Cynodon, Coix, Olyreae, Phareae, Compositae or Leguminosae. For example, the plants can be e.g., corn, rice, wheat, rye, oats, barley, pea, beans, lentil, peanut, yam bean, cowpeas, velvet beans, soybean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, sweetpea, sorghum, millet, sunflower, canola or the like.

Similarly, the library of diverse organisms can incldue a plurality of animals such as non-human mammals, fish, insects, or the like

Optionally, a plurality of selected library members can be crossed by pooling gametes from the selected members and repeatedly crossing any resulting additional reproductively viable organisms to produce a second library of diverse organisms (e.g., by split pairwise pooling and rejoining of the male and female gametes). Here again, the second library can be selected for a desired trait or property, with the resulting selected members forming the basis for additional poolwise breeding and selection.

A feature of the invention is the libraries made by these (or any preceding) method

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1, panels A-D: Scheme for *in vitro* shuffling of genes.

Fig. 2 Scheme for enriching for mismatched sequences using MutS

Fig 3· Alternative scheme for enriching for mismatched sequences using MutS.

Fig. 4. Scheme for evolving growth hormone genes to produce larger fish.

Fig. 5: Scheme for shuffling prokaryotes by protoplast fusion

Fig. 6: Scheme for introducing a sexual cycle into fungi previously incapable of sexual reproduction.

Fig. 7· General scheme for shuffling of fungi by protoplast fusion.

Fig. 8: Shuffling fungi by protoplast fusion with protoplasts generated by use of inhibitors of enzymes responsible for cell wall formation.

Fig. 9· Shuffling fungi by protoplast fusion using fungal strains deficient in cell-wall synthesis that spontaneously form protoplasts.

Fig. 10: YAC-mediated whole genome shuffling of Saccharomyces cerevisiae and related organisms.

Fig. 11: YAC-mediated shuffling of large DNA fragments.

Fig. 12: (A, B, C and D) DNA sequences of a wildtype recA protein and five hyperrecombinogenic variants thereof.

09 JAN 2003

Fig. 13: Amino acid sequences of a wildtype recA protein and five hyperrecombinogenic variants thereof.

Fig 14 illustration of combinatoriality

Fig. 15: Repeated pairwise recombination to access multi-mutant progeny

Fig. 16. graph of fitness versus sequence space for three different mutation strategies.

Fig. 17 graphs of asexual sequential mutagenesis and sexual recursive recombination

Fig. 18: Schematic for non-homologous recombination

Fig. 19: Schematic for split and pool strategy

Fig. 20, panel A. schematic for selectable/ counterselectable marker strategy.

Fig. 20, panel B: schematic for selectable/ counterselectable marker strategy for Rec A.

Fig. 21: plant regeneration strategy for regenerating salt-tolerant plants.

Fig. 22 Whole genome shuffling of parsed (subcloned) genomes

Fig. 23: Schematic for blind cloning of gene homologs.

Fig. 24: High throughput family shuffling.

Fig. 25: Schematic and graph of poolwise recombination.

Fig. 26: Schematic of protoplast fusion

Fig. 27. Schematic assay for poolwise recombination.

Fig. 28: Schematic of halo assay and integrated system.

Fig. 29: Schematic drawing illustrating recursive pooled breeding of fish.

Fig. 30: Schematic drawing illustrating recursive pooled breeding of plants.

Fig. 31: Schematic for shuffling of *S. Colicolor*.

Fig. 32: schematic drawing illustrating HTP actinorohodin assay.

Fig. 33: schematic drawing and table illustrating whole genome shuffling of four parental strains.

Fig. 34: schematic drawing of WGS through organized heteroduplex shuffling.

DETAILED DESCRIPTION

I. GENERAL

A. THE BASIC APPROACH

The invention provides methods for artificially evolving cells to acquire a new or improved property by recursive sequence recombination. Briefly, recursive sequence

recombination entails successive cycles of recombination to generate molecular diversity and screening/selection to take advantage of that molecular diversity That is, a family of nucleic acid molecules is created showing substantial sequence and/or structural identity but differing as to the presence of mutations These sequences are then recombined in any of the described formats so as to optimize the diversity of mutant combinations represented in the resulting recombined library. Typically, any resulting recombinant nucleic acids or genomes are recursively recombined for one or more cycles of recombination to increase the diversity of resulting products. After this recursive recombination procedure, the final resulting products are screened and/or selected for a desired trait or property.

Alternatively, each recombination cycle can followed by at least one cycle of screening or selection for molecules having a desired characteristic. In this embodiment, the molecule(s) selected in one round form the starting materials for generating diversity in the next round.

The cells to be evolved can be bacteria, archaebacteria, or eukaryotic cells and can constitute a homogeneous cell line or mixed culture. Suitable cells for evolution include the bacterial and eukaryotic cell lines commonly used in genetic engineering, protein expression, or the industrial production or conversion of proteins, enzymes, primary metabolites, secondary metabolites, fine, specialty or commodity chemicals. Suitable mammalian cells include those from, e.g., mouse, rat, hamster, primate, and human, both cell lines and primary cultures. Such cells include stem cells, including embryonic stem cells and hemopoietic stem cells, zygotes, fibroblasts, lymphocytes, Chinese hamster ovary (CHO), mouse fibroblasts (NIH3T3), kidney, liver, muscle, and skin cells. Other eukaryotic cells of interest include plant cells, such as maize, rice, wheat, cotton, soybean, sugarcane, tobacco, and arabidopsis; fish, algae, fungi (*penicillium, aspergillus, podospora, neurospora, saccharomyces*), insect (e.g., baculo lepidoptera), yeast (*picchia* and *saccharomyces, Schizosaccharomyces pombe*). Also of interest are many bacterial cell types, both gram-negative and gram-positive, such as *Bacillus subtilis, B. licehniformis, B. cereus, Escherichia coli, Streptomyces, Pseudomonas, Salmonella, Actinomycetes, Lactobacillius, Acetonitcbacter, Deinococcus*, and *Erwinia*. The complete genome sequences of *E. coli* and *Bacillus subtilis* are described by Blattner et al., *Science* 277, 1454-1462 (1997); Kunst et al., *Nature* 390, 249-256 (1997)).

Evolution commences by generating a population of variant cells. Typically, the cells in the population are of the same type but represent variants of a progenitor cell. In

some instances, the variation is natural as when different cells are obtained from different individuals within a species, from different species or from different genera In other instances, variation is induced by mutagenesis of a progenitor cell Mutagenesis can be effected by subjecting the cell to mutagenic agents, or if the cell is a mutator cell (e.g , has mutations in genes involved in DNA replication, recombination and/or repair which favor introduction of mutations) simply by propagating the mutator cells Mutator cells can be generated from successive selections for simple phenotypic changes (e.g., acquisition of rifampicin-resistance, then nalidixic acid resistance then lac- to lac+ (see Mao et al., *J. Bacteriol.* 179, 417-422 (1997)), or mutator cells can be generated by exposure to specific inhibitors of cellular factors that result in the mutator phenotype These could be inhibitors of *mut*S, *mut*L, *mut*D, *rec*D, *mut*Y, *mut*M, *dam*, *uvr*D and the like

More generally, mutations are induced in cell populations using any available mutation technique. Common mechanisms for inducing mutations include, but are not limited to, the use of strains comprising mutations such as those involved in mismatch repair. e.g mutations in *mut*S, *mut*T, *mut*L and *mut*H; exposure to UV light; Chemical mutagenesis, e.g use of inhibitors of MMR, DNA damage inducible genes, or SOS inducers; overproduction/ underproduction/ mutation of any component of the homologous recombination complex/pathway, e.g. RecA, ssb, etc.; overproduction/ underproduction/ mutation of genes involved in DNA synthesis/homeostasis; overproduction/ underproduction/ mutation of recombination-stimulating genes from bacteria, phage (e.g. Lambda Red function), or other organisms; addition of chi sites into/flanking the donor DNA fragments; coating the DNA fragments with RecA/ssb and the like.

In other instances, variation is the result of transferring a library of DNA fragments into the cells (e.g., by conjugation, protoplast fusion, liposome fusion, transformation, transduction or natural competence). At least one, and usually many of the fragments in the library, show some, but not complete, sequence or structural identity with a cognate or allelic gene within the cells sufficient to allow homologous recombination to occur. For example, in one embodiment, homologous integration of a plasmid carrying a shuffled gene or metabolic pathway leads to insertion of the plasmid-borne sequences adjacent to the genomic copy. Optionally, a counter-selectable marker strategy is used to select for recombinants in which recombination occurred between the homologous sequences, leading to elimination of the counter-selectable marker. This strategy is illustrated in Fig. 20A A variety of selectable and counter selectable markers are amply illustrated in the art. For a list of useful

markers, *see,* Berg and Berg (1996), Transposable element tools for microbial genetics Escherichia coli and Salmonella Neidhardt Washington, D.C., ASM Press. 2. 2588-2612, La Rossa, *ibid.*, 2527-2587. This strategy can be recursively repeated to maximize sequence diversity of targeted genes prior to screening/ selection for a desired trait or property.

The library of fragments can derive from one or more sources One source of fragments is a genomic library of fragments from a different species, cell type, organism or individual from the cells being transfected. In this situation, many of the fragments in the library have a cognate or allelic gene in the cells being transformed but differ from that gene due to the presence of naturally occurring species variation, polymorphisms, mutations, and the presence of multiple copies of some homologous genes in the genome Alternatively, the library can be derived from DNA from the same cell type as is being transformed after that DNA has been subject to induced mutation, by conventional methods, such as radiation, error-prone PCR, growth in a mutator organism, transposon mutagenesis, or cassette mutagenesis. Alternatively, the library can derive from a genomic library of fragments generated from the pooled genomic DNA of a population of cells having the desired characteristics Alternatively, the library can derive from a genomic library of fragments generated from the pooled genomic DNA of a population of cells having desired characteristics.

In any of these situations, the genomic library can be a complete genomic library or subgenomic library deriving, for example, from a selected chromosome, or part of a chromosome or an episomal element within a cell. As well as, or instead of these sources of DNA fragments, the library can contain fragments representing natural or selected variants of selected genes of known function (i.e., focused libraries).

The number of fragments in a library can vary from a single fragment to about 10^{10}, with libraries having from 10^3 to 10^8 fragments being common. The fragments should be sufficiently long that they can undergo homologous recombination and sufficiently short that they can be introduced into a cell, and if necessary, manipulated before introduction. Fragment sizes can range from about 10 b to about 20mb. Fragments can be double- or single-stranded.

The fragments can be introduced into cells as whole genomes or as components of viruses, plasmids, YACS, HACs or BACs or can be introduced as they are, in which case all or most of the fragments lack an origin of replication. Use of viral fragments with single-stranded genomes offer the advantage of delivering fragments in single stranded form, which promotes recombination. The fragments can also be joined to a selective marker before

introduction. Inclusion of fragments in a vector having an origin of replication affords a longer period of time after introduction into the cell in which fragments can undergo recombination with a cognate gene before being degraded or selected against and lost from the cell, thereby increasing the proportion of cells with recombinant genomes Optionally, the vector is a suicide vector capable of a longer existence than an isolated DNA fragment but not capable of permanent retention in the cell line Such a vector can transiently express a marker for a sufficient time to screen for or select a cell bearing the vector (e.g., because cells transduced by the vector are the target cell type to be screened in subsequent selection assays), but is then degraded or otherwise rendered incapable of expressing the marker. The use of such vectors can be advantageous in performing optional subsequent rounds of recombination to be discussed below. For example, some suicide vectors express a long-lived toxin which is neutralized by a short-lived molecule expressed from the same vector. Expression of the toxin alone will not allow vector to be established. Jense & Gerdes, *Mol. Microbiol.*, 17, 205-210 (1995); Bernard et al., *Gene* 162, 159-160. Alternatively, a vector can be rendered suicidal by incorporation of a defective origin of replication (e.g. a temperature-sensitive origin of replication) or by omission of an origin of replication Vectors can also be rendered suicidal by inclusion of negative selection markers, such as ura3 in yeast or sacB in many bacteria. These genes become toxic only in the presence of specific compounds. Such vectors can be selected to have a wide range of stabilities. A list of conditional replication defects for vectors which can be used, e.g., to render the vector replication defective is found, e.g., in Berg and Berg (1996), "Transposable element tools for microbial genetics" Escherichia coli and Salmonella Neidhardt. Washington, D.C., ASM Press. 2: 2588-2612. Similarly, a list of counterselectable markers, generally applicable to vector selection is also found in Berg and Berg, *id. See also*, LaRossa (1996) "Mutant selections linking physiology, inhibitors, and genotypes" Escherichia coli and Salmonella F. C. Neidhardt. Washington, D.C., ASM Press. 2: 2527-2587.

After introduction into cells, the fragments can recombine with DNA present in the genome, or episomes of the cells by homologous, nonhomologous or site-specific recombination. For present purposes, homologous recombination makes the most significant contribution to evolution of the cells because this form of recombination amplifies the existing diversity between the DNA of the cells being transfected and the DNA fragments. For example, if a DNA fragment being transfected differs from a cognate or allelic gene at two positions, there are four possible recombination products, and each of these recombination

products can be formed in different cells in the transformed population. Thus, homologous recombination of the fragment doubles the initial diversity in this gene. When many fragments recombine with corresponding cognate or allelic genes, the diversity of recombination products with respect to starting products increases exponentially with the number of mutations Recombination results in modified cells having modified genomes and/or episomes Recursive recombination prior to selection further increases diversity of resulting modified cells.

The variant cells, whether the result of natural variation, mutagenesis, or recombination are screened or selected to identify a subset of cells that have evolved toward acquisition of a new or improved property. The nature of the screen, of course, depends on the property and several examples will be discussed below. Typically, recombination is repeated before initial screening. Optionally, however, the screening can also be repeated before performing subsequent cycles of recombination. Stringency can be increased in repeated cycles of screening.

The subpopulation of cells surviving screening are optionally subjected to a further round of recombination. In some instances, the further round of recombination is effected by propagating the cells under conditions allowing exchange of DNA between cells. For example, protoplasts can be formed from the cells, allowed to fuse, and regenerated. Cells with recombinant genomes are propagated from the fused protoplasts. Alternatively, exchange of DNA can be promoted by propagation of cells or protoplasts in an electric field. For cells having a conjugative transfer apparatus, exchange of DNA can be promoted simply by propagating the cells.

In other methods, the further round of recombination is performed by a split and pool approach. That is, the surviving cells are divided into two pools. DNA is isolated from one pool, and if necessary amplified, and then transformed into the other pool. Accordingly, DNA fragments from the first pool constitute a further library of fragments and recombine with cognate fragments in the second pool resulting in further diversity. An example of this strategy is illustrated in Fig. 19. As shown, a pool of mutant bacteria with improvements in a desired phenotype is obtained and split. Genes are obtained from one half, e.g., by PCR, by cloning of random genomic fragments, by infection with a transducing phage and harvesting transducing particles, or by the introduction of an origin of transfer (OriT) randomly into the relevant chromosome to create a donor population of cells capable of transferring random fragments by conjugation to an acceptor population. These genes are

then shuffled (in vitro by known methods or in vivo as taught herein), or simply cloned into an allele replacement vector (e g , one carrying selectable and counter-selectable markers) The gene pool is then transformed into the other half of the original mutant pool and recombinants are selected and screened for further improvements in phenotype These best variants are used as the starting point for the next cycle Alternatively, recursive recombination by any of the methods noted can be performed prior to screening, thereby increasing the diversity of the population of cells to be screened

In other methods, some or all of the cells surviving screening are transfected with a fresh library of DNA fragments, which can be the same or different from the library used in the first round of recombination In this situation, the genes in the fresh library undergo recombination with cognate genes in the surviving cells If genes are introduced as components of a vector, compatibility of this vector with any vector used in a previous round of transfection should be considered. If the vector used in a previous round was a suicide vector, there is no problem of incompatibility. If, however, the vector used in a previous round was not a suicide vector, a vector having a different incompatibility origin should be used in the subsequent round. In all of these formats, further recombination generates additional diversity in the DNA component of the cells resulting in further modified cells

The further modified cells are subjected to another round of screening/selection according to the same principles as the first round. Screening/selection identifies a subpopulation of further modified cells that have further evolved toward acquisition of the property This subpopulation of cells can be subjected to further rounds of recombination and screening according to the same principles, optionally with the stringency of screening being increased at each round Eventually, cells are identified that have acquired the desired property.

II. DEFINITIONS

The term cognate refers to a gene sequence that is evolutionarily and functionally related between species. For example, in the human genome, the human CD4 gene is the cognate gene to the mouse CD4 gene, since the sequences and structures of these two genes indicate that they are homologous and that both genes encode a protein which functions in signaling T-cell activation through MHC class II-restricted antigen recognition.

Screening is, in general, a two-step process in which one first determines which cells do and do not express a screening marker or phenotype (or a selected level of marker or phenotype), and then physically separates the cells having the desired property. Selection is a

form of screening in which identification and physical separation are achieved simultaneously by expression of a selection marker, which, in some genetic circumstances, allows cells expressing the marker to survive while other cells die (or vice versa) Screening markers include luciferase, β-galactosidase, and green fluorescent protein. Selection markers include drug and toxin resistance genes.

An exogenous DNA segment is one foreign (or heterologous) to the cell or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments can be expressed to yield exogenous polypeptides

The term "gene" is used broadly to refer to any segment of DNA associated with a biological function Thus, genes include coding sequences and/or the regulatory sequences required for their expression. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins

The terms "identical" or "percent identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g* , by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85 2444 (1988), by computerized implementations of algorithms GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7 0, Genetics Computer Group, 575 Science Dr., Madison, WI.

Another example of a useful alignment algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity It also plots a tree or dendogram showing the clustering relationships used to create the alignment PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol* 35 351-360 (1987) The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score

threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues, always > 0) and N (penalty score for mismatching residues; always < 0) For amino acid sequences, a scoring matrix is used to calculate the cumulative score Extension of the word hits in each direction are halted when. the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions

The term "naturally-occurring" is used to describe an object that can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring Generally, the term naturally-occurring refers to an object as present in a non-pathological (undiseased) individual, such as would be typical for the species

Asexual recombination is recombination occurring without the fusion of gametes to form a zygote.

A "mismatch repair deficient strain" can include any mutants in any organism impaired in the functions of mismatch repair. These include mutant gene products of mutS, mutT, mutH, mutL, ovrD, dcm, vsr, umuC, umuD, sbcB, recJ, etc. The impairment is achieved by genetic mutation, allelic replacement, selective inhibition by an added reagent such as a small compound or an expressed antisense RNA, or other techniques Impairment can be of the genes noted, or of homologous genes in any organism.

III VARIATIONS

A. COATING FRAGMENTS WITH *REC*A PROTEIN

The frequency of homologous recombination between library fragments and cognate endogenous genes can be increased by coating the fragments with a recombinogenic protein before introduction into cells. See Pati et al., *Molecular Biology of Cancer* 1, 1 (1996); Sena & Zarling, *Nature Genetics* 3, 365 (1996); Revet et al., *J. Mol. Biol.* 232, 779-791 (1993); Kowalczkowski & Zarling *in Gene Targeting* (CRC 1995), Ch. 7. The recombinogenic protein promotes homologous pairing and/or strand exchange. The best characterized *rec*A protein is from *E. coli* and is available from Pharmacia (Piscataway, NJ). In addition to the wild-type protein, a number of mutant *rec*A-like proteins have been identified (e.g., *rec*A803). Further, many organisms have *rec*A-like recombinases with strand-transfer activities (e.g., Ogawa et al., *Cold Spring Harbor Symposium on Quantitative Biology* 18, 567-576 (1993); Johnson & Symington, *Mol. Cell. Biol.* 15, 4843-4850 (1995); Fugisawa et al., *Nucl. Acids Res.* 13, 7473 (1985); Hsieh et al., *Cell* 44, 885 (1986); Hsieh et al., *J. Biol. Chem.* 264, 5089 (1989); Fishel et al., *Proc. Natl. Acad. Sci. USA* 85, 3683 (1988); Cassuto et al., *Mol. Gen. Genet.* 208, 10 (1987); Ganea et al., *Mol. Cell Biol.* 7, 3124 (1987); Moore et al., *J. Biol. Chem.* 19, 11108 (1990); Keene et al., *Nucl. Acids Res.* 12, 3057 (1984); Kimiec, *Cold Spring Harbor Symp.* 48, 675 (1984); Kimeic, *Cell* 44, 545 (1986); Kolodner et al., *Proc. Natl. Acad. Sci. USA* 84, 5560 (1987); Sugino et al., *Proc.*

Natl. Acad. Sci. USA 85, 3683 (1985); Halbrook et al., *J. Biol. Chem.* 264, 21403 (1989), Eisen et al., *Proc. Natl. Acad. Sci. USA* 85, 7481 (1988); McCarthy et al , *Proc. Natl. Acad. Sci. USA* 85, 5854 (1988), Lowenhaupt et al., *J. Biol. Chem.* 264, 20568 (1989) Examples of such recombinase proteins include *rec*A, *rec*A803, *uvs*X, (Roca, A.I , *Crit. Rev. Biochem. Molec. Biol.* 25, 415 (1990)), *sep*1 (Kolodner et al , *Proc. Natl. Acad. Sci. (U.S.A.)* 84, 5560 (1987), Tishkoff et al., *Molec. Cell. Biol.* 11, 2593), *Ruv*C (Dunderdale et al., *Nature* 354, 506 (1991)), *DST*2, *KEM*1, *XRN*1 (Dykstra et al., *Molec. Cell. Biol.* 11, 2583 (1991)), *STP*α/*DST*1 (Clark et al., *Molec. Cell. Biol.* 11, 2576 (1991)), *HPP*-1 (Moore et al., *Proc. Natl. Acad. Sci.* (U.S.A.) 88, 9067 (1991)), other eukaryotic recombinases (Bishop et al , *Cell* 69, 439 (1992); Shinohara et al., *Cell* 69, 457.

*Rec*A protein forms a nucleoprotein filament when it coats a single-stranded DNA. In this nucleoprotein filament, one monomer of *rec*A protein is bound to about 3 nucleotides. This property of *rec*A to coat single-stranded DNA is essentially sequence independent, although particular sequences favor initial loading of *rec*A onto a polynucleotide (e.g., nucleation sequences) The nucleoprotein filament(s) can be formed on essentially any DNA to be shuffled and can form complexes with both single-stranded and double-stranded DNA in prokaryotic and eukaryotic cells.

Before contacting with *rec*A or other recombinase, fragments are often denatured, e.g., by heat-treatment. *Rec*A protein is then added at a concentration of about 1-10 μM. After incubation, the *rec*A-coated single-stranded DNA is introduced into recipient cells by conventional methods, such as chemical transformation or electroporation. In general, it can be desirable to coat the DNA with a RecA homolog isolated from the organism into which the coated DNA is being delivered. Recombination involves several cellular factors and the host RecA equivalent generally interacts better with other host factors than less closely related RecA molecules The fragments undergo homologous recombination with cognate endogenous genes. Because of the increased frequency of recombination due to recombinase coating, the fragments need not be introduced as components of vectors.

Fragments are sometimes coated with other nucleic acid binding proteins that promote recombination, protect nucleic acids from degradation, or target nucleic acids to the nucleus. Examples of such proteins includes Agrobacterium virE2 (Durrenberger et al., *Proc. Natl. Acad. Sci. USA* 86, 9154-9158 (1989)). Alternatively, the recipient strains are deficient in RecD activity. Single stranded ends can also be generated by 3'-5' exonuclease activity or restriction enzymes producing 5' overhangs.

1 *Mut*S selection

The *E. coli* mismatch repair protein MutS can be used in affinity chromatography to enrich for fragments of double-stranded DNA containing at least one base of mismatch. The MutS protein recognizes the bubble formed by the individual strands about the point of the mismatch. See, e g., Hsu & Chang, WO 9320233 The strategy of affinity enriching for partially mismatched duplexes can be incorporated into the present methods to increase the diversity between an incoming library of fragments and corresponding cognate or allelic genes in recipient cells.

Fig. 2 shows one scheme in which MutS is used to increase diversity The DNA substrates for enrichment are substantially similar to each other but differ at a few sites. For example, the DNA substrates can represent complete or partial genomes (e.g., a chromosome library) from different individuals with the differences being due to polymorphisms. The substrates can also represent induced mutants of a wildtype sequence. The DNA substrates are pooled, restriction digested, and denatured to produce fragments of single-stranded DNA The single-stranded DNA is then allowed to reanneal Some single-stranded fragments reanneal with a perfectly matched complementary strand to generate perfectly matched duplexes Other single-stranded fragments anneal to generate mismatched duplexes. The mismatched duplexes are enriched from perfectly matched duplexes by MutS chromatography (e.g., with MutS immobilized to beads). The mismatched duplexes recovered by chromatography are introduced into recipient cells for recombination with cognate endogenous genes as described above. MutS affinity chromatography increases the proportion of fragments differing from each other and the cognate endogenous gene. Thus, recombination between the incoming fragments and endogenous genes results in greater diversity.

Fig. 3 shows a second strategy for MutS enrichment. In this strategy, the substrates for MutS enrichment represent variants of a relatively short segment, for example, a gene or cluster of genes, in which most of the different variants differ at no more than a single nucleotide. The goal of MutS enrichment is to produce substrates for recombination that contain more variations than sequences occurring in nature. This is achieved by fragmenting the substrates at random to produce overlapping fragments. The fragments are denatured and reannealed as in the first strategy. Reannealing generates some mismatched duplexes which can be separated from perfectly matched duplexes by MutS affinity chromatography. As before, MutS chromatography enriches for duplexes bearing at least a single mismatch. The mismatched duplexes are then reassembled into longer fragments. This is accomplished by

cycles of denaturation, reannealing, and chain extension of partially annealed duplexes (see Section V) After several such cycles, fragments of the same length as the original substrates are achieved, except that these fragments differ from each other at multiple sites These fragments are then introduced into cells where they undergo recombination with cognate endogenous genes

2. Positive Selection For Allelic Exchange

The invention further provides methods of enriching for cells bearing modified genes relative to the starting cells. This can be achieved by introducing a DNA fragment library (e.g., a single specific segment or a whole or partial genomic library) in a suicide vector (i.e., lacking a functional replication origin in the recipient cell type) containing both positive and negative selection markers. Optionally, multiple fragment libraries from different sources (e.g., *B. subtilis, B. licheniformis* and *B. cereus*) can be cloned into different vectors bearing different selection markers. Suitable positive selection markers include *neo*^{R}, kanamycin^R, *hyg*, *his*D, *gpt*, *ble*, *tet*^{R}. Suitable negative selection markers include *hsv*-tk, *hprt*, *gpt*, *Sac*B *ura*3and cytosine deaminase. A variety of examples of conditional replication vectors, mutations affecting vector replication, limited host range vectors, and counterselectable markers are found in Berg and Berg, *supra*, and LaRossa, *ibid.* and the references therein.

In one example, a plasmid with R6K and f1 origins of replication, a positively selectable marker (beta-lactamase), and a counterselectable marker (B. subtilis sacB) was used. M13 transduction of plasmids containing cloned genes were efficiently recombined into the chromosomal copy of that gene in a *rep* mutant *E. coli* strain.

Another strategy for applying negative selection is to include a wildtype *rps*L gene (encoding ribosomal protein S12) in a vector for use in cells having a mutant *rps*L gene conferring streptomycin resistance. The mutant form of *rps*L is recessive in cells having wildtype *rps*L Thus, selection for Sm resistance selects against cells having a wildtype copy of *rps*L. See Skorupski & Taylor, *Gene* 169, 47-52 (1996). Alternatively, vectors bearing only a positive selection marker can be used with one round of selection for cells expressing the marker, and a subsequent round of screening for cells that have lost the marker (e.g., screening for drug sensitivity) The screen for cells that have lost the positive selection marker is equivalent to screening against expression of a negative selection marker. For example, Bacillus can be transformed with a vector bearing a CAT gene and a sequence to be integrated. See Harwood & Cutting, *Molecular Biological Methods for Bacillus*, at pp. 31-33. Selection for chloramphenicol resistance isolates cells that have taken up vector. After a

suitable period to allow recombination, selection for CAT sensitivity isolates cells which have lost the CAT gene. About 50% of such cells will have undergone recombination with the sequence to be integrated

Suicide vectors bearing a positive selection marker and optionally, a negative selection marker and a DNA fragment can integrate into host chromosomal DNA by a single crossover at a site in chromosomal DNA homologous to the fragment Recombination generates an integrated vector flanked by direct repeats of the homologous sequence In some cells, subsequent recombination between the repeats results in excision of the vector and either acquisition of a desired mutation from the vector by the genome or restoration of the genome to wildtype.

In the present methods, after transfer of the gene library cloned in a suitable vector, positive selection is applied for expression of the positive selection marker Because nonintegrated copies of the suicide vector are rapidly eliminated from cells, this selection enriches for cells that have integrated the vector into the host chromosome. The cells surviving positive selection can then be propagated and subjected to negative selection, or screened for loss of the positive selection marker Negative selection selects against cells expressing the negative selection marker Thus, cells that have retained the integrated vector express the negative marker and are selectively eliminated. The cells surviving both rounds of selection are those that initially integrated and then eliminated the vector. These cells are enriched for cells having genes modified by homologous recombination with the vector. This process diversifies by a single exchange of genetic information. However, if the process is repeated either with the same vectors or with a library of fragments generated by PCR of pooled DNA from the enriched recombinant population, resulting in the diversity of targeted genes being enhanced exponentially each round of recombination. This process can be repeated recursively, with selection being performed as desired.

3 Individualized Optimization of Genes

In general, the above methods do not require knowledge of the number of genes to be optimized, their map location or their function. However, in some instances, where this information is available for one or more gene, it can be exploited. For example, if the property to be acquired by evolution is enhanced recombination of cells, one gene likely to be important is *rec*A, even though many other genes, known and unknown, may make additional contributions. In this situation, the *rec*A gene can be evolved, at least in part, separately from other candidate genes. The *rec*A gene can be evolved by any of the methods

of recursive recombination described in Section V Briefly, this approach entails obtaining diverse forms of a *rec*A gene, allowing the forms to recombine, selecting recombinants having improved properties, and subjecting the recombinants to further cycles of recombination and selection. At any point in the individualized improvement of *rec*A, the diverse forms of *rec*A can be pooled with fragments encoding other genes in a library to be used in the general methods described herein. In this way, the library is seeded to contain a higher proportion of variants in a gene known to be important to the property sought to be acquired than would otherwise be the case

In one example (illustrated in Fig 20B), a plasmid is constructed carrying a non-functional (mutated) version of a chromosomal gene such as *URA3*, where the wild-type gene confers sensitivity to a drug (in this case 5-fluoroorotic acid) The plasmid also carries a selectable marker (resistance to another drug such as kanamycin), and a library of *recA* variants Transformation of the plasmid into the cell results in expression of the *recA* variants, some of which will catalyze homologous recombination at an increased rate Those cells in which homologous recombination occurred are resistant to the selectable drug on the plasmid, and to 5-fluoroorotic acid because of the disruption of the chromosomal copy of this gene The *recA* variants which give the highest rates of homologous recombination are the most highly represented in a pool of homologous recombinants. The mutant *recA* genes can be isolated from this pool by PCR, re-shuffled, cloned back into the plasmid and the process repeated Other sequences can be inserted in place of *recA* to evolve other components of the homologous recombination system

4 Harvesting DNA Substrates for Shuffling

In some shuffling methods, DNA substrates are isolated from natural sources and are not easily manipulated by DNA modifying or polymerizing enzymes due to recalcitrant impurities, which poison enzymatic reactions. Such difficulties can be avoided by processing DNA substrates through a harvesting strain. The harvesting strain is typically a cell type with natural competence and a capacity for homologous recombination between sequences with substantial diversity (e.g., sequences exhibiting only 75% sequence identity) The harvesting strain bears a vector encoding a negative selection marker flanked by two segments respectively complementary to two segments flanking a gene or other region of interest in the DNA from a target organism The harvesting strain is contacted with fragments of DNA from the target organism. Fragments are taken up by natural competence, or other methods described herein, and a fragment of interest from the target organism recombines with the

vector of the harvesting strain causing loss of the negative selection marker. Selection against the negative marker allows isolation of cells that have taken up the fragment of interest Shuffling can be carried out in the harvester strain (e.g., a RecE/T strain) or vector can be isolated from the harvester strain for in vitro shuffling or transfer to a different cell type for in vivo shuffling Alternatively, the vector can be transferred to a different cell type by conjugation, protoplast fusion or electrofusion. An example of a suitable harvester strain is *Acinetobacter calcoaceticus mut*S. Melnikov and Youngman, (1999) Nucl Acid Res 27(4):1056-1062 This strain is naturally competent and takes up DNA in a nonsequence-specific manner Also, because of the mutS mutation, this strain is capable of homologous recombination of sequences showing only 75% sequence identity

IV APPLICATIONS

A. RECOMBINOGENICITY

One goal of whole cell evolution is to generate cells having improved capacity for recombination. Such cells are useful for a variety of purposes in molecular genetics including the *in vivo* formats of recursive sequence recombination described in Section V. Almost thirty genes (e.g., *rec*A, *rec*B, *rec*C, *rec*D, *rec*E, *rec*F, *rec*G, *rec*O, *rec*Q, *rec*R, *rec*T, *ruv*A, *ruv*B, *ruv*C, *sbc*B, *ssb*, *top*A, *gyr*A and B, *lig*, *pol*A, *uvr*D, E, *rec*L, *mut*D, *mut*H, *mut*L, *mut*T, *mut*U, *hel*D) and DNA sites (e.g., *chi*, *rec*N, *sbc*C) involved in genetic recombination have been identified in *E. coli*, and cognate forms of several of these genes have been found in other organisms (e.g., rad51, rad55-rad57, Dmc1 in yeast (see Kowalczykowski et al., *Microbiol. Rev.* 58, 401-465 (1994); Kowalczkowski & Zarling, *supra*) and human homologs of Rad51 and Dmc1 have been identified (see Sandler et al., *Nucl. Acids Res.* 24, 2125-2132 (1996)). At least some of the *E. coli* genes, including recA are functional in mammalian cells, and can be targeted to the nucleus as a fusion with SV40 large T antigen nuclear targeting sequence (Reiss et al., *Proc. Natl. Acad. Sci. USA,* 93, 3094-3098 (1996)). Further, mutations in mismatch repair genes, such as *mut*L, *mut*S, *mut*H, *mut*T relax homology requirements and allow recombination between more diverged sequences (Rayssiguier et al., *Nature* 342, 396-401 (1989)). The extent of recombination between divergent strains can be enhanced by impairing mismatch repair genes and stimulating SOS genes. Such can be achieved by use of appropriate mutant strains and/or growth under conditions of metabolic stress, which have been found to stimulate SOS and inhibit mismatch repair genes. Vulic et al., *Proc. Natl. Acad. Sci. USA* 94 (1997). In addition, this can be achieved by impairing the products of mismatch repair genes by exposure to selective inhibitors

Starting substrates for recombination are selected according to the general principles described above That is, the substrates can be whole genomes or fractions thereof containing recombination genes or sites. Large libraries of essentially random fragments can be seeded with collections of fragments constituting variants of one or more known recombination genes, such as recA. Alternatively, libraries can be formed by mixing variant forms of the various known recombination genes and sites

The library of fragments is introduced into the recipient cells to be improved and recombination occurs, generating modified cells. The recipient cells preferably contain a marker gene whose expression has been disabled in a manner that can be corrected by recombination. For example, the cells can contain two copies of a marker gene bearing mutations at different sites, which copies can recombine to generate the wildtype gene. A suitable marker gene is green fluorescent protein A vector can be constructed encoding one copy of GFP having stopcodons near the N-terminus, and another copy of GFP having stopcodons near the C-terminus of the protein The distance between the stop codons at the respective ends of the molecule is 500 bp and about 25% of recombination events result in active GFP. Expression of GFP in a cell signals that a cell is capable of homologous recombination to recombine in between the stop codons to generate a contiguous coding sequence. By screening for cells expressing GFP, one enriches for cells having the highest capacity for recombination. The same type of screen can be used following subsequent rounds of recombination. However, unless the selection marker used in previous round(s) was present on a suicide vector, subsequent round(s) should employ a second disabled screening marker within a second vector bearing a different origin of replication or a different positive selection marker to vectors used in the previous rounds.

B MULTIGENOMIC COPY NUMBER--GENE REDUNDANCY

The majority of bacterial cells in stationary phase cultures grown in rich media contain two, four or eight genomes. In minimal medium the cells contain one or two genomes. The number of genomes per bacterial cell thus depends on the growth rate of the cell as it enters stationary phase. This is because rapidly growing cells contain multiple replication forks, resulting in several genomes in the cells after termination. The number of genomes is strain dependent, although all strains tested have more than one chromosome in stationary phase. The number of genomes in stationary phase cells decreases with time. This appears to be due to fragmentation and degradation of entire chromosomes, similar to apoptosis in mammalian cells. This fragmentation of genomes in cells containing multiple genome copies

results in massive recombination and mutagenesis Useful mutants may find ways to use energy sources that will allow them to continue growing Multigenome or gene-redundant cells are much more resistant to mutagenesis and can be improved for a selected trait faster

Some cell types, such as *Deinococcus radians* (Daly and Minton *J. Bacteriol.* 177, 5495-5505 (1995)) exhibit polyploidy throughout the cell cycle This cell type is highly radiation resistant due to the presence of many copies of the genome High frequency recombination between the genomes allows rapid removal of mutations induced by a variety of DNA damaging agents.

A goal of the present methods is to evolve other cell types to have increased genome copy number akin to that of *Deinoccocus radians*. Preferably, the increased copy number is maintained through all or most of its cell cycle in all or most growth conditions. The presence of multiple genome copies in such cells results in a higher frequency of homologous recombination in these cells, both between copies of a gene in different genomes within the cell, and between a genome within the cell and a transfected fragment. The increased frequency of recombination allows the cells to be evolved more quickly to acquire other useful characteristics

Starting substrates for recombination can be a diverse library of genes only a few of which are relevant to genomic copy number, a focused library formed from variants of gene(s) known or suspected to have a role in genomic copy number or a combination of the two. As a general rule one would expect increased copy number would be achieved by evolution of genes involved in replication and cell septation such that cell septation is inhibited without impairing replication. Genes involved in replication include *tus*, *xer*C, *xer*D, *dif*, *gyr*A, *gyr*B, *par*E, *par*C, *dif*, *Ter*A, *Ter*B, *Ter*C, *Ter*D, *Ter*E, *Ter*F, and genes influencing chromosome partitioning and gene copy number include *min*D, *muk*A (*tol*C), *muk*B, *muk*C, *muk*D, *spo*OJ, *spo*IIIE (Wake & Errington, *Annu. Rev. Genet.* 29, 41-67 (1995)). A useful source of substrates is the genome of a cell type such as *Deinoccocus radians* known to have the desired phenotype of multigenomic copy number. As well as, or instead of, the above substrates, fragments encoding protein or antisense RNA inhibitors to genes known to be involved in cell septation can also be used.

In nature, the existence of multiple genomic copies in a cell type would usually not be advantageous due to the greater nutritional requirements needed to maintain this copy number. However, artificial conditions can be devised to select for high copy number. Modified cells having recombinant genomes are grown in rich media (in which conditions,

multicopy number should not be a disadvantage) and exposed to a mutagen, such as ultraviolet or gamma irradiation or a chemical mutagen, e g , mitomycin, nitrous acid, photoactivated psoralens, alone or in combination, which induces DNA breaks amenable to repair by recombination. These conditions select for cells having multicopy number due to the greater efficiency with which mutations can be excised. Modified cells surviving exposure to mutagen are enriched for cells with multiple genome copies If desired, selected cells can be individually analyzed for genome copy number (e.g., by quantitative hybridization with appropriate controls) Some or all of the collection of cells surviving selection provide the substrates for the next round of recombination. In addition, individual cells can be sorted using a cell sorter for those cells containing more DNA, e.g., using DNA specific fluorescent compounds or sorting for increased size using light dispersion. Eventually cells are evolved that have at least 2, 4, 6, 8 or 10 copies of the genome throughout the cell cycle. In a similar manner, protoplasts can also be recombined.

C. SECRETION

The protein (or metabolite) secretion pathways of bacterial and eukaryotic cells can be evolved to export desired molecules more efficiently, such as for the manufacturing of protein pharmaceuticals, small molecule drugs or specialty chemicals. Improvements in efficiency are particularly desirable for proteins requiring multisubunit assembly (such as antibodies) or extensive posttranslational modification before secretion.

The efficiency of secretion may depend on a number of genetic sequences including a signal peptide coding sequence, sequences encoding protein(s) that cleave or otherwise recognize the coding sequence, and the coding sequence of the protein being secreted. The latter may affect folding of the protein and the ease with which it can integrate into and traverse membranes. The bacterial secretion pathway in *E. coli* include the *Sec*A, *Sec*B, *Sec*E, *Sec*D and *Sec*F genes. In *Bacillus subtilis*, the major genes are secA, secD, secE, secF, secY, ffh, ftsY together with five signal peptidase genes (sipS, sipT, sipU, sipV and sipW) (Kunst et al, *supra*). For proteins requiring posttranslational modification, evolution of genes effecting such modification may contribute to improved secretion. Likewise genes with expression products having a role in assembly of multisubunit proteins (e.g., chaperonins) may also contribute to improved secretion.

Selection of substrates for recombination follows the general principles discussed above. In this case, the focused libraries referred to above comprise variants of the known secretion genes. For evolution of prokaryotic cells to express eukaryotic proteins, the

initial substrates for recombination are often obtained at least in part from eukaryotic sources Incoming fragments can undergo recombination both with chromosomal DNA in recipient cells and with the screening marker construct present in such cells (see below). The latter form of recombination is important for evolution of the signal coding sequence incorporated in the screening marker construct Improved secretion can be screened by the inclusion of marker construct in the cells being evolved The marker construct encodes a marker gene, operably linked to expression sequences, and usually operably linked to a signal peptide coding sequence. The marker gene is sometimes expressed as a fusion protein with a recombinant protein of interest. This approach is useful when one wants to evolve the recombinant protein coding sequence together with secretion genes

In one variation, the marker gene encodes a product that is toxic to the cell containing the construct unless the product is secreted. Suitable toxin proteins include diphtheria toxin and ricin toxin Propagation of modified cells bearing such a construct selects for cells that have evolved to improve secretion of the toxin. Alternatively, the marker gene can encode a ligand to a known receptor, and cells bearing the ligand can be detected by FACS using labeled receptor. Optionally, such a ligand can be operably linked to a phospholipid anchoring sequence that binds the ligand to the cell membrane surface following secretion. (See commonly owned, copending 08/309,345). In a further variation, secreted marker protein can be maintained in proximity with the cell secreting it by distributing individual cells into agar drops. This is done, e g., by droplet formation of a cell suspension. Secreted protein is confined within the agar matrix and can be detected by e.g., FACS. In another variation, a protein of interest is expressed as a fusion protein together with b-lactamase or alkaline phosphatase. These enzymes metabolize commercially available chromogenic substrates (e.g., X-gal), but do so only after secretion into the periplasm. Appearance of colored substrate in a colony of cells therefore indicates capacity to secrete the fusion protein and the intensity of color is related to the efficiency of secretion.

The cells identified by these screening and selection methods have the capacity to secrete increased amounts of protein. This capacity may be attributable to increased secretion and increased expression, or from increased secretion alone.

1 Expression

Cells can also be evolved to acquire increased expression of a recombinant protein. The level of expression is, of course, highly dependent on the construct from which the recombinant protein is expressed and the regulatory sequences, such as the promoter,

enhancer(s) and transcription termination site contained therein. Expression can also be affected by a large number of host genes having roles in transcription, posttranslational modification and translation. In addition, host genes involved in synthesis of ribonucleotide and amino acid monomers for transcription and translation may have indirect effects on efficiency of expression Selection of substrates for recombination follows the general principles discussed above. In this case, focused libraries comprise variants of genes known to have roles in expression. For evolution of prokaryotic cells to express eukaryotic proteins, the initial substrates for recombination are often obtained, at least in part, from eukaryotic sources; that is eukaryotic genes encoding proteins such as chaperonins involved in secretion and/assembly of proteins. Incoming fragments can undergo recombination both with chromosomal DNA in recipient cells and with the screening marker construct present in such cells (see below).

Screening for improved expression can be effected by including a reporter construct in the cells being evolved. The reporter construct expresses (and usually secretes) a reporter protein, such as GFP, which is easily detected and nontoxic. The reporter protein can be expressed alone or together with a protein of interest as a fusion protein. If the reporter gene is secreted, the screening effectively selects for cells having either improved secretion or improved expression, or both.

2 Plant Cells

A further application of recursive sequence recombination is the evolution of plant cells, and transgenic plants derived from the same, to acquire resistance to pathogenic diseases (fungi, viruses and bacteria), insects, chemicals (such as salt, selenium, pollutants, pesticides, herbicides, or the like), including, e.g., atrazine or glyphosate, or to modify chemical composition, yield or the like. The substrates for recombination can again be whole genomic libraries, fractions thereof or focused libraries containing variants of gene(s) known or suspected to confer resistance to one of the above agents. Frequently, library fragments are obtained from a different species to the plant being evolved.

The DNA fragments are introduced into plant tissues, cultured plant cells, plant microspores, or plant protoplasts by standard methods including electroporation (From et al., *Proc. Natl. Acad. Sci. USA* 82, 5824 (1985), infection by viral vectors such as cauliflower mosaic virus (CaMV) (Hohn et al., *Molecular Biology of Plant Tumors*, (Academic Press, New York, 1982) pp. 549-560; Howell, US 4,407,956), high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on

the surface (Klein et al., *Nature* 327, 70-73 (1987)), use of pollen as vector (WO 85/01856), or use of *Agrobacterium tumefaciens* or *A. rhizogenes* carrying a T-DNA plasmid in which DNA fragments are cloned The T-DNA plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and a portion is stably integrated into the plant genome (Horsch et al., *Science* 233, 496-498 (1984), Fraley et al., *Proc. Natl. Acad. Sci. USA* 80, 4803 (1983))

Diversity can also be generated by genetic exchange between plant protoplasts according to the same principles described below for fungal protoplasts. Procedures for formation and fusion of plant protoplasts are described by Takahashi et al., US 4,677,066, Akagi et al., US 5,360,725; Shimamoto et al., Us 5,250,433; Cheney et al., US 5,426,040.

After a suitable period of incubation to allow recombination to occur and for expression of recombinant genes, the plant cells are contacted with the agent to which resistance is to be acquired, and surviving plant cells are collected. Some or all of these plant cells can be subject to a further round of recombination and screening Eventually, plant cells having the required degree of resistance are obtained.

These cells can then be cultured into transgenic plants. Plant regeneration from cultured protoplasts is described in Evans et al., "Protoplast Isolation and Culture," *Handbook of Plant Cell Cultures* 1, 124-176 (MacMillan Publishing Co., New York, 1983); Davey, "Recent Developments in the Culture and Regeneration of Plant Protoplasts," *Protoplasts*, (1983) pp. 12-29, (Birkhauser, Basal 1983); Dale, "Protoplast Culture and Plant Regeneration of Cereals and Other Recalcitrant Crops," *Protoplasts* (1983) pp. 31-41, (Birkhauser, Basel 1983); Binding, "Regeneration of Plants," *Plant Protoplasts*, pp. 21-73, (CRC Press, Boca Raton, 1985).

In a variation of the above method, one or more preliminary rounds of recombination and screening can be performed in bacterial cells according to the same general strategy as described for plant cells. More rapid evolution can be achieved in bacterial cells due to their greater growth rate and the greater efficiency with which DNA can be introduced into such cells. After one or more rounds of recombination/screening, a DNA fragment library is recovered from bacteria and transformed into the plant cells. The library can either be a complete library or a focused library. A focused library can be produced by amplification from primers specific for plant sequences, particularly plant sequences known or suspected to have a role in conferring resistance.

3. Example. Concatemeric Assembly of Atrazine-Catabolizing Plasmid

Pseudomonas atrazine catabolizing genes AtzA and AtzB were subcloned from pMD1 (deSouza et al., *Appl. Environ. Microbiol.* 61, 3373-3378 (1995); de Souza et al., *J. Bacteriol.* 178, 4894-4900 (1996)) into pUC18. A 1 9 kb AvaI fragment containing AtzA was end-filled and inserted into an AvaI site of pUC18. A 3.9 kb ClaI fragment containing AtzB was end-filled and cloned into the HincII site of pUC18 AtzA was then excised from pUC18 with EcoRI and BamHI, AzB with BamHI and HindIII, and the two inserts were co-ligated into pUC18 digested with EcoRI and HindIII The result was a 5.8 kb insert containing AtzA and AtzB in pUC18 (total plasmid size 8 4 kb)

Recursive sequence recombination was performed as follows. The entire 8.4 kb plasmid was treated with DNaseI in 50 mM Tris-Cl pH 7.5, 10 mM $MnCl_2$ and fragments between 500 and 2000 bp were gel purified. The fragments were assembled in a PCR reaction using Tth-XL enzyme and buffer from Perkin Elmer, 2.5 mM MgOAc, 400 μM dNTPs and serial dilutions of DNA fragments. The assembly reaction was performed in an MJ Research "DNA Engine" programmed with the following cycles: 1) 94°C, 20 seconds; 2) 94°C, 15 seconds, 3) 40°C, 30 seconds; 4) 72°C, 30 seconds + 2 seconds per cycle; 5) go to step 2, 39 more times; 6) 4°C.

The AtzA and AtzB genes were not amplified from the assembly reaction using the polymerase chain reaction, so instead DNA was purified from the reaction by phenol extraction and ethanol precipitation, then digested the assembled DNA with a restriction enzyme that linearized the plasmid (KpnI: the KpnI site in pUC18 was lost during subcloning, leaving only the KpnI site in AtzA). Linearized plasmid was gel-purified, self-ligated overnight and transformed into *E. coli* strain NM522. (The choice of host strain was relevant very little plasmid of poor quality was obtained from a number of other commercially available strains including TG1, DH10B, DH12S.)

Serial dilutions of the transformation reaction were plated onto LB plates containing 50 μg/ml ampicillin, the remainder of the transformation was made 25% in glycerol and frozen at -80°C. Once the transformed cells were titered, the frozen cells were plated at a density of between 200 and 500 on 150 mm diameter plates containing 500 μg/ml atrazine and grown at 37°C.

Atrazine at 500μg/ml forms an insoluble precipitate. The products of the AtzA and AtzB genes transform atrazine into a soluble product. Cells containing the wild type AtzA and AtzB genes in pUC18 will thus be surrounded by a clear halo where the atrazine has been degraded The more active the AtzA and AtzB enzymes, the more rapidly a clear halo will

form and grow on atrazine-containing plates Positives were picked as those colonies that most rapidly formed the largest clear zones The (approximately) 40 best colonies were picked, pooled, grown in the presence of 50 μg/ml ampicillin and plasmid prepared from them The entire process (from DNase-treatment to plating on atrazine plates) was repeated 4 times with 2000-4000 colonies/cycle.

A modification was made in the fourth round. Cells were plated on both 500 μg/ml atrazine, and 500 μg/ml of the atrazine analogue terbutylazine, which was undegradable by the wild type AtzA and AtzB genes Positives were obtained that degraded both compounds The atrazine chlorohydrolase (product of AtzA gene) was 10-100 fold higher than that produced by the wildtype gene

D. PLANT GENOME SHUFFLING

Plant genome shuffling allows recursive cycles to be used for the introduction and recombination of genes or pathways that confer improved properties to desired plant species. Any plant species, including weeds and wild cultivars, showing a desired trait, such as herbicide resistance, salt tolerance, pest resistance, or temperature tolerance, can be used as the source of DNA that is introduced into the crop or horticultural host plant species.

Genomic DNA prepared from the source plant is fragmented (e g. by DNaseI, restriction enzymes, or mechanically) and cloned into a vector suitable for making plant genomic libraries, such as pGA482 (An. G., 1995, Methods Mol. Biol. 44:47-58). This vector contains the *A. tumefaciens* left and right borders needed for gene transfer to plant cells and antibiotic markers for selection in *E. coli, Agrobacterium,* and plant cells. A multicloning site is provided for insertion of the genomic fragments A cos sequence is present for the efficient packaging of DNA into bacteriophage lambda heads for transfection of the primary library into *E. coli*. The vector accepts DNA fragments of 25-40 kb.

The primary library can also be directly electroporated into an *A. tumefaciens* or *A. rhizogenes* strain that is used to infect and transform host plant cells (Main, GD et al., 1995, Methods Mol. Biol. 44:405-412). Alternatively, DNA can be introduced by electroporation or PEG-mediated uptake into protoplasts of the recipient plant species (Bilang et al. (1994) Plant Mol Biol Manual, Kluwer Academic Publishers, A1:1-16) or by particle bombardment of cells or tissues (Christou, ibid, A2.1-15). If necessary, antibiotic markers in the T-DNA region can be eliminated, as long as selection for the trait is possible, so that the final plant products contain no antibiotic genes.

Stably transformed whole cells acquiring the trait are selected on solid or liquid media containing the agent to which the introduced DNA confers resistance or tolerance If the trait in question cannot be selected for directly, transformed cells can be selected with antibiotics and allowed to form callus or regenerated to whole plants and then screened for the desired property.

The second and further cycles consist of isolating genomic DNA from each transgenic line and introducing it into one or more of the other transgenic lines. In each round, transformed cells are selected or screened for incremental improvement. To speed the process of using multiple cycles of transformation, plant regeneration can be deferred until the last round. Callus tissue generated from the protoplasts or transformed tissues can serve as a source of genomic DNA and new host cells. After the final round, fertile plants are regenerated and the progeny are selected for homozygosity of the inserted DNAs Ultimately, a new plant is created that carries multiple inserts which additively or synergistically combine to confer high levels of the desired trait Alternatively, microspores can be isolated as homozygotes generated from spontaneous diploids

In addition, the introduced DNA that confers the desired trait can be traced because it is flanked by known sequences in the vector. Either PCR or plasmid rescue is used to isolate the sequences and characterize them in more detail. Long PCR (Foord, OS and Rose, EA, 1995, PCR Primer: A Laboratory Manual, CSHL Press, pp 63-77) of the full 25-40 kb insert is achieved with the proper reagents and techniques using as primers the T-DNA border sequences. If the vector is modified to contain the *E. coli* origin of replication and an antibiotic marker between the T-DNA borders, a rare cutting restriction enzyme, such as NotI or SfiI, that cuts only at the ends of the inserted DNA is used to create fragments containing the source plant DNA that are then self-ligated and transformed into *E. coli* where they replicate as plasmids. The total DNA or subfragment of it that is responsible for the transferred trait can be subjected to in vitro evolution by DNA shuffling. The shuffled library can be reiteratively recombined by any method herein and then introduced into host plant cells and screened for improvement of the trait. In this way, single and multigene traits can be transferred from one species to another and optimized for higher expression or activity leading to whole organism improvement. This entire process can also be reiteratively repeated.

Alternatively, the cells can be transformed microspores with the regenerated haploid plants being screened directly for improved traits as noted below.

E MICROSPORE MANIPULATION

Microspores are haploid (1n) male spores that develop into pollen grains Anthers contain a large numbers of microspores in early-uninucleate to first-mitosis stages Microspores have been successfully induced to develop into plants for most species, such as, e.g., rice (Chen, CC 1977 In Vitro 13. 484-489), tobacco (Atanassov, I et al. 1998 Plant Mol Biol. 38:1169-1178), Tradescantia (Savage JRK and Papworth DG 1998 Mutat Res. 422:313-322), Arabidopsis (Park SK et al. 1998 Development. 125:3789-3799), sugar beet (Majewska-Sawka A and Rodrigues-Garcia MI 1996 J Cell Sci. 109:859-866), Barley (Olsen FL 1991 Hereditas 115:255-266) and oilseed rape (Boutillier KA et al. 1994 Plant Mol Biol 26.1711-1723)

The plants derived from microspores are predominantly haploid or diploid (infrequently polyploid and aneuploid). The diploid plants are homozygous and fertile and can be generated in a relatively short time Microspores obtained from F1 hybrid plants represent great diversity, thus being an excellent model for studying recombination. In addition, microspores can be transformed with T-DNA introduced by agrobacterium or other available means and then regenerated into individual plants. Furthermore, protoplasts can be made from microspores and they can be fused similar to what occur in fungi and bacteria.

Microspores, due to their complex ploidy and regenerating ability, provide a tool for plant whole genome shuffling. For example, if pollens from 4 parents are collected and pooled, and then used to randomly pollinate the parents, the progenies should have 2^4 = 16 possible combinations. Assuming this plant has 7 chromosomes, microspores collected from the 16 progenies will represent 2^7x16 = 2048 possible chromosomal combinations. This number is even greater if meiotic processes occur. When diploid, homozygous embryos are generated from these microspores, in many cases, they are screened for desired phenotypes, such as herbicide- or disease- resistant. In addition, for plant oil composition these embryos can be dissected into two halves: one for analysis the other for regeneration into a viable plant.

Protoplasts generated from microspores (especially the haploid ones) are pooled and fused. Microspores obtained from plants generated by protoplast fusion are pooled and fused again, increasing the genetic diversity of the resulting microspores.

Microspores can be subjected to mutagenesis in various ways, such as by chemical mutagenesis, radiation-induced mutagenesis and, e.g., t-DNA transformation, prior to fusion or regeneration. New mutations which are generated can be recombined through the recursive processes described above and herein.

F. EXAMPLE: ACQUISITION OF SALT TOLERANCE

As depicted in Fig. 21, DNA from a salt tolerant plant is isolated and used to create a genomic library Protoplasts made from the recipient species are transformed/transfected with the genomic library (e.g., by electroporation, agrobacterium, etc) Cells are selected on media with a normally inhibitory level of NaCl. Only the cells with newly acquired salt tolerance will grow into callus tissue. The best lines are chosen and genomic libraries are made from their pooled DNA These libraries are transformed into protoplasts made from the first round transformed calli. Again, cells are selected on increased salt concentrations After the desired level of salt tolerance is achieved, the callus tissue can be induced to regenerate whole plants. Progeny of these plants are typically analyzed for homozygosity of the inserts to ensure stability of the acquired trait. At the indicated steps, plant regeneration or isolation and shuffling of the introduced genes can be added to the overall protocol.

G TRANSGENIC ANIMALS

1 Transgene Optimization

One goal of transgenesis is to produce transgenic animals, such as mice, rabbits, sheep, pigs, goats, and cattle, secreting a recombinant protein in the milk. A transgene for this purpose typically comprises in operable linkage a promoter and an enhancer from a milk-protein gene (e.g., α, β, or γ casein, β-lactoglobulin, acid whey protein or α-lactalbumin), a signal sequence, a recombinant protein coding sequence and a transcription termination site. Optionally, a transgene can encode multiple chains of a multichain protein, such as an immunoglobulin, in which case, the two chains are usually individually operably linked to sets of regulatory sequences. Transgenes can be optimized for expression and secretion by recursive sequence recombination. Suitable substrates for recombination include regulatory sequences such as promoters and enhancers from milk-protein genes from different species or individual animals. Cycles of recombination can be performed *in vitro* or *in vivo* by any of the formats discussed in Section V. Screening is performed *in vivo* on cultures of mammary-gland derived cells, such as HC11 or MacT, transfected with transgenes and reporter constructs such as those discussed above. After several cycles of recombination and screening, transgenes resulting in the highest levels of expression and secretion are extracted from the mammary gland tissue culture cells and used to transfect embryonic cells, such as zygotes and embryonic stem cells, which are matured into transgenic animals.

2 Whole Animal Optimization

In this approach, libraries of incoming fragments are transformed into embryonic cells, such as ES cells or zygotes The fragments can be variants of a gene known to confer a desired property, such as growth hormone Alternatively, the fragments can be partial or complete genomic libraries including many genes

Fragments are usually introduced into zygotes by microinjection as described by Gordon et al., *Methods Enzymol.* 101, 414 (1984); Hogan et al., *Manipulation of the Mouse Embryo: A Laboratory Manual* (C.S.H.L. N.Y., 1986) (mouse embryo); and Hammer et al., *Nature* 315, 680 (1985) (rabbit and porcine embryos); Gandolfi et al , *J. Reprod. Fert.* 81, 23-28 (1987); Rexroad et al., *J. Anim. Sci.* 66, 947-953 (1988) (ovine embryos) and Eyestone et al., *J. Reprod. Fert.* 85, 715-720 (1989); Camous et al., *J. Reprod. Fert.* 72, 779-785 (1984); and Heyman et al., *Theriogenology* 27, 5968 (1987) (bovine embryos). Zygotes are then matured and introduced into recipient female animals which gestate the embryo and give birth to a transgenic offspring.

Alternatively, transgenes can be introduced into embryonic stem cells (ES). These cells are obtained from preimplantation embryos cultured *in vitro*. Bradley et al., *Nature* 309, 255-258 (1984) Transgenes can be introduced into such cells by electroporation or microinjection. Transformed ES cells are combined with blastocysts from a non-human animal. The ES cells colonize the embryo and in some embryos form the germ line of the resulting chimeric animal. *See* Jaenisch, *Science*, 240, 1468-1474 (1988).

Regardless whether zygotes or ES are used, screening is performed on whole animals for a desired property, such as increased size and/or growth rate. DNA is extracted from animals having evolved toward acquisition of the desired property. This DNA is then used to transfect further embryonic cells These cells can also be obtained from animals that have acquired toward the desired property in a split and pool approach. That is, DNA from one subset of such animals is transformed into embryonic cells prepared from another subset of the animals. Alternatively, the DNA from animals that have evolved toward acquisition of the desired property can be transfected into fresh embryonic cells. In either alternative, transfected cells are matured into transgenic animals, and the animals subjected to a further round of screening for the desired property.

Fig. 4 shows the application of this approach for evolving fish toward a larger size. Initially, a library is prepared of variants of a growth hormone gene. The variants can be natural or induced. The library is coated with recA protein and transfected into fertilized fish eggs. The fish eggs then mature into fish of different sizes. The growth hormone gene

fragment of genomic DNA from large fish is then amplified by PCR and used in the next round of recombination Alternatively, fish α-IFN is evolved to enhance resistance to viral infections as described below

3 Evolution of improved hormones for expression in transgenic animals (e g., Fish) to create animals with improved traits.

Hormones and cytokines are key regulators of size, body weight, viral resistance and many other commercially important traits. DNA shuffling is used to rapidly evolve the genes for these proteins using in vitro assays. This was demonstrated with the evolution of the human alpha interferon genes to have potent antiviral activity on murine cells Large improvements in activity were achieved in two cycles of family shuffling of the human IFN genes.

In general, a method of increasing resistance to virus infection in cells can be performed by first introducing a shuffled library comprising at least one shuffled interferon gene into animal cells to create an initial library of animal cells or animals. The initial library is then challenged with the virus Animal cells or animals are selected from the initial library which are resistant to the virus and a plurality of transgenes from a plurality of animal cells or animals which are resistant to the virus are recovered. The plurality of transgenes is recovered to produce an evolved library of animal cells or animals which is again challenged with the virus. Cells or animals are selected from the evolved library the which are resistant to the virus.

For example, genes evolved with in vitro assays are introduced into the germplasm of animals or plants to create improved strains. One limitation of this procedure is that in vitro assays are often only crude predictors of in vivo activity. However, with improving methods for the production of transgenic plants and animals, one can now marry whole organism breeding with molecular breeding. The approach is to introduce shuffled libraries of hormone genes into the species of interest. This can be done with a single gene per transgenic or with pools of genes per transgenic. Progeny are then screened for the phenotype of interest. In this case, shuffled libraries of interferon genes (alpha IFN for example) are introduced into transgenic fish. The library of transgenic fish are challenged with a virus The most resistant fish are identified (i.e. either survivors of a lethal challenge; or those that are deemed most □healthy' after the challenge). The IFN transgenes are recovered by PCR and shuffled in either a poolwise or a pairwise fashion. This generates an evolved library of IFN genes. A second library of transgenic fish is created and the process is repeated. In this way, IFN is evolved for improved antiviral activity in a whole organism assay.

This procedure is general and can be applied to any trait that is affected by a gene or gene family of interest and which can be quantitatively measured

Fish interferon sequence data is available for the Japanese flatfish (*Paralichthys olivaceus*) as mRNA sequence (Tamai *et al.* (1993) "Cloning and expression of flatfish (Paralichthys olivaceus) interferon cDNA." Biochem Biophys Acta 1174, 182-186; *see also*, Tami *et al.* (1993) "Purification and characterization of interferon-like antiviral protein derived from flatfish (*Paralichthys olivaceus*) lymphocytes immortalized by oncogenes." Cytotechnology 1993; 1 1 (2):121-131). This sequence can be used to clone out IFN genes from this species. This sequence can also be used as a probe to clone homologous interferons from additional species of fish. As well, additional sequence information can be utilized to clone out more species of fish interferons Once a library of interferons has been cloned, these can be family shuffled to generate a library of variants.

A Protein sequence of flatfish interferon is

MIRSTNSNKS DILMNCHHLIIR YDDNSAPSGGSL FRKMIMLLKL LKLITFGQLRVV ELFVKSNTSKTS TVLSIDGSNLISL LDAPKDILDKPSCNSF QLDLLLASSAWTLLT ARLLNYPYPA VLLSAGVASVVLVQVP.

In one embodiment, BHK-21 (A fibroblast cell line from hamster) can be transfected with the shuffled IFN-expression plasmids Active recombinant IFN is produced and then purified by WGA agarose affinity chromatography (Tamai, et al. 1993 Biochim Ciophys Acta. *supra*). The antiviral activity of IFN can be measured on fish cells challenged by rhabdoviurs Tami *et al.* (1993) "Purification and characterization of interferon-like antiviral protein derived from flatfish (*Paralichthys olivaceus*) lymphocytes immortalized by oncogenes." Cytotechnology 1993, 1 1 (2):121-131).

H. WHOLE GENOME SHUFFLING IN HIGHER ORGANISMS-- POOLWISE RECURSIVE BREEDING

The present invention provides a procedure for generating large combinatorial libraries of higher eukaryotes, plants, fish, domesticated animals, etc. In addition to the procedures outlined above, poolwise combination of male and female gametes can also be used to generate large diverse molecular libraries.

In one aspect, the process includes recursive poolwise matings for several generations without any deliberate screening. This is similar to classical breeding, except that pools of organisms, rather than pairs of organisms, are mated, thereby accelerating the generation of genetic diversity.

This method is similar to recursive fusion of a diverse population of bacterial protoplasts resulting in the generation of multiparent progeny harboring genetic information from all of the starting population of bacteria The process described here is to perform analogous artificial or natural matings of large populations of natural isolates, imparting a split pool mating strategy. Before mating, all of the male gametes i.e. pollen, sperm, etc., are isolated from the starting population and pooled. These are then used to "self" fertilize a mixed pool of the female gametes from the same population.

The process is repeated with the subsequent progeny for several generations, with the final progeny being a combinatorial organism library with each member having genetic information originating from many if not all of the starting "parents." This process generates large diverse organism libraries on which many selections and or screens can be imparted, and it does not require sophisticated *in vitro* manipulation of genes However, it results in the creation of useful new strains (perhaps well diluted in the population) in a much shorter time frame than such organisms could be generated using a classical targeted breeding approach.

These libraries are generated relatively quickly (e.g., typically in less than three years for most plants of commercial interest, with six cycles or less of recursive breeding being sufficient to generate desired diversity).

An additional benefit of these methods is that the resulting libraries provide organismal diversity in areas, such as agriculture, aquaculture, and animal husbandry, that are currently genetically homogeneous.

Examples of these methods for several organisms are described below.

1 Plants

A population of plants, for example all of the different corn strains in a commercial seed/germplasm collection, are grown and the pollen from the entire population is harvested and pooled. This mixed pollen population is then used to "self" fertilize the same population. Self pollination is prevented, so that the fertilization is combinatorial. The cross results in all pairwise crosses possible within the population, and the resulting seeds result in many of the possible outcomes of each of these pairwise crosses. The seeds from the fertilized plants are then harvested, pooled, planted, and the pollen is again harvested, pooled, and used to "self" fertilize the population. After only several generations, the resulting population is a very diverse combinatorial library of corn. The seeds from this library are harvested and screened for desirable traits, e.g., salt tolerance, growth rate, productivity, yield, disease

resistance, etc. Essentially any plant collection can be modified by this approach Important commercial crops include both monocots and dicots Monocots include plants in the grass family (*Gramineae*), such as plants in the sub families *Fetucoideae* and *Poacoideae*, which together include several hundred genera including plants in the genera *Agrostis*, *Phleum*, *Dactylis*, *Sorgum*, *Setaria*, *Zea* (e.g., corn), *Oryza* (e.g , rice), *Triticum* (e.g., wheat), *Secale* (e.g., rye), *Avena* (e g., oats), *Hordeum* (e.g , barley), *Saccharum*, *Poa*, *Festuca*, *Stenotaphrum*, *Cynodon*, *Coix*, the *Olyreae*, *Phareae* and many others. Plants in the family *Gramineae* are a particularly preferred target plants for the methods of the invention Additional preferred targets include other commercially important crops, e.g., from the families *Compositae* (the largest family of vascular plants, including at least 1,000 genera, including important commercial crops such as sunflower), and *Leguminosae* or "pea family," which includes several hundred genera, including many commercially valuable crops such as pea, beans, lentil, peanut, yam bean, cowpeas, velvet beans, soybean, clover, alfalfa, lupine, vetch, *lotus*, sweet clover, wisteria, and sweetpea Common crops applicable to the methods of the invention include *Zea mays*, rice, soybean, sorghum, wheat, oats, barley, millet, sunflower, and canola

This process can also be carried out using pollen from different species or more divergent strains (e.g., crossing the ancient grasses with corn). Different plant species can be forced to cross. Only a few plants from an initial cross would have to result in order to make the process viable. These few progeny, e.g., from a cross between soy bean and corn, would generate pollen and eggs, each of which would represent a different meiotic outcome from the recombination of the two genomes. The pollen would be harvested and used to "self" pollinate the original progeny. This process would then be carried out recursively. This would generate a large family shuffled library of two or more species, which could be subsequently screened

The above strategy is illustrated schematically in Figure 30.

2. Fish

The natural tendency of fish to lay their eggs outside of the body and to have a male cover those eggs with sperm provides another opportunity for a split pooled breeding strategy. The eggs from many different fish, e.g., salmon from different fisheries about the world, can be harvested, pooled, and then fertilized with similarly collected and pooled salmon sperm. The fertilization will result in all of the possible pairwise matings of the starting population. The resulting progeny is then grown and again the sperm and eggs are harvested,

and pooled, with each egg and sperm representing a different meiotic outcome of the different crosses The pooled sperm are then used to fertilize the pooled eggs and the process is carried out recursively After several generations the resulting progeny can then be subjected to selections and screens for desired properties, such as size, disease resistance, etc

The above strategy is illustrated schematically in Figure 29

3. Animals

The advent of *in vitro* fertilization and surrogate motherhood provides a means of whole genome shuffling in animals such as mammals As with fish, the eggs and the sperm from a population, for example from all slaughter cows, are collected and pooled. The pooled eggs are then *in vitro* fertilized with the pooled sperm. The resulting embryos are then returned to surrogate mothers for development. As above, this process is repeated recursively until a large diverse population is generated that can be screened for desirable traits.

A technically feasible approach would be similar to that used for plants. In this case, sperm from the males of the starting population is collected and pooled, and then this pooled sample is used to artificially inseminate multiple females from each of the starting populations. Only one (or a few) sperm would succeed in each animal, but these should be different for each fertilization. The process is reiterated by harvesting the sperm from all of the male progeny, pooling it, and using it to fertilize all of the female progeny. The process is carried out recursively for several generations to generate the organism library, which can then be screened.

I. RAPID EVOLUTION AS A PREDICTIVE TOOL

Recursive sequence recombination can be used to simulate natural evolution of pathogenic microorganisms in response to exposure to a drug under test. Using recursive sequence recombination, evolution proceeds at a faster rate than in natural evolution One measure of the rate of evolution is the number of cycles of recombination and screening required until the microorganism acquires a defined level of resistance to the drug. The information from this analysis is of value in comparing the relative merits of different drugs and in particular, in predicting their long term efficacy on repeated administration.

The pathogenic microorganisms used in this analysis include the bacteria that are a common source of human infections, such as *chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptocci, pneumonococci, meningococci* and *conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis,* and Lymes disease bacteria.

Evolution is effected by transforming an isolate of bacteria that is sensitive to a drug under test with a library of DNA fragments The fragments can be a mutated version of the genome of the bacteria being evolved If the target of the drug is a known protein or nucleic acid, a focused library containing variants of the corresponding gene can be used. Alternatively, the library can come from other kinds of bacteria, especially bacteria typically found inhabiting human tissues, thereby simulating the source material available for recombination *in vivo* The library can also come from bacteria known to be resistant to the drug. After transformation and propagation of bacteria for an appropriate period to allow for recombination to occur and recombinant genes to be expressed, the bacteria are screened by exposing them to the drug under test and then collecting survivors. Surviving bacteria are subject to further rounds of recombination. The subsequent round can be effected by a split and pool approach in which DNA from one subset of surviving bacteria is introduced into a second subset of bacteria. Alternatively, a fresh library of DNA fragments can be introduced into surviving bacteria. Subsequent round(s) of selection can be performed at increasing concentrations of drug, thereby increasing the stringency of selection

A similar strategy can be used to simulate viral acquisition of drug resistance. The object is to identify drugs for which resistance can be acquired only slowly, if at all. The viruses to be evolved are those that cause infections in humans for which at least modestly effective drugs are available. Substrates for recombination can come from induced mutants, natural variants of the same viral strain or different viruses. If the target of the drug is known (e.g., nucleotide analogs which inhibit the reverse transcriptase gene of HIV), focused libraries containing variants of the target gene can be produced. Recombination of a viral genome with a library of fragments is usually performed *in vitro*. However, in situations in which the library of fragments constitutes variants of viral genomes or fragments that can be encompassed in such genomes, recombination can also be performed *in vivo*, e.g., by transfecting cells with multiple substrate copies (see Section V). For screening, recombinant viral genomes are introduced into host cells susceptible to infection by the virus and the cells are exposed to a drug effective against the virus (initially at low concentration). The cells can be spun to remove any noninfected virus. After a period of infection, progeny viruses can be collected from the culture medium, the progeny viruses being enriched for viruses that have acquired at least partial resistance to the drug. Alternatively, virally infected cells can be plated in a soft agar lawn and resistant viruses isolated from plaques. Plaque size provides some indication of the degree of viral resistance.

Progeny viruses surviving screening are subject to additional rounds of recombination and screening at increased stringency until a predetermined level of drug resistance has been acquired The predetermined level of drug resistance may reflect the maximum dosage of a drug practical to administer to a patient without intolerable side effects The analysis is particularly valuable for investigating acquisition of resistance to various combination of drugs, such as the growing list of approved anti-HIV drugs (e.g., AZT, ddI, ddC, d4T, TIBO 82150, nevaripine, 3TC, crixivan and ritonavir).

J THE EVOLUTIONARY IMPORTANCE OF RECOMBINATION

Strain improvement is the directed evolution of an organism to be more "fit" for a desired task. In nature, adaptation is facilitated by sexual recombination. Sexual recombination allows a population to exploit the genetic diversity within it, e.g., by consolidating useful mutations and discarding deleterious ones. In this way, adaptation and evolution can proceed in leaps. In the absence of a sexual cycle, members of a population must evolve independently by accumulating random mutations sequentially. Many useful mutations are lost while deleterious mutations can accumulate. Adaptation and evolution in this way proceeds slowly as compared to sexual evolution.

As shown in Fig. 17, asexual evolution is a slow and inefficient process Populations move as individuals rather than as groups A diverse population is generated by the mutagenesis of a single parent resulting in a distribution of fit and unfit individuals. In the absence of a sexual cycle, each piece of genetic information of the surviving population remains in the individual mutants. Selection of the "fittest" results in many "fit" individuals being discarded along with the useful genetic information they carry. Asexual evolution proceeds one genetic event at a time and is thus limited by the intrinsic value of a single genetic event. Sexual evolution moves more quickly and efficiently. Mating within a population consolidates genetic information within the population and results in useful mutations being combined together. The combining of useful genetic information results in progeny that are much more fit than their parents. Sexual evolution thus proceeds much faster by multiple genetic events

Years of plant and animal breeding has demonstrated the power of employing sexual recombination to effect the rapid evolution of complex genomes towards a particular task. This general principle is further demonstrated by using DNA shuffling to recombine DNA molecules *in vitro* to accelerate the rate of directed molecular evolution The strain improvement efforts of the fermentation industry rely on the directed evolution of

microorganisms by sequential random mutagenesis Incorporation of recombination into this iterative process greatly accelerates the strain improvement process, which in turn increases the profitability of current fermentation processes and facilitates the development of new products

K DNA SHUFFLING VS NATURAL RECOMBINATION - THE UTILITY OF POOLWISE RECOMBINATION.

DNA shuffling includes the recursive recombination of DNA sequences A significant difference between DNA shuffling and natural sexual recombination is that DNA shuffling can produce DNA sequences originating from multiple parental sequences while sexual recombination produces DNA sequences originating from only two parental sequences (Fig. 25)

As shown in figure 25, the rate of evolution is in part limited by the number of useful mutations that a member of a population can accumulate between selection events. In sequential random mutagenesis, useful mutations are accumulated one per selection event Many useful mutations are discarded each cycle in favor of the best performer, and neutral or deleterious mutations which survive are as difficult to lose as they were to gain and thus accumulate. In sexual evolution pairwise recombination allows mutations from two different parents to segregate and recombine in different combinations. Useful mutations can accumulate and deleterious mutations can be lost Poolwsie recombination, such as that effected by DNA shuffling, has the same advantages as pairwise recombination but allows mutations from many parents to consolidate into a single progeny Thus poolwise recombination provides a means for increasing the number of useful mutations that can accumulate each selection event The graph in Fig 25 shows a plot of the potential number of mutations an individual can accumulate by each of these processes. Recombination is exponentially superior to sequential random mutagenesis, and this advantage increases exponentially with the number of parents that can recombine. Sexual recombination is thus more conservative. In nature, the pairwise nature of sexual recombination may provide important stability within a population by impeding the large changes in DNA sequence that can result from poolwise recombination. For the purposes of directed evolution, however, poolwise recombination is more efficient.

The potential diversity that can be generated from a population is greater as a result of poolwise recombination as compared to that resulting from pairwise recombination. Further, poolwise recombination enables the combining of multiple beneficial mutations originating from multiple parental sequences.

To demonstrate the importance of poolwise recombination vs pairwise recombination in the generation of molecular diversity consider the breeding of ten independent DNA sequences each containing only one unique mutation. There are $2^{10} = 1024$ different combinations of those ten mutations ranging from a single sequence having no mutations (the consensus) to that having all ten mutations. If this pool were recombined together by pairwise recombination, a population containing the consensus, the parents, and the 45 different combinations of any two of the mutations would result in 56 or ca. 5% of the possible 1024 mutant combinations. Alternatively, if the pool were recombined together in a poolwise fashion, all 1024 would be theoretically generated, resulting in an approximately 20 fold increase in library diversity When looking for a unique solution to a problem in molecular evolution, the more complex the library, the more complex the possible solution. Indeed, the most fit member of a shuffled library often contains several mutations originating from several independent starting sequences.

1 DNA Shuffling Provides Recursive Pairwise Recombination

In vitro DNA shuffling results in the efficient production of combinatorial genetic libraries by catalyzing the recombination of multiple DNA sequences. While the result of DNA shuffling is a population representing the poolwise recombination of multiple sequences, the process does not rely on the recombination of multiple DNA sequences simultaneously, but rather on their recursive pairwise recombination. The assembly of complete genes from a mixed pool of small gene fragments requires multiple annealing and elongation cycles, the thermal cycles of the primerless PCR reaction. During each thermal cycle many pairs of fragments anneal and are extended to form a combinatorial population of larger chimeric DNA fragments. After the first cycle of reassembly, chimeric fragments contain sequence originating from predominantly two different parent genes, with all possible pairs of "parental" sequence theoretically represented. This is similar to the result of a single sexual cycle within a population. During the second cycle, these chimeric fragments anneal with each other or with other small fragments, resulting in chimeras originating from up to four of the different starting sequences, again with all possible combinations of the four parental sequences theoretically represented. This second cycle is analogous to the entire population resulting from a single sexual cross, both parents and offspring, inbreeding.

Further cycles result in chimeras originating from 8, 16, 32, etc parental sequences and are analogous to further inbreedings of the preceding population. This could be considered similar to the diversity generated from a small population of birds that are isolated

on an island, breeding with each other for many generations. The result mimics the outcome of "poolwise" recombination, but the path is via recursive pairwise recombination. For this reason, the DNA molecules generated from *in vitro* DNA shuffling are not the "progeny" of the starting "parental" sequences, but rather the great, great ,great, great$_n$,. (n = number of thermal cycles) grand progeny of the starting "ancestor" molecules

L FERMENTATION

The fermentation of microorganisms for the production of natural products is the oldest and most sophisticated application of biocatalysis. Industrial microorganisms effect the multistep conversion of renewable feedstocks to high value chemical products in a single reactor and in so doing catalyze a multi-billion dollar industry Fermentation products range from fine and commodity chemicals such as ethanol, lactic acid, amino acids and vitamins, to high value small molecule pharmaceuticals, protein pharmaceuticals, and industrial enzymes *See, e.g.*, McCoy (1998) C&EN 13-19) for an introduction to biocatalysis.

Success in bringing these products to market and success in competing in the market depends on continuous improvement of the whole cell biocatalysts. Improvements include increased yield of desired products, removal of unwanted co-metabolites, improved utilization of inexpensive carbon and nitrogen sources, and adaptation to fermenter conditions, increased production of a primary metabolite, increased production of a secondary metabolite, increased tolerance to acidic conditions, increased tolerance to basic conditions, increased tolerance to organic solvents, increased tolerance to high salt conditions and increased tolerance to high or low temperatures. Shortcomings in any of these areas can result in high manufacturing costs, inability to capture or maintain market share, and failure of bringing promising products to market. For this reason, the fermentation industry invests significant financial and personnel resources in the improvement of production strains.

Current strategies for strain improvement rely on the empirical and iterative modification of fermenter conditions and genetic manipulation of the producing organism. While advances in the molecular biology of established industrial organisms have been made, rational metabolic engineering is information intensive and is not broadly applicable to less characterized industrial strains. The most widely practiced strategy for strain improvement employs random mutagenesis of the producing strain and screening for mutants having improved properties. For mature strains, those subjected to many rounds of improvement, these efforts routinely provide a 10% increase in product titre per year. Although effective, this classic strategy is slow, laborious, and expensive Technological advances in this area are

aimed at automation and increasing sample screening throughput in hopes of reducing the cost of strain improvement. However, the real technical barrier resides in the intrinsic limitation of single mutations to effect significant strain improvement The methods herein overcome this limitation and provide access to multiple useful mutations per cycle which can be used to complement automation technologies and catalyze strain improvement processes

The methods herein allow biocatalysts to be improved at a faster pace than conventional methods. Whole genome shuffling can at least double the rate of strain improvement for microorganisms used in fermentation as compared to traditional methods. This provides for a relative decrease in the cost of fermentation processes New products can enter the market sooner, producers can increase profits as well as market share, and consumers gain access to more products of higher quality and at lower prices Further, increased efficiency of production processes translates to less waste production and more frugal use of resources. Whole genome shuffling provides a means of accumulating multiple useful mutation per cycle and thus eliminate the inherent limitation of current strain improvement programs (SIPs)

DNA shuffling provides recursive mutagenesis, recombination, and selection of DNA sequences. A key difference between DNA shuffling-mediated recombination and natural sexual recombination is that DNA shuffling effects both the pairwise (two parents) and the poolwise (multiple parents) recombination of parent molecules, as described *supra*. Natural recombination is more conservative and is limited to pairwise recombination. In nature, pairwise recombination provides stability within a population by preventing large leaps in sequences or genomic structure that can result from poolwise recombination. However, for the purposes of directed evolution, poolwise recombination is appealing since the beneficial mutations of multiple parents can be combined during a single cross to produce a superior offspring. Poolwise recombination is analogous to the crossbreeding of inbred strains in classic strain improvement, except that the crosses occur between many strains at once. In essence, poolwise recombination is a sequence of events that effects the recombination of a population of nucleic acid sequences that results in the generation of new nucleic acids that contains genetic information from more than two of the original nucleic acids. The power of *in vitro* DNA shuffling is that large combinatorial libraries can be generated from a small pool of DNA fragments reassembled by recursive pairwise annealing and extension reactions, "matings." Many of the *in vivo* recombination formats described (such as plasmid-plasmid, plasmid-chromosome, phage-phage, phage-chromosome, phage-plasmid, conjugal DNA-

chromosome, exogenous DNA-chromosome, chromosome-chromosome, with the DNA being introduced into the cell by natural and non-natural competence, transduction, transfection, conjugation, protoplast fusion, etc) result primarily in the pairwise recombination of two DNA molecules Thus, these formats when executed for only a single cycle of recombination are inherently limited in their potential to generate molecular diversity To generate the level of diversity obtained by *in vitro* DNA shuffling methods, pairwise mating formats must be carried out recursively, i.e for many generations, prior to screening for improved sequences Thus a pool of DNA sequences, such as four independent chromosomes, must be recombined, for example by protoplast fusion, and the progeny of that recombination (each representing a unique outcome of the pairwise mating) must then be pooled, without selection, and then recombined again, and again, and again. This process should be repeated for a sufficient number of cycles to result in progeny having the desired complexity. Only once sufficient diversity has been generated, should the resulting population be screened for new and improved sequences

There are a few general methods for effecting efficient recombination in prokaryotes. Bacteria have no known sexual cycle *per se*, but there are natural mechanisms by which the genomes of these organisms undergo recombination. These mechanisms include natural competence, phage-mediated transduction, and cell-cell conjugation Bacteria that are naturally competent are capable of efficiently taking up naked DNA from the environment. If homologous, this DNA undergoes recombination with the genome of the cell, resulting in genetic exchange. *Bacillus subtilis*, the primary production organism of the enzyme industry, is known for the efficiency with which it carries out this process.

In generalized transduction, a bacteriophage mediates genetic exchange. A transducing phage will often package headfulls of the host genome These phage can infect a new host and deliver a fragment of the former host genome which is frequently integrated via homologous recombination. Cells can also transfer DNA between themselves by conjugation Cells containing the appropriate mating factors transfer episomes as well as entire chromosomes to an appropriate acceptor cell where it can recombine with the acceptor genome. Conjugation resembles sexual recombination for microbes and can be intraspecific, interspecific, and intergeneric. For example, an efficient means of transforming *Streptomyces* sp., a genera responsible for producing many commercial antibiotics, is by the conjugal transfer of plasmids from *Echerichia coli*.

For many industrial microorganisms, knowledge of competence, transducing phage, or fertility factors is lacking Protoplast fusion has been developed as a versatile and general alternative to these natural methods of recombination. Protoplasts are prepared by removing the cell wall by treating cells with lytic enzymes in the presence of osmotic stabilizers In the presence of a fusogenic agent, such as polyethylene glycol (PEG), protoplasts are induced to fuse and form transient hybrids or "fusants " During this hybrid state, genetic recombination occurs at high frequency allowing the genomes to reassort The final crucial step is the successful segregation and regeneration of viable cells from the fused protoplasts Protoplast fusion can be intraspecific, interspecific, and intergeneric and has been applied to both prokaryotes and eukaryotes. In addition, it is possible to fuse more than two cells, thus providing a mechanism for effecting poolwise recombination. While no fertility factors, transducing phages or competency development is needed for protoplast fusion, a method for the formation, fusing, and regeneration of protoplasts is typically optimized for each organism. Protoplast fusion as applied to poolwise recombination is described in more detail, *supra*.

One key to SIP is having an assay that can be dependably used to identify a few mutants out of thousands that have subtle increases in product yield. The limiting factor in many assay formats is the uniformity of cell growth This variation is the source of baseline variability in subsequent assays Inoculum size and culture environment (temperature/humidity) are sources of cell growth variation Automation of all aspects of establishing initial cultures and state-of-the-art temperature and humidity controlled incubators are useful in reducing variability

Mutant cells or spores are separated on solid media to produce individual sporulating colonies. Using an automated colony picker (Q-bot, Genetix, U.K.), colonies are identified, picked, and 10,000 different mutants inoculated into 96 well microtitre dishes containing two 3 mm glass balls/well. The Q-bot does not pick an entire colony but rather inserts a pin through the center of the colony and exits with a small sampling of cells (or mycelia) and spores. The time the pin is in the colony, the number of dips to inoculate the culture medium, and the time the pin is in that medium each effect inoculum size, and each can be controlled and optimized. The uniform process of the Q-bot decreases human handling error and increases the rate of establishing cultures (roughly 10,000/4 hours). These cultures are then shaken in a temperature and humidity controlled incubator. The glass balls act to promote uniform aeration of cells and the dispersal of mycelial fragments similar to the blades

of a fermenter. An embodiment of this procedure is further illustrated in Fig. 28, including an integrated system for the assay.

1 Prescreen

The ability to detect a subtle increase in the performance of a mutant over that of a parent strain relies on the sensitivity of the assay The chance of finding the organisms having an improvement is increased by the number of individual mutants that can be screened by the assay. To increase the chances of identifying a pool of sufficient size a prescreen that increases the number of mutants processed by 10-fold can be used. The goal of the primary screen will be to quickly identify mutants having equal or better product titres than the parent strain(s) and to move only these mutants forward to liquid cell culture

The primary screen is an agar plate screen is analyzed by the Q-bot colony picker. Although assays can be fundamentally different, many result, e.g., in the production of colony halos. For example, antibiotic production is assayed on plates using an overlay of a sensitive indicator strain, such as *B. subtilis*. Antibiotic production is typically assayed as a zone of clearing (inhibited growth of the indicator organism) around the producing organism. Similarly, enzyme production can be assayed on plates containing the enzyme substrate, with activity being detected as a zone of substrate modification around the producing colony. Product titre is correlated with the ratio of halo area to colony area.

The Q-bot or other automated system is instructed to only pick colonies having a halo ratio in the top 10% of the population i.e. 10,000 mutants from the 100,000 entering the plate prescreen. This increases the number of improved clones in the secondary assay and eliminates the wasted effort of screening knock-out and low producers. This improves the "hit rate" of the secondary assay.

M. PROMOTION OF GENETIC EXCHANGE

1 General

Some methods of the invention effect recombination of cellular DNA by propagating cells under conditions inducing exchange of DNA between cells. DNA exchange can be promoted by generally applicable methods such as electroporation, biolistics, cell fusion, or in some instances, by conjugation, transduction, or agrobacterium mediated transfer and meiosis. For example, *Agrobacterium* can transform *S. cerevisiae* with T-DNA, which is incorporated into the yeast genome by both homologous recombination and a gap repair mechanism. (Piers et al., *Proc. Natl. Acad. Sci. USA* 93(4), 1613-8 (1996)).

In some methods, initial diversity between cells (i.e., before genome exchange) is induced by chemical or radiation-induced mutagenesis of a progenitor cell type, optionally followed by screening for a desired phenotype In other methods, diversity is natural as where cells are obtained from different individuals, strains or species

In some shuffling methods, induced exchange of DNA is used as the sole means of effecting recombination in each cycle of recombination. In other methods, induced exchange is used in combination with natural sexual recombination of an organism. In other methods, induced exchange and/or natural sexual recombination are used in combination with the introduction of a fragment library. Such a fragment library can be a whole genome, a whole chromosome, a group of functionally or genetically linked genes, a plasmid, a cosmid, a mitochondrial genome, a viral genome (replicative and nonreplicative) or specific or random fragments of any of these The DNA can be linked to a vector or can be in free form. Some vectors contain sequences promoting homologous or nonhomologous recombination with the host genome. Some fragments contain double stranded breaks such as caused by shearing with glass beads, sonication, or chemical or enzymatic fragmentation, to stimulate recombination.

In each case, DNA can be exchanged between cells after which it can undergo recombination to form hybrid genomes. Generally, cells are recursively subject to recombination to increase the diversity of the population prior to screening. Cells bearing hybrid genomes, e.g., generated after at least one, and usually several cycles of recombination are screened for a desired phenotype, and cells having this phenotype are isolated. These cells can additionally form starting materials for additional cycles of recombination in a recursive recombination/selection scheme.

One means of promoting exchange of DNA between cells is by fusion of cells, such as by protoplast fusion. A protoplast results from the removal from a cell of its cell wall, leaving a membrane-bound cell that depends on an isotonic or hypertonic medium for maintaining its integrity. If the cell wall is partially removed, the resulting cell is strictly referred to as a spheroplast and if it is completely removed, as a protoplast. However, here the term protoplast includes spheroplasts unless otherwise indicated.

Protoplast fusion is described by Shaffner et al., *Proc. Natl. Acad. Sci. USA* 77, 2163 (1980) and other exemplary procedures are described by Yoakum et al., US 4,608,339, Takahashi et al., US 4,677,066 and Sambrooke et al., at Ch. 16. Protoplast fusion has been reported between strains, species, and genera (e.g., yeast and chicken erythrocyte).

Protoplasts can be prepared for both bacterial and eukaryotic cells, including mammalian cells and plant cells, by several means including chemical treatment to strip cell walls. For example, cell walls can be stripped by digestion with a cell wall degrading enzyme such as lysozyme in a 10-20% sucrose, 50 mM EDTA buffer. Conversion of cells to spherical protoplasts can be monitored by phase-contrast microscopy. Protoplasts can also be prepared by propagation of cells in media supplemented with an inhibitor of cell wall synthesis, or use of mutant strains lacking capacity for cell wall formation. Preferably, eukaryotic cells are synchronized in G1 phase by arrest with inhibitors such as α-factor, *K. lactis* killer toxin, leflonamide and adenylate cyclase inhibitors. Optionally, some but not all, protoplasts to be fused can be killed and/or have their DNA fragmented by treatment with ultraviolet irradiation, hydroxylamine or cupferon (Reeves et al., *FEMS Microbiol. Lett.* 99, 193-198 (1992)). In this situation, killed protoplasts are referred to as donors, and viable protoplasts as acceptors Using dead donors cells can be advantageous in subsequently recognizing fused cells with hybrid genomes, as described below. Further, breaking up DNA in donor cells is advantageous for stimulating recombination with acceptor DNA Optionally, acceptor and/or fused cells can also be briefly, but nonlethally, exposed to UV irradiation further to stimulate recombination.

Once formed, protoplasts can be stabilized in a variety of osmolytes and compounds such as sodium chloride, potassium chloride, sodium phosphate, potassium phosphate, sucrose, sorbitol in the presence of DTT. The combination of buffer, pH, reducing agent, and osmotic stabilizer can be optimized for different cell types. Protoplasts can be induced to fuse by treatment with a chemical such as PEG, calcium chloride or calcium propionate or electrofusion (Tsoneva, *Acta Microbiologica Bulgaria* 24, 53-59 (1989)). A method of cell fusion employing electric fields has also been described. See Chang US, 4,970,154. Conditions can be optimized for different strains.

The fused cells are heterokaryons containing genomes from two or more component protoplasts. Fused cells can be enriched from unfused parental cells by sucrose gradient sedimentation or cell sorting. The two nuclei in the heterokaryons can fuse (karyogamy) and homologous recombination can occur between the genomes. The chromosomes can also segregate asymmetrically resulting in regenerated protoplasts that have lost or gained whole chromosomes. The frequency of recombination can be increased by treatment with ultraviolet irradiation or by use of strains overexpressing recA or other recombination genes, or the yeast rad genes, and cognate variants thereof in other species, or

by the inhibition of gene products of *Mut*S, *Mut*L, or *Mut*D Overexpression can be either the result of introduction of exogenous recombination genes or the result of selecting strains, which as a result of natural variation or induced mutation, overexpress endogenous recombination genes. The fused protoplasts are propagated under conditions allowing regeneration of cell walls, recombination and segregation of recombinant genomes into progeny cells from the heterokaryon and expression of recombinant genes This process can be reiteratively repeated to increase the diversity of any set of protoplasts or cells After, or occasionally before or during, recovery of fused cells, the cells are screened or selected for evolution toward a desired property.

Thereafter a subsequent round of recombination can be performed by preparing protoplasts from the cells surviving selection/screening in a previous round The protoplasts are fused, recombination occurs in fused protoplasts, and cells are regenerated from the fused protoplasts This process can again be reiteratively repeated to increase the diversity of the starting population. Protoplasts, regenerated or regenerating cells are subject to further selection or screening.

Subsequent rounds of recombination can be performed on a split pool basis as described above. That is, a first subpopulation of cells surviving selection/screening from a previous round are used for protoplast formation. A second subpopulation of cells surviving selection/screening from a previous round are used as a source for DNA library preparation. The DNA library from the second subpopulation of cells is then transformed into the protoplasts from the first subpopulation. The library undergoes recombination with the genomes of the protoplasts to form recombinant genomes. This process can be repeated several times in the absence of a selection event to increase the diversity of the cell population. Cells are regenerated from protoplasts, and selection/screening is applied to regenerating or regenerated cells. In a further variation, a fresh library of nucleic acid fragments is introduced into protoplasts surviving selection/screening from a previous round.

An exemplary format for shuffling using protoplast fusion is shown in Fig. 5. The figure shows the following steps: protoplast formation of donor and recipient strains, heterokaryon formation, karyogamy, recombination, and segregation of recombinant genomes into separate cells. Optionally, the recombinant genomes, if having a sexual cycle, can undergo further recombination with each other as a result of meiosis and mating. Recursive cycles of protoplast fusion, or recursive mating/meiosis is often used to increase the diversity of a cell population. After achieving a sufficiently diverse population via one of these forms of

recombination, cells are screened or selected for a desired property. Cells surviving selection/screening can then used as the starting materials in a further cycle of protoplasting or other recombination methods as noted herein

2 Selection For Hybrid Strains

The invention provides selection strategies to identify cells formed by fusion of components from parental cells from two or more distinct subpopulations Selection for hybrid cells is usually performed before selecting or screening for cells that have evolved (as a result of genetic exchange) to acquisition of a desired property A basic premise of most such selection schemes is that two initial subpopulations have two distinct markers. Cells with hybrid genomes can thus be identified by selection for both markers.

In one such scheme, at least one subpopulation of cells bears a selective marker attached to its cell membrane. Examples of suitable membrane markers include biotin, fluorescein and rhodamine. The markers can be linked to amide or thiol groups or through more specific derivatization chemistries, such as iodo-acetates, iodoacetamides, maleimides For example, a marker can be attached as follows. Cells or protoplasts are washed with a buffer (e.g., PBS), which does not interfere with the chemical coupling of a chemically active ligand which reacts with amino groups of lysines or N-terminal aminogroups of membrane proteins. The ligand is either amine reactive itself (e.g , isothiocyanates, succinimidyl esters, sulfonyl chlorides) or is activated by a heterobifunctional linker (e.g. EMCS, SIAB, SPDP, SMB) to become amine reactive. The ligand is a molecule which is easily bound by protein derivatized magnetic beads or other capturing solid supports. For example, the ligand can be succinimidyl activated biotin (Molecular Probes Inc.: B-1606, B-2603, S-1515, S-1582). This linker is reacted with aminogroups of proteins residing in and on the surface of a cell. The cells are then washed to remove excess labelling agent before contacting with cells from the second subpopulation bearing a second selective marker.

The second subpopulation of cells can also bear a membrane marker, albeit a different membrane marker from the first subpopulation. Alternatively, the second subpopulation can bear a genetic marker. The genetic marker can confer a selective property such as drug resistance or a screenable property, such as expression of green fluorescent protein.

After fusion of first and second subpopulations of cells and recovery, cells are screened or selected for the presence of markers on both parental subpopulations. For example, fusants are enriched for one population by adsorbtion to specific beads and these are

then sorted by FACS for those expressing a marker. Cells surviving both screens for both markers are those having undergone protoplast fusion, and are therefore more likely to have recombined genomes Usually, the markers are screened or selected separately. Membrane-bound markers, such as biotin, can be screened by affinity enrichment for the cell membrane marker (e.g., by panning fused cells on an affinity matrix). For example, for a biotin membrane label, cells can be affinity purified using streptavidin-coated magnetic beads (Dynal). These beads are washed several times to remove the non-fused host cells. Alternatively, cells can be panned against an antibody to the membrane marker. In a further variation, if the membrane marker is fluorescent, cells bearing the marker can be identified by FACS. Screens for genetic markers depend on the nature of the markers, and include capacity to grow on drug-treated media or FACS selection for green fluorescent protein. If first and second cell populations have fluorescent markers of different wavelengths, both markers can be screened simultaneously by FACS sorting.

In a further selection scheme for hybrid cells, first and second populations of cells to be fused express different subunits of a heteromultimeric enzyme. Usually, the heteromultimeric enzyme has two different subunits, but heteromultimeric enzymes having three, four or more different subunits can be used. If an enzyme has more than two different subunits, each subunit can be expressed in a different subpopulation of cells (e.g., three subunits in three subpopulations), or more than one subunit can be expressed in the same subpopulation of cells (e.g., one subunit in one subpopulation, two subunits in a second subpopulation). In the case where more than two subunits are used, selection for the poolwise recombination of more than two protoplasts can be achieved.

Hybrid cells representing a combination of genomes of first, second or more subpopulation component cells can then be recognized by an assay for intact enzyme. Such an assay can be a binding assay, but is more typically a functional assay (e.g., capacity to metabolize a substrate of the enzyme). Enzymatic activity can be detected for example by processing of a substrate to a product with a fluorescent or otherwise easily detectable absorbance or emission spectrum. The individual subunits of a heteromultimeric enzyme used in such an assay preferably have no enzymic activity in dissociated form, or at least have significantly less activity in dissociated form than associated form. Preferably, the cells used for fusion lack an endogenous form of the heteromultimeric enzyme, or at least have significantly less endogenous activity than results from heteromultimeric enzyme formed by fusion of cells.

Penicillin acylase enzymes, cephalosporin acylase and penicillin acyltransferase are examples of suitable heteromultimeric enzymes. These enzymes are encoded by a single gene, which is translated as a proenzyme and cleaved by posttranslational autocatalytic proteolysis to remove a spacer endopeptide and generate two subunits, which associate to form the active heterodimeric enzyme. Neither subunit is active in the absence of the other subunit. However, activity can be reconstituted if these separated gene portions are expressed in the same cell by co-transformation. Other enzymes that can be used have subunits that are encoded by distinct genes (e.g., faoA and faoB genes encode 3-oxoacyl-CoA thiolase of *Pseudonmonas fragi* (*Biochem. J* 328, 815-820 (1997)).

An exemplary enzyme is penicillin G acylase from *Escherichia coli*, which has two subunits encoded by a single gene. Fragments of the gene encoding the two subunits operably linked to appropriate expression regulation sequences are transfected into first and second subpopulations of cells, which lack endogenous penicillin acylase activity. A cell formed by fusion of component cells from the first and second subpopulations expresses the two subunits, which assemble to form functional enzyme, e.g., penicillin acylase. Fused cells can then be selected on agar plates containing penicillin G, which is degraded by penicillin acylase.

In another variation, fused cells are identified by complementation of auxotrophic mutants. Parental subpopulations of cells can be selected for known auxotrophic mutations. Alternatively, auxotrophic mutations in a starting population of cells can be generated spontaneously by exposure to a mutagenic agent. Cells with auxotrophic mutations are selected by replica plating on minimal and complete media. Lesions resulting in auxotrophy are expected to be scattered throughout the genome, in genes for amino acid, nucleotide, and vitamin biosynthetic pathways. After fusion of parental cells, cells resulting from fusion can be identified by their capacity to grow on minimal media. These cells can then be screened or selected for evolution toward a desired property. Further steps of mutagenesis generating fresh auxotrophic mutations can be incorporated in subsequent cycles of recombination and screening/selection.

In variations of the above method, de novo generation of auxotrophic mutations in each round of shuffling can be avoided by reusing the same auxotrophs. For example, auxotrophs can be generated by transposon mutagenesis using a transposon bearing selective marker. Auxotrophs are identified by a screen such as replica plating. Auxotrophs are pooled, and a generalized transducing phage lysate is prepared by growth of phage on a

population of auxotrophic cells. A separate population of auxtrophic cells is subjected to genetic exchange, and complementation is used to selected cells that have undergone genetic exchange and recombination These cells are then screened or selected for acquisition of a desired property. Cells surviving screening or selection then have auxotrophic markers regenerated by introduction of the transducing transposon library The newly generated auxotrophic cells can then be subject to further genetic exchange and screening/selection

In a further variation, auxotrophic mutations are generated by homologous recombination with a targeting vector comprising a selective marker flanked by regions of homology with a biosynthetic region of the genome of cells to be evolved. Recombination between the vector and the genome inserts the positive selection marker into the genome causing an auxotrophic mutation. The vector is in linear form before introduction of cells Optionally, the frequency of introduction of the vector can be increased by capping its ends with self-complementarity oligonucleotides annealed in a hair pin formation. Genetic exchange and screening/selection proceed as described above. In each round, targeting vectors are reintroduced regenerating the same population of auxotrophic markers.

In another variation, fused cells are identified by screening for a genomic marker present on one subpopulation of parental cells and an episomal marker present on a second subpopulation of cells. For example, a first subpopulation of yeast containing mitochondria can be used to complement a second subpopulation of yeast having a petite phenotype (i.e., lacking mitochondria).

In a further variation, genetic exchange is performed between two subpopulations of cells, one of which is dead. Cells are preferably killed by brief exposure to DNA fragmenting agents such as hydroxylamine, cupferon, or irradiation. Viable cells are then screened for a marker present on the dead parental subpopulation.

3 Liposome-mediated transfers

In the methods noted above, in which nucleic acid fragment libraries are introduced into protoplasts, the nucleic acids are sometimes encapsulated in liposomes to facilitate uptake by protoplasts. Lipsome-mediated uptake of DNA by protoplasts is described in Redford et al., *Mol. Gen. Genet.* 184, 567-569 (1981). Liposomes can efficiently deliver large volumes of DNA to protoplasts (see Deshayes et al., *EMBO J.* 4, 2731-2737 (1985)). *See also,* Philippot and Schuber (eds) (1995) Liposomes as Tools in Basic Research and Industry CRC press, Boca Raton, e.g., Chapter 9, Remy *et al.* "Gene Transfer with Cationic Amphiphiles." Further, the DNA can be delivered as linear fragments, which are often more

recombinogenic that whole genomes. In some methods, fragments are mutated prior to encapsulation in liposomes In some methods, fragments are combined with RecA and homologs, or nucleases (e g , restriction endonucleases) before encapsulation in liposomes to promote recombination. Alternatively, protoplasts can be treated with lethal doses of nicking reagents and then fused Cells which survive are those which are repaired by recombination with other genomic fragments, thereby providing a selection mechansim to select for recombinant (and therefore desirably diverse) protoplasts

4. Shuffling filamentous fungi

Filamentous fungi are particularly suited to performing the shuffling methods described above. Filamentous fungi are divided into four main classifications based on their structures for sexual reproduction: *Phycomycetes*, *Ascomycetes*, *Basidiomycetes* and the *Fungi Imperfecti* *Phycomycetes* (e.g., *Rhizopus, Mucor*) form sexual spores in sporangium. The spores can be uni or multinucleate and often lack septated hyphae (coenocytic). *Ascomycetes* (e.g., *Aspergillus*, *Neurospora*, *Penicillum*) produce sexual spores in an ascus as a result of meiotic division. Asci typically contain 4 meiotic products, but some contain 8 as a result of additional mitotic division. *Basidiomycetes* include mushrooms, and smuts and form sexual spores on the surface of a basidium. In *holobasidiomycetes*, such as mushrooms, the basidium is undivided. In *hemibasidiomycetes,* such as ruts (*Uredinales*) and smut fungi (*Ustilaginales*), the basidium is divided. *Fungi imperfecti,* which include most human pathogens, have no known sexual stage.

Fungi can reproduce by asexual, sexual or parasexual means. Asexual reproduction, involves vegetative growth of mycelia, nuclear division and cell division without involvement of gametes and without nuclear fusion. Cell division can occur by sporulation, budding or fragmentation of hyphae.

Sexual reproduction provides a mechanism for shuffling genetic material between cells. A sexual reproductive cycle is characterized by an alteration of a haploid phase and a diploid phase. Diploidy occurs when two haploid gamete nuclei fuse (karyogamy). The gamete nuclei can come from the same parental strains (self-fertile), such as in the homothallic fungi. In heterothallic fungi, the parental strains come from strains of different mating type.

A diploid cell converts to haploidy via meiosis, which essentially consists of two divisions of the nucleus accompanied by one division of the chromosomes. The products of one meiosis are a tetrad (4 haploid nuclei). In some cases, a mitotic division occurs after meiosis, giving rise to eight product cells. The arrangement of the resultant cells (usually

enclosed in spores) resembles that of the parental strains. The length of the haploid and diploid stages differs in various fungi: for example, the Basidiomycetes and many of the *Ascomycetes* have a mostly hapolid life cycle (that is, meiosis occurs immediately after karyogamy), whereas others (e.g., *Saccharomyces cerevisiae*) are diploid for most of their life cycle (karyogamy occurs soon after meiosis). Sexual reproduction can occur between cells in the same strain (selfing) or between cells from different strains (outcrossing).

Sexual dimorphism (dioecism) is the separate production of male and female organs on different mycelia. This is a rare phenomenon among the fungi, although a few examples are known. Heterothallism (one locus-two alleles) allows for outcrossing between crosscompatable strains which are self-incompatable. The simplest form is the two allele-one locus system of mating types/factors, illustrated by the following organisms: A and *a* in *Neurospora;* a and α in *Saccharomyces;* plus and minus in *Schizzosaccharomyces* and *Zygomycetes;* a_1 and a_2 in *Ustilago.*

Multiple-allelomorph heterothallism is exhibited by some of the higher *Basidiomycetes* (e.g. *Gasteromycetes* and *Hymenomycetes*), which are heterothallic and have several mating types determined by multiple alleles. Heterothallism in these organisms is either bipolar with one mating type factor, or tetrapolar with two unlinked factors, *A* and *B*. Stable, fertile heterokaryon formation depends on the presence of different A factors and, in the case of tetrapolar organisms, of different *B* factors as well. This system is effective in the promotion of outbreeding and the prevention of self-breeding. The number of different mating factors may be very large (i e. thousands) (Kothe, *FEMS Microbiol. Rev.* 18, 65-87 (1996)), and non-parental mating factors may arise by recombination.

Parasexual reproduction provides a further means for shuffling genetic material between cells. This process allows recombination of parental DNA without involvement of mating types or gametes. Parasexual fusion occurs by hyphal fusion giving rise to a common cytoplasm containing different nuclei. The two nuclei can divide independently in the resulting heterokaryon but occasionally fuse. Fusion is followed by haploidization, which can involve loss of chromosomes and mitotic crossing over between homolgous chromosomes. Protoplast fusion is a form of parasexual reproduction.

Within the above four classes, fungi are also classified by vegetative compatibility group. Fungi within a vegetative compatibility group can form heterokaryons with each other. Thus, for exchange of genetic material between different strains of fungi, the fungi are usually prepared from the same vegetative compatibility group. However, some

genetic exchange can occur between fungi from different incompatibility groups as a result of parasexual reproduction (see Timberlake et al., US 5,605,820). Further, as discussed elsewhere, the natural vegetative compatibility group of fungi can be expanded as a result of shuffling.

Several isolates of Aspergillus nidulans, A. flavus, A fumigatus, Penicillium chrysogenum, P. notatum, Cephalosporium chrysogenum, Neurospora crassa, Aureobasidium pullulans have been karyotyped. Genome sizes generally range between 20 and 50 Mb among the Aspergilli. Differences in karyotypes often exist between similar strains and are also caused by transformation with exogenous DNA Filamentous fungal genes contain introns, usually ~50-100 bp in size, with similar consensus 5' and 3' splice sequences. Promotion and termination signals are often cross-recognizable, enabling the expression of a gene/pathway from one fungus (e.g. A. nidulans) in another (e.g. P. chrysogenum).

The major components of the fungal cell wall are chitin (or chitosan), β-glucan, and mannoproteins. Chitin and β-glucan form the scaffolding, mannoproteins are interstitial components which dictate the wall's porosity, antigenicity and adhesion. Chitin synthetase catalyzes the polymerization of β-(1,4)-linked N-acetylglucosamine (GIcNAc) residues, forming linear strands running antiparallel; β-(1,3)-glucan synthetase catalyze the homopolymerization of glucose.

One general goal of shuffling is to evolve fungi to become useful hosts for genetic engineering, in particular for the shuffling of unrelated genes. *A. nidulans* and *neurospora* are generally the fungal organisms of choice to serve as a hosts for such manipulations because of their sexual cycles and well-established use in classical and molecular genetics. Another general goal is to improve the capacity of fungi to make specific compounds (e.g. antibacterials (penicillins, cephalosporins), antifungals (e.g. echinocandins, aureobasidins), and wood-degrading enzymes). There is some overlap between these general goals, and thus, some desired properties are useful for achieving both goals.

One desired property is the introduction of meiotic apparatus into fungi presently lacking a sexual cycle (see Sharon et al., *Mol. Gen. Genet.* 251, 60-68 (1996)). A scheme for introducing a sexual cycle into the fungi *P. chrysogenum* (a fungus imperfecti) is shown in Fig. 6. Subpopulations of protoplasts are formed from *A. nidulans* (which has a sexual cycle) and *P. chrysogenum*, which does not. The two strains preferably bear different markers. The *A. nidulans* protoplasts are killed by treatment with UV or hydroxylamine. The two subpopulations are fused to form heterokaryons. In some heterokaryons, nuclei fuse, and

some recombination occurs Fused cells are cultured under conditions to generate new cell walls and then to allow sexual recombination to occur. Cells with recombinant genomes are then selected (e.g., by selecting for complementation of auxotrophic markers present on the respective parent strains). Cells with hybrid genomes are more likely to have acquired the genes necessary for a sexual cycle Protoplasts of cells can then be crossed with killed protoplasts of a further population of cells known to have a sexual cycle (the same or different as the previous round) in the same manner, followed by selection for cells with hybrid genomes.

Another desired property is the production of a mutator strain of fungi. Such a fungus can be produced by shuffling a fungal strain containing a marker gene with one or more mutations that impair or prevent expression of a functional product Shufflants are propagated under conditions that select for expression of the positive marker (while allowing a small amount of residual growth without expression). Shufflants growing fastest are selected to form the starting materials for the next round of shuffling.

Another desired property is to expand the host range of a fungus so it can form heterokaryons with fungi from other vegetative compatibility groups. Incompatability between species results from the interactions of specific alleles at different incompatability loci (such as the *"het"* loci). If two strains undergo hyphal anastomosis, a lethal cytoplasmic incompatability reaction may occur if the strains differ at these loci. Strains must carry identical loci to be entirely compatible. Several of these loci have been identified in various species, and the incompatibility effect is somewhat additive (hence, "partial incompatibility" can occur). Some tolerant and *het*-negative mutants have been described for these organisms (e.g. Dales & Croft, *J. Gen. Microbiol.* 136, 1717-1724 (1990)). Further, a tolerance gene (tol) has been reported, which suppresses mating-type heterokaryon incompatibility. Shuffling is performed between protoplasts of strains from different incompatibility groups. A preferred format uses a live acceptor strain and a UV-irradiated dead acceptor strain. The UV irradiation serves to introduce mutations into DNA inactivating het genes. The two strains should bear different genetic markers. Protoplasts of the strain are fused, cells are regenerated and screened for complementation of markers. Subsequent rounds of shuffling and selection can be performed in the same manner by fusing the cells surviving screening with protoplasts of a fresh population of donor cells. Similar to other procedures noted herein, the cells resulting from regeneration of the protoplasts are optionally refused by protoplasting and

regenerated into cells one or more times prior to any selection step to increase the diversity of the resulting population of cells to be screened.

Another desired property is the introduction of multiple-allelomorph heterothallism into *Ascomycetes* and *Fungi imperfecti*, which do not normally exhibit this property. This mating system allows outbreeding without self-breeding Such a mating system can be introduced by shuffling *Ascomycetes* and *Fungi imperfecti* with DNA from *Gasteromycetes* or *Hymenomycetes*, which have such a system.

Another desired property is spontaneous formation of protoplasts to facilitate use of a fungal strain as a shuffling host. Here, the fungus to be evolved is typically mutagenized Spores of the fungus to be evolved are briefly treated with a cell-wall degrading agent for a time insufficient for complete protoplast formation, and are mixed with protoplasts from other strain(s) of fungi. Protoplasts formed by fusion of the two different subpopulations are identified by genetic or other selection/or screening as described above. These protoplasts are used to regenerate mycelia and then spores, which form the starting material for the next round of shuffling. In the next round, at least some of the surviving spores are treated with cell-wall removing enzyme but for a shorter time than the previous round. After treatment, the partially stripped cells are labeled with a first label. These cells are then mixed with protoplasts, which may derive from other cells surviving selection in a previous round, or from a fresh strain of fungi. These protoplasts are physically labeled with a second label. After incubating the cells under conditions for protoplast fusion fusants with both labels are selected. These fusants are used to generate mycelia and spores for the next round of shuffling, and so forth. Eventually, progeny that spontaneously form protoplasts (i.e , without addition of cell wall degrading agent) are identified. As with other procedures noted herein, cells or protoplasts can be reiteratively fused and regnerated prior to performing any selection step to increase the diversity of the resulting cells or protoplasts to be screened. Similarly, selected cells or protoplasts can be reiteratively fused and regenerated for one or several cycles without imposing selection on the resulting cellular or protoplast populations, thereby increasing the diversity of cells or protoplasts which are eventually screened. This process of performing multiple cycles of recombination interspersed with selection steps can be reiteratively repeated as desired.

Another desired property is the acquisition and/or improvement of genes encoding enzymes in biosynthetic pathways, genes encoding transporter proteins, and genes encoding proteins involved in metabolic flux control. In this situation, genes of the pathway

can be introduced into the fungus to be evolved either by genetic exchange with another strain of fungus possessing the pathway or by introduction of a fragment library from an organism possessing the pathway Genetic material of these fungi can then be subjected to further shuffling and screening/selection by the various procedures discussed in this application Shufflant strains of fungi are selected/screened for production of the compound produced by the metabolic pathway or precursors thereof

Another desired property is increasing the stability of fungi to extreme conditions such as heat. In this situation, genes conferring stability can be acquired by exchanging DNA with or transforming DNA from a strain that already has such properties. Alternatively, the strain to be evolved can be subjected to random mutagenesis. Genetic material of the fungus to be evolved can be shuffled by any of the procedures described in this application, with shufflants being selected by surviving exposure to extreme conditions

Another desired property is capacity of a fungus to grow under altered nutritional requirements (e.g., growth on particular carbon or nitrogen sources). Altering nutritional requirements is particularly valuable, e.g., for natural isolates of fungi that produce valuable commercial products but have esoteric and therefore expensive nutritional requirement. The strain to be evolved undergoes genetic exchange and/or transformation with DNA from a strain that has the desired nutritional requirements. The fungus to be evolved can then optionally be subjected to further shuffling as described in this application and with recombinant strains being selected for capacity to grow in the desired nutritional circumstances. Optionally, the nutritional circumstances can be varied in successive rounds of shuffling starting at close to the natural requirements of the fungus to be evolved and in subsequent rounds approaching the desired nutritional requirements.

Another desired property is acquisition of natural competence in a fungus The procedure for acquisition of natural competence by shuffling is generally described in PCT/US97/04494. The fungus to be evolved typically undergoes genetic exchange or transformation with DNA from a bacterial strain or fungal strain that already has this property. Cells with recombinant genomes are then selected by capacity to take up a plasmid bearing a selective marker. Further rounds of recombination and selection can be performed using any of the procedures described above.

Another desired property is reduced or increased secretion of proteases and DNase. In this situation, the fungus to be evolved can acquire DNA by exchange or transformation from another strain known to have the desired property Alternatively, the

fungus to be evolved can be subject to random mutagenesis The fungus to be evolved is shuffled as above The presence of such enzymes, or lack thereof, can be assayed by contacting the culture media from individual isolates with a fluorescent molecule tethered to a support via a peptide or DNA linkage Cleavage of the linkage releases detectable fluorescence to the media

Another desired property is producing fungi with altered transporters (e.g., MDR). Such altered transporters are useful, for example, in fungi that have been evolved to produce new secondary metabolites, to allow entry of precursors required for synthesis of the new secondary metabolites into a cell, or to allow efflux of the secondary metabolite from the cell. Transporters can be evolved by introduction of a library of transporter variants into fungal cells and allowing the cells to recombine by sexual or parasexual recombination. To evolve a transporter with capacity to transport a precursor into the cells, cells are propagated in the present of precursor, and cells are then screened for production of metabolite. To evolve a transporter with capacity to export a metabolite, cells are propagated under conditions supporting production of the metabolite, and screened for export of metabolite to culture medium.

A general method of fungal shuffling is shown in Fig. 7 Spores from a frozen stock, a lyophilized stock, or fresh from an agar plate are used to inoculate suitable liquid medium (1). Spores are germinated resulting in hyphal growth (2). Mycelia are harvested, and washed by filtration and/or centrifugation. Optionally the sample is pretreated with DTT to enhance protoplast formation (3). Protoplasting is performed in an osmotically stabling medium (e.g., 1 m NaCl/20mM MgSO4, pH 5 8) by the addition of cell wall-degrading enzyme (e.g., Novozyme 234) (4). Cell wall degrading enzyme is removed by repeated washing with osmotically stabilizing solution (5). Protoplasts can be separated from mycelia, debris and spores by filtration through miracloth, and density centrifugation (6). Protoplasts are harvested by centrifugation and resuspended to the appropriate concentration. This step may lead to some protoplast fusion (7). Fusion can be stimulated by addition of PEG (e.g., PEG 3350), and/or repeated centrifugation and resuspension with or without PEG. Electrofusion can also be performed (8). Fused protoplasts can optionally be enriched from unfused protoplasts by sucrose gradient sedimentation (or other methods of screening described above). Fused protoplasts can optionally be treated with ultraviolet irradiation to stimulate recombination (9). Protoplasts are cultured on osmotically stabilized agar plates to

regenerate cell walls and form mycelia (10) The mycelia are used to generate spores (11), which are used as the starting material in the next round of shuffling (12).

Selection for a desired property can be performed either on regenerated mycelia or spores derived therefrom

In an alternative method, protoplasts are formed by inhibition of one or more enzymes required for cell wall synthesis (see Fig. 8). The inhibitor should be fungistatic rather than fungicidal under the conditions of use. Examples of inhibitors include antifungal compounds described by (e.g., Georgopapadakou & Walsh, *Antimicrob. Ag. Chemother.* 40, 279-291 (1996); Lyman & Walsh, *Drugs* 44, 9-35 (1992)) Other examples include chitin synthase inhibitors (polyoxin or nikkomycin compounds) and/or glucan synthase inhibitors (e.g. echinocandins, papulocandins, pneumocandins). Inhibitors should be applied in osmotically stabilized medium. Cells stripped of their cell walls can be fused or otherwise employed as donors or hosts in genetic transformation/strain development programs. A possible scheme utilizing this method reiteratively is outlined in Figure 8.

In a further variation, protoplasts are prepared using strains of fungi, which are genetically deficient or compromised in their ability to synthesize intact cell walls (see Fig. 9) Such mutants are generally referred to as fragile, osmotic-remedial, or cell wall-less, and are obtainable from strain depositories. Examples of such strains include *Neurospora crassa os* mutants (Selitrennikoff, *Antimicrob. Agents. Chemother.* 23, 757-765 (1983)). Some such mutations are temperature-sensitive. Temperature-sensitive strains can be propagated at the permissive temperature for purposes of selection and amplification and at a nonpermissive temperature for purposes of protoplast formation and fusion. A temperature sensitive strain *Neurospora crassa* os strain has been described which propagates as protoplasts when growth in osmotically stabilizing medium containing sorbose and polyoxin at nonpermissive temperature but generates whole cells on transfer to medium containing sorbitol at a permissive temperature. See US 4,873,196.

Other suitable strains can be produced by targeted mutagenesis of genes involved in chitin synthesis, glucan synthesis and other cell wall-related processes. Examples of such genes include CHT1, CHT2 and CALI (or CSD2) of *Saccharomyces cerevisiae* and *Candida spp.* (Georgopapadakou & Walsh 1996); *ETGI/FKSI/CNDI/ CWH53/PB RI* and homologs in *S. cerevisiae, Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, ChvAINdvA Agrobacterium* and *Rhizobium*. Other examples are *MA*, *orl*B, *orl*C, *MD*, *ts*E, and *bim*G of *Aspergillus nidulans* (Borgia, *J. Bacteriol.* 174, 377-389 (1992)).

Strains of *A. nidulans* containing *OrlA1* or *tse1* mutations lyse at restrictive temperatures Lysis of these strains may be prevented by osmotic stabilization, and the mutations may be complemented by the addition of N-acetylglucosimine (GlcNac). *BimG11* mutations are ts for a type 1 protein phosphatase (germlines of strains carrying this mutation lack chitin, and condia swell and lyse) Other suitable genes are *chs*A, *chs*B, *chs*C, *chs*D and *chs*E of *Aspergillus fumigatus*; *chs1 and chs2* of *Neurospora crassa* ; *Phycomyces blakesleeanus MM* and chs1, 2 and 3 of *S. cerevisiae*. Chs1 is a non-essential repair enzyme; chs2 is involved in septum formation and chs3 is involved in cell wall maturation and bud ring formation.

Other useful strains include *S. cerevisiae* CLY (cell lysis) mutants such as ts strains (Paravicini et al , *Mol. Cell Biol.* 12, 4896-4905 (1992)), and the CLY 15 strain which harbors a PKC 1 gene deletion Other useful strains include strain VY 1160 containing a ts mutation in srb (encoding actin) (Schade et al. *Acta Histochem. Suppl.* 41, 193-200 (1991)), and a strain with an *ses* mutation which results in increased sensitivity to cell-wall digesting enzymes isolated from snail gut (Metha & Gregory, *Appl. Environ. Microbiol.* 41, 992-999 (1981)) Useful strains *of C. albicans* include those with mutations in *chs*1, *chs*2, or *chs*3 (encoding chitin synthetases), such as osmotic remedial conditional lethal mutants described by Payton & de Tiani, *Curr. Genet.* 17, 293-296 (1990); *C. utilis* mutants with increased sensitivity to cell-wall digesting enzymes isolated from snail gut (Metha & Gregory, 1981, *supra*); and *N. crassa* mutants *os-1*, *os-2*, *os-3*, *os-4*, *os-5*, amd *os-6*. *See,* Selitrennikoff, *Antimicrob. Agents Chemother.* 23, 757-765 (1983). Such mutants grow and divide without a cell wall at 37°C, but at 22°C produce a cell wall.

Targeted mutagenesis can be achieved by transforming cells with a positive-negative selection vector containing homologous regions flanking a segment to be targeted, a positive selection marker between the homologous regions and a negative selection marker outside the homologous regions (see Capecchi, US 5,627,059). In a variation, the negative selection marker can be an antisense transcript of the positive selection marker (see US 5,527,674).

Other suitable cells can be selected by random mutagenesis or shuffling procedures in combination with selection. For example, a first subpopulation of cells are mutagenized, allowed to recover from mutagenesis, subjected to incomplete degradation of cell walls and then contacted with protoplasts of a second subpopulation of cells. Hybrids cells bearing markers from both subpopulations are identified (as described above) and used as the starting materials in a subsequent round of shuffling. This selection scheme selects both

for cells with capacity for spontaneous protoplast formation and for cells with enhanced recombinogenicity.

In a further variation, cells having capacity for spontaneous protoplast formation can be crossed with cells having enhanced recombinogenicity evolved using other methods of the invention The hybrid cells are particularly suitable hosts for whole genome shuffling.

Cells with mutations in enzymes involved in cell wall synthesis or maintenance can undergo fusion simply as a result of propagating the cells in osmotic-protected culture due to spontaneous protoplast formation If the mutation is conditional, cells are shifted to a nonpermissive condition Protoplast formation and fusion can be accelerated by addition of promoting agents, such as PEG or an electric field (See Philipova & Venkov, *Yeast* 6, 205-212 (1990); Tsoneva et al., *FEMS Microbiol. Lett.* 51, 61-65 (1989))

5. Targeted Shuffling—Hot Spots

In one aspect, targeted homologous genes are cloned into specific regions of the genome (e.g., by homologous recombination or other targeting procedures) which are known to be recombination "hot spots" (i.e., regions showing elevated levels of recombination compared to the average level of recombination observed across an entire genome), or known to be proximal to such hot spots. The resulting recombinant strains are mated recursively. During meiotic recombination, homologous recombinant genes recombine, thereby increasing the diversity of the genes After several cycles of recombination by recursive mating, the resulting cells are screened

6 Shuffling Methods in Yeast

Yeasts are subspecies of fungi that grow as single cells. Yeasts are used for the production of fermented beverages and leavening, for production of ethanol as a fuel, low molecular weight compounds, and for the heterologous production of proteins and enzymes (see accompanying list of yeast strains and their uses). Commonly used strains of yeast include *Saccharomyces cerevisiae, Pichia sp., Canidia sp.* and *Schizosaccharomyces pombe.*

Several types of vectors are available for cloning in yeast including integrative plasmid (YIp), yeast replicating plasmid (YRp, such as the 2μ circle based vectors), yeast episomal plasmid (YEp), yeast centromeric plasmid (YCp), or yeast artificial chromosome (YAC). Each vector can carry markers useful to select for the presence of the plasmid such as LUE2, URA3, and H1S3, or the absence of the plasmid such as URA3 (a gene that is toxic to cells grown in the presence of 5-fluoro orotic acid.

Many yeasts have a sexual cycle and asexual (vegetative) cycles. The sexual cycle involves the recombination of the whole genome of the organism each time the cell passes through meiosis For example, when diploid cells of *S. cerevisiae* are exposed to nitrogen and carbon limiting conditions, diploid cells undergo meiosis to form asci Each ascus holds four haploid spores, two of mating type "a" and two of mating type "α " Upon return to rich medium, haploid spores of opposite mating type mate to form diploid cells once again Asiospores of opposite mating type can mate within the ascus, or if the ascus is degraded, for example with zymolase, the haploid cells are liberated and can mate with spores from other asci. This sexual cycle provides a format to shuffle endogenous genomes of yeast and/or exogenous fragment libraries inserted into yeast vectors. This process results in swapping or accumulation of hybrid genes, and for the shuffling of homologous sequences shared by mating cells.

Yeast strains having mutations in several known genes have properties useful for shuffling. These properties include increasing the frequency of recombination and increasing the frequency of spontaneous mutations within a cell These properties can be the result of mutation of a coding sequence or altered expression (usually overexpression) of a wildtype coding sequence. The HO nuclease effects the transposition of HMLa/α and HMRa/α to the MAT locus resulting in mating type switching. Mutants in the gene encoding this enzyme do not switch their mating type and can be employed to force crossing between strains of defined genotype, such as ones that harbor a library or have a desired phenotype and to prevent in breeding of starter strains. PMS1, MLH1, MSH2, MSH6 are involved in mismatch repair. Mutations in these genes all have a mutator phenotype (Chambers et al., *Mol. Cell. Biol.* 16, 6110-6120 (1996)). Mutations in TOP3 DNA topoisomerase have a 6-fold enhancement of interchromosomal homologous recombination (Bailis et al., *Molecular and Cellular Biology* 12, 4988-4993 (1992)). The RAD50-57 genes confer resistance to radiation. Rad3 functions in excision of pyrimidine dimers. RAD52 functions in gene conversion. RAD5O, MRE11, XRS2 function in both homologous recombination and illegitimate recombination. HOP1, RED1 function in early meiotic recombination (Mao-Draayer, *Genetics* 144, 71-86) Mutations in either HOP1 or RED1 reduce double stranded breaks at the HIS2 recombination hotspot. Strains deficient in these genes are useful for maintaining stability in hyper recombinogenic constructs such as tandem expression libraries carried on YACs. Mutations in HPR 1 are hyperrecombinogenic. HDF1 has DNA end binding activity and is involved in double stranded break repair and V(D)J recombination.

Strains bearing this mutation are useful for transformation with random genomic fragments by either protoplast fusion or electroporation Kar-1 is a dominant mutation that prevents karyogamy Kar-1 mutants are useful for the directed transfer of single chromosomes from a donor to a recipient strain This technique has been widely used in the transfer of YACs between strains, and is also useful in the transfer of evolved genes/chromosomes to other organisms (Markie, *YAC Protocols*, (Humana Press, Totowa, NJ, 1996) HOT1 is an *S. cerevisiae* recombination hotspot within the promoter and enhancer region of the rDNA repeat sequences This locus induces mitotic recombination at adjacent sequences- presumably due to its high level transcription Genes and/or pathways inserted under the transcriptional control of this region undergo increased mitotic recombination. The regions surrounding the arg 4 and his 4 genes are also recombination hot spots, and genes cloned in these regions have an increased probability of undergoing recombination during meiosis. Homologous genes can be cloned in these regions and shuffled in vivo by recursively mating the recombinant strains. CDC2 encodes polymerase δ and is necessary for mitotic gene conversion. Overexpression of this gene can be used in a shuffler or mutator strain A temperature sensitive mutation in CDC4 halts the cell cycle at G1 at the restrictive temperature and could be used to synchronize protoplasts for optimized fusion and subsequent recombination

As with filamentous fungi, the general goals of shuffling yeast include improvement in yeast as a host organism for genetic manipulation, and as a production apparatus for various compounds. One desired property in either case is to improve the capacity of yeast to express and secrete a heterologous protein. The following example describes the use of shuffling to evolve yeast to express and secrete increased amounts of RNase A.

RNase A catalyzes the cleavage of the P-O5' bond of RNA specifically after pyrimidine nucleotides. The enzyme is a basic 124 amino acid polypeptide that has 8 half cystine residues, each required for catalysis YEpWL-RNase A is a vector that effects the expression and secretion of RNaseA from the yeast *S. cerevisiae*, and yeast harboring this vector secrete 1-2 mg of recombinant RNase A per liter of culture medium (del Cardayré et al., *Protein Engineering* 8(3):26, 1-273 (1995)). This overall yield is poor for a protein heterologously expressed in yeast and can be improved at least 10-100 fold by shuffling The expression of RNaseA is easily detected by several plate and microtitre plate assays (del Cardayré & Raines, *Biochemistry* 33, 6031-6037 1994)). Each of the described formats for

whole genome shuffling can be used to shuffle a strain of *S. cerevisiae* harboring YEpWL RNase A, and the resulting cells can be screened for the increased secretion of RNase A into the medium The new strains are cycled recursively through the shuffling format, until sufficiently high levels of RNase A secretion is observed The use of RNase A is particularly useful since it not only requires proper folding and disulfide bond formation but also proper glycosylation Thus numerous components of the expression, folding, and secretion systems can be optimized. The resulting strain is also evolved for improved secretion of other heterologous proteins.

Another goal of shuffling yeast is to increase the tolerance of yeast to ethanol. Such is useful both for the commercial production of ethanol, and for the production of more alcoholic beers and wines The yeast strain to be shuffled acquires genetic material by exchange or transformation with other strain(s) of yeast, which may or may not be know to have superior resistance to ethanol The strain to be evolved is shuffled and shufflants are selected for capacity to survive exposure to ethanol. Increasing concentrations of ethanol can be used in successive rounds of shuffling The same principles can be used to shuffle baking yeasts for improved osmotolerance.

Another desired property of shuffling yeast is capacity to grow under desired nutritional conditions. For example, it is useful to yeast to grow on cheap carbon sources such as methanol, starch, molases, cellulose, cellobiose, or xylose depending on availability. The principles of shuffling and selection are similar to those discussed for filamentous fungi.

Another desired property is capacity to produce secondary metabolites naturally produced by filamentous fungi or bacteria, Examples of such secondary metabolites are cyclosporin A, taxol, and cephalosporins The yeast to be evolved undergoes genetic exchange or is transformed with DNA from organism(s) that produce the secondary metabolite. For example, fungi producing taxol include *Taxomyces andreanae* and *Pestalotopis microspora* (Stierle et al., *Science* 260, 214-216 (1993); Strobel et al., *Microbiol.* 142, 435-440 (1996)). DNA can also be obtained from trees that naturally produce taxol, such as *Taxus brevifolia.* DNA encoding one enzyme in the taxol pathway, taxadiene synthase, which it is believed catalyzes the committed step in taxol biosynthesis and may be rate limiting in overall taxol production, has been cloned (Wildung & Croteau, *J. Biol. Chem.* 271, 9201-4 (1996). The DNA is then shuffled, and shufflants are screened/selected for production of the secondary metabolite. For example, taxol production can be monitored using antibodies to taxol, by mass spectroscopy or UV spectrophotometry. Alternatively,

production of intermediates in taxol synthesis or enzymes in the taxol synthetic pathway can be monitored Concetti & Ripani, *Biol. Chem. Hoppe Seyler* 375, 419-23 (1994) Other examples of secondary metabolites are polyols, amino acids, polyketides, non-ribosomal polypeptides, ergosterol, carotenoids, terpinoids, sterols, vitamin E, and the like

Another desired property is to increase the flocculence of yeast to facilitate separation in preparation of ethanol Yeast can be shuffled by any of the procedures noted above with selection for shuffled yeast forming the largest clumps.

7 Exemplary procedure for yeast protoplasting

Protoplast preparation in yeast is reviewed by Morgan, in *Protoplasts* (Birkhauser Verlag, Basel, 1983). Fresh cells (~10^8) are washed with buffer, for example 0.1 M potassium phosphate, then resuspended in this same buffer containing a reducing agent, such as 50 mM DTT, incubated for 1 h at 30°C with gentle agitation, and then washed again with buffer to remove the reducing agent. These cells are then resuspended in buffer containing a cell wall degrading enzyme, such as Novozyme 234 (1 mg/mL), and any of a variety of osmotic stabilizers, such as sucrose, sorbitol, NaCl, KCl, $MgSO_4$, $MgCl_2$, or NH_4Cl at any of a variety of concentrations These suspensions are then incubated at 30°C with gentle shaking (~60 rpm) until protoplasts are released. To generate protoplasts that are more likely to produce productive fusants several strategies are possible

Protoplast formation can be increased if the cell cycle of the protoplasts have been synchronized to be halted at G1. In the case of *S. cerevisiae* this can be accomplished by the addition of mating factors, either a or α (Curran & Carter, *J. Gen. Microbiol.* 129, 1589-1591 (1983)) These peptides act as adenylate cyclase inhibitors which by decreasing the cellular level of cAMP arrest the cell cycle at G1. In addition, sex factors have been shown to induce the weakening of the cell wall in preparation for the sexual fusion of a and α cells (Crandall & Brock, *Bacteriol. Rev.* 32, 139-163 (1968), Osumi et al., *Arch. Microbiol.* 97, 27-38 (1974)). Thus in the preparation of protoplasts, cells can be treated with mating factors or other known inhibitors of adenylate cyclase, such as leflunomide or the killer toxin from *K. lactis*, to arrest them at G1 (Sugisaki et al., *Nature* 304, 464-466 (1983)). Then after fusing of the protoplasts (step 2), cAMP can be added to the regeneration medium to induce S-phase and DNA synthesis. Alternatively, yeast strains having a temperature sensitive mutation in the CDC4 gene can be used, such that cells could be synchronized and arrested at G1. After fusion cells are returned to the permissive temperature so that DNA synthesis and growth resumes.

Once suitable protoplasts have been prepared, it is necessary to induce fusion by physical or chemical means. An equal number of protoplasts of each cell type is mixed in phosphate buffer (0 2 M, pH 5 8, 2 x 10^8 cells/mL) containing an osmotic stabilizer, for example 0 8 M NaCl, and PEG 6000 (33% w/v) and then incubated at 30°C for 5 min while fusion occurs. Polyols, or other compounds that bind water, can be employed. The fusants are then washed and resuspended in the osmotically stabilized buffer lacking PEG, and transferred to osmotically stabilized regeneration medium on/in which the cells can be selected or screened for a desired property.

8. Shuffling Methods Using Artificial Chromosomes

Yeast artificial chromosomes (Yacs) are yeast vectors into which very large DNA fragments (e.g., 50-2000 kb) can be cloned (see, e g., Monaco & Larin, *Trends. Biotech.* 12(7), 280-286 (1994); Ramsay, *Mol. Biotechnol.* 1(2), 181-201 1994; Huxley, *Genet. Eng.* 16, 65-91 (1994); Jakobovits, *Curr. Biol.* 4(8), 761-3 (1994), Lamb & Gearhart, *Curr. Opin. Genet. Dev.* 5(3), 342-8 (1995); Montoliu et al., *Reprod. Fertil. Dev.* 6, 577-84 (1994)) These vectors have telomeres (Tel), a centromere (Cen), an autonomously replicating sequence (ARS), and can have genes for positive (e.g., TRP1) and negative (e.g., URA3) selection. YACs are maintained, replicated, and segregate as other yeast chromosomes through both meiosis and mitosis thereby providing a means to expose cloned DNA to true meiotic recombination.

YACs provide a vehicle for the shuffling of libraries of large DNA fragments *in vivo*. The substrates for shuffling are typically large fragments from 20 kb to 2 Mb. The fragments can be random fragments or can be fragments known to encode a desirable property. For example, a fragment might include an operon of genes involved in production of antibiotics. Libraries can also include whole genomes or chromosomes. Viral genomes and some bacterial genomes can be cloned intact into a single YAC. In some libraries, fragments are obtained from a single organism. Other libraries include fragment variants, as where some libraries are obtained from different individuals or species Fragment variants can also be generated by induced mutation Typically, genes within fragments are expressed from naturally associated regulatory sequences within yeast However, alternatively, individual genes can be linked to yeast regulatory elements to form an expression cassette, and a concatemer of such cassettes, each containing a different gene, can be inserted into a YAC.

In some instances, fragments are incorporated into the yeast genome, and shuffling is used to evolve improved yeast strains. In other instances, fragments remain as

components of YACs throughout the shuffling process, and after acquisition of a desired property, the YACs are transferred to a desired recipient cell

9 Methods of Evolving Yeast Strains

Fragments are cloned into a YAC vector, and the resulting YAC library is transformed into competent yeast cells Transformants containing a YAC are identified by selecting for a positive selection marker present on the YAC. The cells are allowed to recover and are then pooled Thereafter, the cells are induced to sporulate by transferring the cells from rich medium, to nitrogen and carbon limiting medium. In the course of sporulation, cells undergo meiosis. Spores are then induced to mate by return to rich media. Optionally, asci are lysed o liberate spores, so that the spores can mate with other spores originating from other asci Mating results in recombination between YACs bearing different inserts, and between YACs and natural yeast chromosomes. The latter can be promoted by irradiating spores with ultra violet light Recombination can give rise to new phenotypes either as a result of genes expressed by fragments on the YACs or as a result of recombination with host genes, or both.

After induction of recombination between YACs and natural yeast chromosomes, YACs are often eliminated by selecting against a negative selection marker on the YACs. For example, YACs containing the marker URA3 can be selected against by propagation on media containing 5-fluro-orotic acid. Any exogenous or altered genetic material that remains is contained within natural yeast chromosomes. Optionally, further rounds of recombination between natural yeast chromosomes can be performed after elimination of YACs. Optionally, the same or different library of YACs can be transformed into the cells, and the above steps repeated. By recursively repeating this process, the diversity of the population is increased prior to screening.

After elimination of YACs, yeast are then screened or selected for a desired property. The property can be a new property conferred by transferred fragments, such as production of an antibiotic. The property can also be an improved property of the yeast such as improved capacity to express or secrete an exogenous protein, improved recombinogenicity, improved stability to temperature or solvents, or other property required of commercial or research strains of yeast.

Yeast strains surviving selection/screening are then subject to a further round of recombination. Recombination can be exclusively between the chromosomes of yeast surviving selection/screening. Alternatively, a library of fragments can be introduced into the

yeast cells and recombined with endogenous yeast chromosomes as before This library of fragments can be the same or different from the library used in the previous round of transformation For example, the YACs could contain a library of genomic DNA isolated from a pool of the improved strains obtained in the earlier steps YACs are eliminated as before, followed by additional rounds of recombination and/or transformation with further YAC libraries. Recombination is followed by another round of selection/screening, as above Further rounds of recombination/screening can be performed as needed until a yeast strain has evolved to acquire the desired property

An exemplary scheme for evolving yeast by introduction of a YAC library is shown in Fig. 10. The first part of the figure shows yeast containing an endogenous diploid genome and a YAC library of fragments representing variants of a sequence The library is transformed into the cells to yield 100-1000 colonies per μg DNA Most transformed yeast cells now harbor a single YAC as well as endogenous chromosomes. Meiosis is induced by growth on nitrogen and carbon limiting medium. In the course of meiosis the YACs recombine with other chromosomes in the same cell Haploid spores resulting from meiosis mate and regenerated diploid forms. The diploid forms now harbor recombinant chromosomes, parts of which come from endogenous chromosomes and parts from YACs Optionally, the YACs can now be cured from the cells by selecting against a negative selection marker present on the YACS. Irrespective whether YACS are selected against, cells are then screened or selected for a desired property Cells surviving selection/screening are transformed with another YAC library to start another shuffling cycle

10. Method of Evolving YACs for Transfer to Recipient Strain

These methods are based in part on the fact that multiple YACs can be harbored in the same yeast cell, and YAC-YAC recombination is known to occur (Green & Olson, *Science* 250, 94-98 1990)). Inter-YAC recombination provides a format for which families of homologous genes harbored on fragments of >20 kb can be shuffled *in vivo*. The starting population of DNA fragments show sequence similarity with each other but differ as a result of for example, induced, allelic or species diversity. Often DNA fragments are known or suspected to encode multiple genes that function in a common pathway.

The fragments are cloned into a Yac and transformed into yeast, typically with positive selection for transformants. The transformants are induced to sporulate, as a result of which chromosomes undergo meiosis. The cells are then mated. Most of the resulting diploid cells now carry two YACs each having a different insert. These are again induced to sporulate

and mated. The resulting cells harbor YACs of recombined sequence The cells can then be screened or selected for a desired property. Typically, such selection occurs in the yeast strain used for shuffling However, if fragments being shuffled are not expressed in yeast, YACs can be isolated and transferred to an appropriate cell type in which they are expressed for screening. Examples of such properties include the synthesis or degradation of a desired compound, increased secretion of a desired gene product, or other detectable phenotype.

Preferably, the YAC library is transformed into haploid a and haploid α cells. These cells are then induced to mate with each other, i.e , they are pooled and induced to mate by growth on rich medium. The diploid cells, each carrying two YACs, are then transferred to sporulation medium. During sporulation, the cells undergo meiosis, and homologous chromosomes recombine In this case, the genes harbored in the YACs will recombine, diversifying their sequences The resulting haploid acospores are then liberated from the asci by enzymatic degradation of the asci wall or other available means and the pooled liberated haploid acospores are induced to mate by transfer to rich medium. This process is repeated for several cycles to increase the diversity of the DNA cloned into the YACs. The resulting population of yeast cells, preferably in the haploid state, are either screened for improved properties, or the diversified DNA is delivered to another host cell or organism for screening.

Cells surviving selection/screening are subjected to successive cycles of pooling, sporulation, mating and selection/screening until the desired phenotype has been observed. Recombination can be achieved simply by transferring cells from rich medium to carbon and nitrogen limited medium to induce sporulation, and then returning the spores to rich media to induce mating. Asci can be lysed to stimulate mating of spores originating from different asci.

After YACs have been evolved to encode a desired property they can be transferred to other cell types. Transfer can be by protoplast fusion, or retransformation with isolated DNA. For example, transfer of YACs from yeast to mammalian cells is discussed by Monaco & Larin, *Trends in Biotechnology* 12, 280-286 (1994); Montoliu et al., *Reprod. Fertil. Dev.* 6, 577-84 (1994); Lamb et al., *Curr. Opin. Genet. Dev.* 5, 342-8 (1995).

An exemplary scheme for shuffling a YAC fragment library in yeast is shown in Fig. 11. A library of YAC fragments representing genetic variants are transformed into yeast that have diploid endogenous chromosomes. The transformed yeast continue to have diploid endogenous chromosomes, plus a single YAC. The yeast are induced to undergo meiosis and sporulate. The spores contain haploid genomes and are selected for those which contain a

YAC, using the YAC selective marker. The spores are induced to mate generating diploid cells. The diploid cells now contain two YACs bearing different inserts as well as diploid endogenous chromosomes The cells are again induced to undergo meiosis and sporulate during meiosis, recombination occurs between the YAC inserts, and recombinant YACs are segregated to ascoytes Some ascoytes thus contain haploid endogenous chromosomes plus a YAC chromosome with a recombinant insert The ascoytes mature to spores, which can mate again generating diploid cells. Some diploid cells now possess a diploid complement of endogenous chromosomes plus two recombinant YACs These cells can then be taken through further cycles of meiosis, sporulation and mating. In each cycle, further recombination occurs between YAC inserts and further recombinant forms of inserts are generated. After one or several cycles of recombination has occurred, cells can be tested for acquisition of a desired property. Further cycles of recombination, followed by selection, can then be performed in similar fashion

11 *In vivo* Shuffling of Genes by the Recursive Mating of Yeast Cells Harboring Homologous Genes in Identical Loci.

A goal of DNA shuffling is to mimic and expand the combinatorial capabilities of sexual recombination. *In vitro* DNA shuffling succeeds in this process. However, by changing the mechanism of recombination and altering the conditions under which recombination occurs, naturally *in vitro* recombination methods may jeopardize intrinsic information in a DNA sequence that renders it "evolvable."

Shuffling *in vivo* by employing the natural crossing over mechanisms that occur during meiosis may access inherent natural sequence information and provide a means of creating higher quality shuffled libraries. Described here is a method for the *in vivo* shuffling of DNA that utilizes the natural mechanisms of meiotic recombination and provides an alternative method for DNA shuffling.

The basic strategy is to clone genes to be shuffled into identical loci within the haploid genome of yeast. The haploid cells are then recursively induced to mate and to sporulate. The process subjects the cloned genes to recursive recombination during recursive cycles of meiosis The resulting shuffled genes are then screened in *in situ* or isolated and screened under different conditions.

For example, if one wished to shuffle a family of five lipase genes, the following provides a means of doing so *in vivo*.

The open reading frame of each lipase is amplified by the PCR such that each ORF is flanked by identical 3' and 5' sequences. The 5' flanking sequence is identical to a

region within the 5' coding sequence of the *S. cerevisiae ura* 3 gene and the 3' flanking sequence is identical to a region within the 3' of the *ura* 3 gene The flanking sequences are chosen such that homologous recombination of the PCR product with the *ura* 3 gene results in the incorporation of the lipase gene and the disruption of the *ura* 3 ORF. Both *S. cerevisiae* a and α haploid cells are then transformed with each of the PCR amplified lipase ORFs, and cells having incorporated a lipase gene into the *ura* 3 locus are selected by growth on 5 fluoro orotic acid (5FOA is lethal to cells expressing functional URA3). The result is 10 cell types, two different mating types each harboring one of the five lipase genes in the disrupted *ura* 3 locus. These cells are then pooled and grown under conditions where mating between the a and α cells are favored, e.g. in rich medium

Mating results in a combinatorial mixture of diploid cells having all 32 possible combinations of lipase genes in the two *ura* 3 loci. The cells are then induced to sporulate by growth under carbon and nitrogen limited conditions During sporulation the diploid cells undergo meiosis to form four (two a and two α) haploid ascospores housed in an ascus During meiosis II of the sporulation process sister chromatids align and crossover. The lipase genes cloned into the *ura*3 loci will also align and recombine. Thus the resulting haploid ascospores will represent a library of cells each harboring a different possible chimeric lipase gene, each a unique result of the meiotic recombination of the two lipase genes in the original diploid cell. The walls of asci are degraded by treatment with zymolase to liberate and allow the mixing of the individual ascospores This mixture is then grown under conditions that promote the mating of the a and α haploid cells. It is important to liberate the individual ascospores, since mating will otherwise occur between the ascospores within an ascus. Mixing of the haploid cells allows recombination between more than two lipase genes, enabling "poolwise recombination." Mating brings together new combinations of chimeric genes that can then undergo recombination upon sporulation. The cells are recursively cycled through sporulation, ascospore mixing, and mating until sufficient diversity has been generated by the recursive pairwise recombination of the five lipase genes. The individual chimeric lipase genes either can be screened directly in the haploid yeast cells or transferred to an appropriate expression host.

The process is described above for lipases and yeast; however, any sexual organisms into which genes can be directed can be employed, and any genes, of course, could be substituted for lipases. This process is analogous to the method of shuffling whole

genomes by recursive pairwise mating The diversity, however, in the whole genome case is distributed throughout the host genome rather than localized to specific loci

12 Use of YACs to Clone Unlinked Genes

Shuffling of YACs is particularly amenable to transfer of unlinked but functionally related genes from one species to another, particularly where such genes have not been identified. Such is the case for several commercially important natural products, such as taxol. Transfer of the genes in the metabolic pathway to a different organism is often desirable because organisms naturally producing such compounds are not well suited for mass culturing

Clusters of such genes can be isolated by cloning a total genomic library of DNA from an organisms producing a useful compound into a YAC library. The YAC library is then transformed into yeast The yeast is sporulated and mated such that recombination occurs between YACs and/or between YACs and natural yeast chromosomes Selection/screening is then performed for expression of the desired collection of genes. If the genes encode a biosynthetic pathway, expression can be detected from the appearance of product of the pathway. Production of individual enzymes in the pathway, or intermediates of the final expression product or capacity of cells to metabolize such intermediates indicates partial acquisition of the synthetic pathway. The original library or a different library can be introduced into cells surviving/selection screening, and further rounds of recombination and selection/screening can be performed until the end product of the desired metabolic pathway is produced.

13 YAC-YAC Shuffling

If a phenotype of interest can be isolated to a single stretch of genomic DNA less than 2 megabases in length, it can be cloned into a YAC and replicated in *S. cerevisiae.* The cloning of similar stretches of DNA from related hosts into an identical YAC results in a population of yeast cells each harboring a YAC having a homologous insert effecting a desired phenotype. The recursive breeding of these yeast cells allows the homologous regions of these YACs to recombine during meiosis, allowing genes, pathways, and clusters to recombine during each cycle of meiosis After several cycles of mating and segregation, the YAC inserts are well shuffled. The now very diverse yeast library could then be screened for phenotypic improvements resulting from the shuffling of the YAC inserts.

14 YAC-Chromosome Shuffling

"Mitotic" recombination occurs during cell division and results from the recombination of genes during replication This type of recombination is not limited to that between sister chromatids and can be enhanced by agents that induce recombination machinery, such as nicking chemicals and ultraviolet irradiation Since it is often difficult to directly mate across a species barrier, it is possible to induce the recombination of homologous genes originating from different species by providing the target genes to a desired host organism as a YAC library The genes harbored in this library are then induced to recombine with homologous genes on the host chromosome by enhanced mitotic recombination This process is carried out recursively to generate a library of diverse organisms and then screened for those having the desired phenotypic improvements. The improved subpopulation is then mated recursively as above to identify new strains having accumulated multiple useful genetic alterations

15 Accumulation of Multiple YACs Harboring Useful Genes

The accumulation of multiple unlinked genes that are required for the acquisition or improvement of a given phenotype can be accomplished by the shuffling of YAC libraries. Genomic DNA from organisms having desired phenotypes, such as ethanol tolerance, thermotolerance, and the ability to ferment pentose sugars are pooled, fragmented and cloned into several different YAC vectors, each having a different selective marker (his, ura, ade, etc). *S. cerevisiae* are transformed with these libraries, and selected for their presence (using selective media i.e uracil dropout media for the YAC containing the Ura3 selective marker) and then screened for having acquired or improved a desired phenotype. Surviving cells are pooled, mated recursively, and selected for the accumulation of multiple YACs (by propagation in medium with multiple nutritional dropouts). Cells that acquire multiple YACs harboring useful genomic inserts are identified by further screening. Optimized strains can be used directly, however, due to the burden a YAC may pose to a cell, the relevant YAC inserts can be minimized, subcloned, and recombined into the host chromosome, to generate a more stable production strain.

16. Choice of Host SSF Organism

One example use for the present invention is to create an improved yeast for the production of ethanol from lignocellulosic biomass Specifically, a yeast strain with improved ethanol tolerance and thermostability/thermotolerance is desirable. Parent yeast strains known for good behavior in a Simultaneous Saccharification and Fermentation (SSF)

process are identified. These strains are combined with others known to possess ethanol tolerance and/or thermostability

S. cerevisiae is highly amenable to development for optimized SSF processes It inherently possesses several traits for this use, including the ability to import and ferment a variety of sugars such as sucrose, glucose, galactose, maltose and maltriose Also, yeast has the capability to flocculate, enabling recovery of the yeast biomass at the end of a fermentation cycle, and allowing its re-use in subsequent bioprocesses This is an important property in that it optimizes the use of nutrients in the growth medium *S. cerevisiae* is also highly amenable to laboratory manipulation, has highly characterized genetics and possesses a sexual reproductive cycle. *S. cerevisiae* may be grown under either aerobic or anaerobic conditions, in contrast to some other potential SSF organisms that are strict anaerobes (e.g. *Clostridium* spp.), making them very difficult to handle in the laboratory. *S. cerevisiae* are also "generally regarded as safe" ("GRAS"), and, due to its widespread use for the production of important comestibles for the general public (e.g. beer, wine, bread, etc), is generally familiar and well known. *S. cerevisiae* is commonly used in fermentative processes, and the familiarity in its handling by fermentation experts eases the introduction of novel improved yeast strains into the industrial setting.

S. cerevisiae strains that previously have been identified as particularly good SSF organisms, for example, *S. cerevisiae* D_5A (ATCC200062) (South CR and Lynd LR. (1994) Appl. Biochem. Biotechnol. **45/46**: 467-481; Ranatunga TD et al. (1997) Biotechnol. Lett. **19**:1125-1127) can be used for starting materials. In addition, other industrially used *S. cerevisiae* strains are optionally used as host strains, particularly those showing desirable fermentative characteristics, such as *S. cerevisiae* Y567 (ATCC24858) (Sitton OC et al. (1979) Process Biochem. **14**(9): 7-10; Sitton OC et al. (1981) Adv. Biotechnol. **2**. 231-237, McMurrough I et al. (1971) Folia Microbiol. **16**: 346-349) and *S. cerevisiae* ACA 174 (ATCC 60868) (Benitez T et al. (1983) Appl. Environ. Microbiol. **45**: 1429-1436; Chem. Eng. J. **50**: B17-B22, 1992), which have been shown to have desirable traits for large- scale fermentation

17. Choice of Ethanol Tolerant Strains

Many strains of *S. cerevisiae* have been isolated from high-ethanol environments, and have survived in the ethanol-rich environment by adaptive evolution. For example, strains from Sherry wine aging ("Flor" strains) have evolved highly functional mitochondria to enable their survival in a high-ethanol environment. It has been shown that transfer of these wine yeast mitochondria to other strains increases the recipient's resistance to

high ethanol concentration, as well as thermotolerance (Jimenez, J. and Benitez, T (1988) Curr Genet. **13**: 461-469). There are several flor strains deposited in the ATCC, for example *S. cerevisiae* MY91 (ATCC 201301), MY138 (ATCC 201302), C5 (ATCC 201298), ET7 (ATCC 201299), LA6 (ATCC 201300), OSB21 (ATCC 201303), F23 (*S. globosus* ATCC 90920) Also, several flor strains of *S. uvarum* and *Torulaspora pretoriensis* have been deposited. Other ethanol-tolerant wine strains include *S. cerevisiae* ACA 174 (ATCC 60868), 15% ethanol, and *S. cerevisiae* A54 (ATCC 90921), isolated from wine containing 18% (v/v) ethanol, and NRCC 202036 (ATCC 46534), also a wine yeast. Other *S. cerevisiae* ethanologens that additionally exhibit enhanced ethanol tolerance include ATCC 24858, ATCC 24858, G 3706 (ATCC 42594), NRRL Y-265 (ATCC 60593), and ATCC 24845 - ATCC 24860. A strain of *S. pastorianus* (*S. carlsbergensis* ATCC 2345) has high ethanol-tolerance (13% v/v). *S. cerevisiae* Sa28 (ATCC 26603), from Jamaican cane juice sample, produces high levels of alcohol from molasses, is sugar tolerant, and produces ethanol from wood acid hydrolyzate

Several of the listed strains, as well as additional strains can be used as starting materials for breeding ethanol tolerance.

18. Choice of Temperature Tolerant Strains

A few temperature tolerant strains have been reported, including the highly flocculent strain *S. pastorianus* SA 23 (*S. carlsbergensis* ATCC 26602), which produces ethanol at elevated temperatures, and. *S. cerevisiae* Kyokai 7 (*S. sake*, ATCC 26422), a sake yeast tolerant to brief heat and oxidative stress. Ballesteros et al ((1991) Appl. Biochem. Biotechnol. **28/29**. 307-315) examined 27 strains of yeast for their ability to grow and ferment glucose in the 32-45°C temperature range, including *Saccharomyces*, *Kluyveromyces* and *Candida* spp. Of these, the best thermotolerant clones were *Kluyveromyces marxianus* LG and *Kluyveromyces fragilis* 2671 (Ballesteros et al (1993) Appl. Biochem. Biotechnol. **39/40**: 201-211). *S. cerevisiae-pretoriensis* FDHI was somewhat thermotolerant, however was poor in ethanol tolerance. Recursive recombination of this strain with others that display ethanol tolerance can be used to acquire the thermotolerant characteristics of the strain in progeny which also display ethanol tolerance.

Candida acidothermophilum (*Issatchenkia orientalis*, ATCC 20381) is a good SSF strain that also exhibits improved performance in ethanol production from lignocellulosic biomass at higher SSF temperatures than *S. cerevisiae* D_5A (Kadam, KL, Schmidt, SL (1997)

Appl Microbiol. Biotechnol. **48** 709-713) This strain can also be a genetic contributor to an improved SSF strain

19 Shuffling of Strains

In those instances where strains are highly related, a recursive mating strategy may be pursued For example, a population of haploid *S. cerevisiae* (a and alpha) are mutagenized and screened for improved EtOH or thermal tolerance The improved haploid subpopulation are mixed together and mated as a pool and induced to sporulate. The resulting haploid spores are freed by degrading the asci wall and mixed. The freed spores are then induced to mate and sporulate recursively This process is repeated a sufficient number of times to generate all possible mutant combinations The whole genome shuffled population (haploid) is then screened for further EtOH or thermal tolerance.

When strains are not sufficiently related for recursive mating, formats based on protoplast fusion may be employed. Recursive and poolwise protoplast fusion can be performed to generate chimeric populations of diverse parental strains. The resultant pool of progeny is selected and screened to identify improved ethanol and thermal tolerant strains.

Alternatively, a YAC-based Whole Genome Shuffling format can be used In this format, YACs are used to shuttle large chromosomal fragments between strains. As detailed earlier, recombination occurs between YACs or between YACs, and the host chromosomes. Genomic DNA from organisms having desired phenotypes are pooled, fragmented and cloned into several different YAC vectors, each having a different selective marker (his, ura, ade, etc) *S. cerevisiae* are transformed with these libraries, and selected for their presence (using selective media, i.e. uracil dropout media for the YAC containing the Ura3 selective marker) and then screened for having acquired or improved a desired phenotype Surviving cells are pooled, mated recursively (as above), and selected for the accumulation of multiple YACs (by propagation in medium with multiple nutritional dropouts). Cells that acquire multiple YACs harboring useful genomic inserts are identified by further screening (see below)

20. Selection for Improved Strains

Having produced large libraries of novel strains by mutagenesis and recombination, a first task is to isolate those strains that possess improvements in the desired phenotypes. Identification of the organism libraries is facilitated where the desired key traits are selectable phenotypes. For example, ethanol has different effects on the growth rate of a yeast population, viability, and fermentation rate. Inhibition of cell growth and viability

increases with ethanol concentration, but high fermentative capacity is only inhibited at higher ethanol concentrations. Hence, selection of growing cells in ethanol is a viable approach to isolate ethanol-tolerant strains Subsequently, the selected strains may be analyzed for their fermentative capacity to produce ethanol. Provided that growth and media conditions are the same for all strains (parents and progeny), a hierarchy of ethanol tolerance may be constructed

Simple selection schemes for identification of thermal tolerant and ethanol tolerant strains are available and, in this case, are based on those previously designed to identify potentially useful SSF strains. Selection of ethanol tolerance is performed by exposing the population to ethanol, then plating the population and looking for growth. Colonies capable of growing after exposure to ethanol can be re-exposed to a higher concentration of ethanol and the cycle repeated until the most tolerant strains are selected. In order to discern strains possessing heritable ethanol tolerance from with temporarily acquired adaptations, these cycles may be punctuated with cycles of growth in the absence of selection (e.g. no ethanol)

Alternatively, the mixed population can be grown directly at increasing concentrations of ethanol, and the most tolerant strains enriched (Aguilera and Benitez, 1986, Arch Microbiol 4:337-44). For example this enrichment could be carried out in a chemostat or turbidostat. Similar selections can be developed for thermal tolerance, in which strains are identified by their ability to grow after a heat treatment, or directly for growth at elevated temperatures (Ballesteros et al., 1991, Applied Biochem and Biotech, 28:307-315). The best strains identified by these selections will be assayed more thoroughly in subsequent screens for ethanol, thermal tolerance or other properties of interest

In one aspect, organisms having increased ethanol tolerance are selected for. A population of natural *S. cerevisae* isolates are mutagenized. This population is then grown under fermentor conditions under low initial ethanol concentrations. Once the culture has reached saturation, the culture is diluted into fresh medium having a slightly higher ethanol content. This process of successive dilution into medium of incrementally increasing ethanol concentration is continued until a threshold of ethanol tolerance is reached. The surviving mutant population having the highest ethanol tolerance are then pooled and their genomes recombined by any method noted herein. Enrichment could also be achieved by a continuos culture in a chemostat or turbidostat in which temperature or ethanol concentrations are progressively elevated. The resulting shuffled population are then exposed once again to the

enrichment strategy but at a higher starting medium ethanol concentration. This strategy is optionally applied for the enrichment of thermotolerant cells and for the enrichment of cells having combined thermo- and ethanol tolerance

21 Screening for Improved Strains

Strains showing viability in initial selections are assayed more quantitatively for improvements in the desired properties before being reshuffled with other strains.

Progeny resulting from mutagenesis of a strain, or those pre-selected for their ethanol tolerance and/or thermostability, can be plated on non-selective agar Colonies can be picked robotically into microtiter dishes and grown. Cultures are replicated to fresh microtiter plates, and the replicates are incubated under the appropriate stress condition(s). The growth or metabolic activity of individual clones may be monitored and ranked Indicators of viabili can range from the size of growing colonies on solid media, density of growing cultures, or color change of a metabolic activity indicator added to liquid media. Strains that show the greatest viability are then mixed and shuffled, and the resulting progeny are rescreened under more stringent conditions

22. Development of an Ethanologen Capable of Converting Cellulose to Ethanol

Once a strain of yeast exhibiting thermotolerance and ethanol tolerance is developed, the degradation of cellulose to monomeric sugars is provided by the inclusion to the host strain of an efficient cellulase degradation pathway

Additional desirable characteristic can be useful to enhance the production of ethanol by the host. For example, inclusion of heterologous enzymes and pathways that broaden the substrate sugar range may be performed "Tuning" of the strain can be accomplished by the addition of various other traits, or the restoration of certain endogenous traits that are desirable, but lost during the recombination procedures.

23 Conferring of Cellulase Activity

A vast number of cellulases and cellulase degradation systems have been characterized from fungi, bacteria and yeast (see reviews by Beguin, P and Aubert, J-P (1994) FEMS Microbiol. Rev. **13**: 25-58; Ohima, K. et al. (1997) Biotechnol. Genet. Eng. Rev. **14**: 365-414). An enzymatic pathway required for efficient saccharification of cellulose involves the synergistic action of endoglucanases (endo-1,4-β-D-glucanases, EC 3.2.1.4), exocellobiohydrolases (exo-1,4-β-D-glucanases, EC 3.2.1.91), and β-glucosidases (cellobiases,

1,4-β-D-glucanases EC 3.2.1.21) (Fig. 9). The heterologous production of cellulase enzymes in the ethanologen would enable the saccharification of cellulose, producing monomeric sugars that may be used by the organism for ethanol production. There are several advantages to the heterologous expression of a functional cellulase pathway in the ethanologen. For example, the SSF process would eliminate the need for a separate bioprocess step for saccharification, and would ameliorate end-product inhibition of cellulase enzymes by accumulated intermediate and product sugars.

Naturally occurring cellulase pathways are inserted into the ethanologen, or one may choose to use custom improved "hybrid" cellulase pathways, employing the coordinate action of cellulases derived from different natural sources, including thermophiles.

Several cellulases from non-*Saccharomyces* have been produced and secreted from this organism successfully, including bacterial, fungal, and yeast enzymes, for example *T. reesei* CBH I ((Shoemaker (1994), in "The Cellulase System of *Trichoderma reesei: Trichoderma* strain improvement and Expression of *Trichoderma cellulases in Yeast*," Online, Pinner, UK, 593-600). It is possible to employ straightforward metabolic engineering techniques to engender cellulase activity in *Saccharomyces*. Also, yeast have been forced to acquire elements of cellulose degradation pathways by protoplast fusion (e.g. intergeneric hybrids of *Saccharomyces cerevisiae* and *Zygosaccharomyces fermentati*, a cellobiase-producing yeast, have been created (Pina A, et. al. (1986) Appl. Environ. Microbiol. **51**: 995-1003). In general, any cellulase component enzyme that derives from a closely related yeast organism could be transferred by protoplast fusion. Cellobiases produced by a somewhat broader range of yeast may be accessed by whole genome shuffling in one of its many formats (e.g. whole, fragmented, YAC-based).

Optimally, the cellulase enzymes to be used should exhibit good synergy, an appropriate level of expression and secretion from the host, good specific activity (i.e. resistance to host degradation factors and enzyme modification) and stability in the desired SSF environment. An example of a hybrid cellulose degradation pathway having excellent synergy includes the following enzymes: CBH I exocellobiohydrolase of *Trichoderma reesei*, the *Acidothermus cellulolyticus* E1 endoglucanase, and the *Thermomonospera fusca* E3 exocellulase (Baker, et. al. (1998) Appl. Biochem. Biotechnol **70-72**: 395-403).

It is suggested here that these enzymes (or improved mutants thereof) be considered for use in the SSF organism, along with a cellobiase (β-glucosidase), such as that from *Candida peltata*. Other possible cellulase systems to be considered should possess

particularly good activity against crystalline cellulose, such as the *T. reesei* cellulase system (Teeri, TT, et. al. (1998) Biochem Soc Trans. **26** 173-178), or possess particularly good thermostability characteristics (e g cellulase systems from thermophilic organisms, such as *Thermomonospora fusca* (Zhang, S., et. al. (1995) Biochem. **34**. 3386-335)

A rational approach to the cloning of cellulases in the ethanologenic yeast host could be used For example, known cellulase genes are cloned into expression cassettes utilizing *S. cerevisiae* promoter sequences, and the resultant linear fragments of DNA may be transformed into the recipient host by placing short yeast sequences at the termini to encourage site-specific integration into the genome. This is preferred to plasmidic transformation for reasons of genetic stability and maintenance of the transforming DNA.

If an entire cellulose degradative pathway were introduced, a selection could be implemented in an agar-plate-based format, and a large number of clones could be assayed for cellulase activity in a short period of time. For example, selection for an exocellulase may be accessible by providing a soluble oligocellulose substrate or carboxymethylcellulose (CMC) as a sole carbon source to the host, otherwise unable to grow on agar containing this sole carbon source. Clones producing active cellulase pathways would grow by virtue of their ability to produce glucose.

Alternatively, if the different cellulases were to be introduced sequentially, it would be useful to first introduce a cellobiase, enabling a selection using commercially available cellobiose as a sole carbon source. Several strains of *S. cerevisiae* that are able to grow on cellobiose have been created by introduction of a cellobiase gene (e.g. Rajoka MI, et al. (1998) Floia Microbiol. (Praha) **43**, 129-135; Skory, CD, et. al. (1996) Curr. Genet. **30**, 417-422; D'Auria, S, et. al. (1996) Appl. Biochem. Biotechnol. **61**, 157-166; Adam, AC, et. al. (1995) Yeast **11**, 395-406; Adam, AC (1991) Curr. Genet. **20**, 5-8).

Subsequent transformation of this organism with CBHI exocellulase can be selected for by growth on a cellulose substrate such as carboxymethylcellulose (CMC). Finally, addition of an endoglucanase creates a yeast strain with improved crystalline degradation capacity.

24. Conferring of Pentose Sugar Utilization

Inclusion of pentose sugar utilization pathways is an important facet to a potentially useful SSF organism. The successful expression of xylose sugar utilization pathways for ethanol production has been reported in *Saccharomyces* (e.g. Chen, ZD and Ho, NWY (1993) Appl. Biochem. Biotechnol. **39/40** 135-147).

It would also be useful to accomplish L-arabinose substrate utilization for ethanol production in the *Saccharomyces* host Yeast strains that utilize L-arabinose include some *Candida* and *Pichia* spp. (McMillan JD and Boynton BL (1994) Appl Biochem. Biotechnol **45-46** 569-584; Dien BS, et al (1996) Appl. Biochem Biotechnol. **57-58**. 233-242). Genes necessary for arabinose fermentation in *E. coli* could also be inroduced by rational means (e.g as has been performed previously in *Z. mobilis* (Deanda K, et. al (1996) Appl. Environ Microbiol **62**. 4465-4470))

25. Conferring of Other Useful Activities

Several other traits that are important for optimization of an SSF strain have been shown to be transferable to *S. cerevisiae*. Like thermal tolerance, cellulase activity and pentose sugar utilization, these traits may not normally be exhibited by *Saccharomyces* (or the particular strain of *Saccharomyces* being used as a host), and may be added by genetic means For example, expression of human muscle acylphosphatase in *S. cerevisiae* has been suggested to increase ethanol production (Rougei, G., et. al. (1996) Biotechnol. App. Biochem. **23**: 273-278).

It can occur that evolved stress-tolerant SSF strain acquire some undesirable mutations in the course of the evolution strategy. Indeed, this is a pervasive problem in strain improvement strategies that rely on mutagenesis techniques, and can result in highly unstable or fragile production strains It is possible to restore some of these desirable traits by rational methods such as cloning of specific genes that have been knocked out or negatively influenced in the previous rounds of strain improvement. The advantage to this approach is specificity- the offending gene may be targeted directly. The disadvantage is that it may be time-consuming and repetitious if several genes have been compromised, and it only addresses problems that have been characterized. A preferred (and more traditional) approach to the removal of undesirable/deleterious mutations is to back-cross the evolved strain to a desirable parent strain (e.g. the original "host" SSF strain). This strategy has been employed successfully throughout strain improvement where accessible (i.e. for organisms that have sexual cycles of reproduction). When lacking the advantage of a sexual process, it has been accomplished by using other methods, such as parasexual recombination or protoplast fusion For example, the ability to flocculate was conferred on a non-flocculating strain of *S. cerevisiae* by protoplast fusion with a flocculation competent *S. cerevisiae* (Watari, J., et. al (1990) Agric. Biol. Chem. **54**: 1677-1681)

N. IN VITRO WHOLE GENOME SHUFFLING

The shuffling of large DNA sequences, such as eukaryotic chrmosomes, is difficult by prior art *in vitro* shuffling methods A method for overcoming this limitation is described herein.

The cells of related eukaryotic species are gently lysed and the intact chromosomes are liberated. The liberated chromosomes are then sorted by FACS or similar method (such as pulse field electrophoresis) with chromosomes of similar size being sequestered together. Each size fraction of the sorted chromosomes generally will represent a pool of analogous chromosomes, for example the Y chromosome of related mammals The i goal is to isolate intact chromosomes that have not been irreversibly damaged.

The fragmentation and reassembly of such large complex pieces of DNA employing DNA polymerases is difficult and would likely introduce an unacceptably high level of random mutations. An alternative approach that employs restriction enzymes and DNA ligase provides a feasible less destructive solution. A chromosomal fraction is digested with one or more restriction enzymes that recognize long DNA sequences (~15-20bp), such as the intron and intein encoded endonucleases (I-*Ppo* I, I-*Ceu* I, PI-*Psp* I, PI-*Tli* I, PI-*Sce* I (VDE) These enzymes each cut, at most, a few times within each chromosome, resulting in a combinatorial mixture of large fragments, each having overhanging single stranded termini that are complementary to other sites cleaved by the same enzyme.

The digest is further modified by very short incubation with a single stranded exonuclease. The polarity of the nuclease chosen is dependent on the single stranded overhang resulting from the restriction enzyme chosen. 5'-3' exonuclease for 3'-overhangs, and 3'-5'- exonuclease for 5'overhangs. This digestion results in significantly long regions of ssDNA overhang on each dsDNA termini. The purpose of this incubation is to generate regions of DNA that define specific regions of DNA where recombination can occur. The fragments are then incubated under condition where the ends of the fragments anneal with other fragments having homologous ssDNA termini Often, the two fragments annealing will have originated from different chromosomes and in the presence of DNA ligase are covalently linked to form a chimeric chromosome. This generates genetic diversity mimicking the crossing over of homologous chromosomes. The complete ligation reaction will contain a combinatorial mixture of all possible ligations of fragments having homologous overhanging termini. A subset of this population will be complete chimeric chromosomes.

To screen the shuffled library, the chromosomes are delivered to a suitable host in a manner allowing for the uptake and expression of entire chromosomes. For example,

YACs (yeast artificial chromosomes) can be delivered to eukaryotic cells by protoplast fusion Thus, the shuffle library could be encapsulated in liposomes and fused with protoplasts of the appropriate host cell The resulting transformants would be propagated and screened for the desired cellular improvements. Once an improved population was identified, the chromosomes would be isolated, shuffled, and screened recursively.

O WHOLE GENOME SHUFFLING OF NATURALLY COMPETENT MICROORGANISMS

Natural competence is a phenomenon observed for some microbial species whereby individual cells take up DNA from the environment and incorporate it into their genome by homologous recombination. *Bacillus subtilis* and *Acetinetobacter spp.* are known to be particularly efficient at this process. A method for the whole genome shuffling (WGS) of these and analogous organisms is described employing this process.

One goal of whole genome shuffling is the rapid accumulation of useful mutations from a population of individual strains into one superior strain. If the organisms to be evolved are naturally competent, then a split pooled strategy for the recursive transformation of naturally competent cells with DNA originating from the pool will effect this process. An example procedure is as follows.

A population of naturally competent organisms that demonstrates a variety of useful traits (such as increased protein secretion) is identified. The strains are pooled, and the pool is split One half of the pool is used as a source of gDNA, while the other is used to generate a pool of naturally competent cells.

The competent cells are grown in the presence of the pooled gDNA to allow DNA uptake and recombination. Cells of one genotype uptake and incorporate gDNA from cells of a different type generating cells having chimeric genomes. The result is a population of cells representing a combinatorial mixture of the genetic variations originating in the original pool These cells are pooled again and transformed with the same source of DNA again. This process is carried out recursively to increase the diversity of the genomes of cells resulting from transformation. Once sufficient diversity has been generated, the cell population is screened for new chimeric organisms demonstrating desired improvements

This process is enhanced by increasing the natural competence of the host organism COMS is a protein that, when expressed in *B. subtilis,* enhances the efficiency of natural competence mediated transformation more than an order of magnitude.

It was demonstrated that approximately 100% of the cells harboring the plasmid pCOMS uptake and recombine genomic DNA fragments into their genomes. In

general, approximately 10% of the genome is recombined into any given transformed cell This observation was demonstrated by the following

A strain of *B. subtilis* pCOMS auxotrophic for two nutritional markers was transformed with genomic DNA (gDNA) isolated from a prototrophic strain of the same organism. 10% of the cells exposed to the DNA were prototrophic for one of the two nutrient markers. The average size of the DNA strand taken up by *B. subtilis* is approximately 50kb or ~2% of the genome. Thus 1 of every ten cells had recombined a marker that was represented 1 in every fifty molecules of uptaken gDNA Thus, most of the cells take up and recombine with approximately five 50kb molecules or 10% of the genome This method represents a powerful tool for rapidly and efficiently recombining whole microbial genomes

In the absence of pCOMS, only 0.3% of the cells prepared for natural competency uptake and integrate a specific marker. This suggested that about 15% of the cells actually underwent recombination with a single genomic fragment. Thus, a recursive transformation strategy as described above produces a whole genome shuffled library, even in the absence of pCOMS. In the absence of pCOMS, however, the complex genomes will represent a smaller, but still screenable percentage of the transformed or shuffled population

P. CONGRESSION

Congression is the integration of two independent unlinked markers into a cell. 0.3% of naturally competent *B. subtilis* cells integrate a single marker (described above). Of these, about 10% have taken up an additional marker. Thus, if one selects or screens for the integration of one specific marker, 10% of the resulting population will have integrated another specific marker. This provides a way of enriching for specific integration events.

For example, if one is looking for the integration of a gene for which there is no easy screen or selection, it will exist as 0.3% of the cell population. If the population is first selected for a specific integration event, then the desired integration will be found in 10% of the population. This represents a significant (~30-fold) enrichment for the desired event. This enrichment is defines as the "congression effect." The congression effect is not influenced by the presence of pCOMS, thus the "pCOMS effect" is simply to increase the percentage of naturally competent cells that are truly naturally competent from about 15% in its absence to 100% in its presence. All competent cells still uptake about the same amount of DNA or ~10% of the Bacillus genome.

The congression effect can be used in the following examples to enhance whole genome shuffling as well, as the targeted integration of shuffled genes to the chromosome.

Q *B. SUBTILIS* SHUFFLING

A population of *B. subtilis* cells having desired properties are identified, pooled and shuffled as described above with one exception once the pooled population is split, half of the population is transformed with an antibiotic selection marker that is flanked by sequence that targets its integration and disruption of a specific nutritional gene, for example, one involved in amino biosynthesis Transformants resistant to the drug are auxotrophic for that nutrient The resistant population is pooled and grown under conditions rendering them naturally competent (or optionally first transformed with pCOMS).

The competent cells are then transformed with gDNA isolated from the original pool, and prototrophs are selected. The prototrophic population will have undergone recombination with genomic fragments encoding a functional copy of the nutritional marker, and thus will be enriched for cells having undergone recombination at other genetic loci by the congression effect.

R. TARGETING OF GENES AND GENE LIBRARIES TO THE CHROMOSOME

It is useful to be able to efficiently deliver genes or gene libraries directly to a specific location in a cells chromosome. As above, target cells are transformed with a positive selection marker flanked by sequences that target its homologous recombination into the chromosome. Selected cells harboring the marker are made naturally competent (with or without pCOMS, but preferably the former) and transformed with a mixture of two sets of DNA fragments. The first set contains a gene or a shuffled library of genes each flanked with sequence to target its integration to a specific chromosomal loci. The second set contains a positive selection marker (different from that first integrated into the cells) flanked by sequence that will target its integration and replacement of the first positive selection marker. Under optimal conditions, the mixture is such that the gene or gene library is in molar excess over the positive selection marker Transformants are then selected for cells containing the new positive marker. These cells are enriched for cells having integrated a copy of the desired gene or gene library by the congression effect and can be directly screened for cells harboring the gene or gene variants of interest. This process was carried out using PCR fragments <10kb, and it was found that, employing the congression effect, a population can be enriched such that 50% of the cells are congregants. Thus, one in two cells contained a gene or gene variant.

Alternatively, the expression host can be absent of the first positive selection marker, and the competent cells are transformed with a mixture of the target genes and a limiting amount of the first positive selection marker fragment Cells selected for the positive marker are screened for the desired properties in the targeted genes. The improved genes are amplified by the PCR, shuffled again, and then returned to the original host again with the first positive selection marker. This process is carried out recursively until the desired function of the genes are obtained. This process obviates the need to construct a primary host strain and the need for two positive markers.

S. CONJUGATION-MEDIATED GENETIC EXCHANGE

Conjugation can be employed in the evolution of cell genomes in several ways Conjugative transfer of DNA occurs during contact between cells. See Guiney (1993) in *Bacterial Conjugation* (Clewell, ed., Plenum Press, New York), pp. 75-104; Reimmann & Haas *in Bacterial Conjugation* (Clewell, ed., Plenum Press, New York 1993), at pp.137-188 (incorporated by reference in their entirety for all purposes). Conjugation occurs between many types of gram negative bacteria, and some types of gram positive bacteria. Conjugative transfer is also known between bacteria and plant cells (Agrobacterium tumefaciens) or yeast As discussed in patent 5,837,458, the genes responsible for conjugative transfer can themselves be evolved to expand the range of cell types (e.g., from bacteria to mammals) between which such transfer can occur.

Conjugative transfer is effected by an origin of transfer (oriT) and flanking genes (MOB A, B and C), and 15-25 genes, termed tra, encoding the structures and enzymes necessary for conjugation to occur. The transfer origin is defined as the site required in cis for DNA transfer. Tra genes include tra A, B, C, D, E, F, G, H, I, J, K, L, M, N, P, Q, R, S, T, U, V, W, X, Y, Z, vir AB (alleles 1-11), C, D, E, G, IHF, and FinOP Tra genes can be expressed in cis or trans to oriT. Other cellular enzymes, including those of the RecBCD pathway, RecA, SSB protein, DNA gyrase, DNA polI, and DNA ligase, are also involved in conjugative transfer. RecE or recF pathways can substitute for RecBCD.

One structural protein encoded by a tra gene is the sex pilus, a filament constructed of an aggregate of a single polypeptide protruding from the cell surface. The sex pilus binds to a polysaccharide on recipient cells and forms a conjugative bridge through which DNA can transfer. This process activates a site-specific nuclease encoded by a MOB gene, which specifically cleaves DNA to be transferred at oriT. The cleaved DNA is then threaded through the conjugation bridge by the action of other tra enzymes.

Mobilizable vectors can exist in episomal form or integrated into the chromosome. Episomal mobilizable vectors can be used to exchange fragments inserted into the vectors between cells Integrated mobilizable vectors can be used to mobilize adjacent genes from the chromosome

T USE OF INTEGRATED MOBILIZABLE VECTORS TO PROMOTE EXCHANGE OF GENOMIC DNA

The F plasmid of E. coli integrates into the chromosome at high frequency and mobilizes genes unidirectional from the site of integration (Clewell, 1993, *supra;* Firth et al , *in Escherichia coli and Salmonella Cellular and Molecular Biology* 2, 2377-2401 (1996), Frost et al., *Microbiol. Rev.* 58, 162-210 (1994)). Other mobilizable vectors do not spontaneously integrate into a host chromosome at high efficiency, but can be induced to do so by growth under particular conditions (e.g., treatment with a mutagenic agent, growth at a nonpermissive temperature for plasmid replication). *See* Reimann & Haas *in Bacterial Conjugation* (ed. Clewell, Plenum Press, NY 1993), Ch. 6. Of particular interest is the IncP group of conjugal plasmids which are typified by their broad host range (Clewell, 1993, *supra.*

Donor "male" bacteria which bear a chromosomal insertion of a conjugal plasmid, such as the *E. coli* F factor can efficiently donate chromosomal DNA to recipient "female" enteric bacteria which lack F (F^-). Conjugal transfer from donor to recipient is initiated at *oriT.* Transfer of the nicked single strand to the recipient occurs in a 5' to 3' direction by a rolling circle mechanisms which allows mobilization of tandem chromosomal copies. Upon entering the recipient, the donor strand is discontinuously replicated. The linear, single-stranded donor DNA strand is a potent substrate for initiation of recA-mediated homologous recombination within the recipient. Recombination between the donor strand and recipient chromosomes can result in the inheritance of donor traits. Accordingly, strains which bear a chromosomal copy of F are designated Hfr (for high frequency of recombination) (Low, 1996 *in Escherichia coli and Salmonella Cellular and Molecular Biology* Vol. 2, pp. 2402-2405; Sanderson, *in Escherichia coli and Salmonella Cellular and Molecular Biology* 2, 2406-2412 (1996)).

The ability of strains with integrated mobilizable vector to transfer chromosomal DNA provides a rapid and efficient means of exchanging genetic material between a population of bacteria thereby allowing combination of positive mutations and dilution of negative mutations. Such shuffling methods typically start with a population of strains with an integrated mobilizable vector encompassing at least some genetic diversity. The genetic diversity can be the result of natural variation, exposure to a mutagenic agent or

introduction of a fragment library The population of cells is cultured without selection to allow genetic exchange, recombination and expression of recombinant genes The cells are then screened or selected for evolution toward a desired property The population surviving selection/screening can then be subject to a further round of shuffling by HFR-mediated genetic exchange, or otherwise.

The natural efficiency of Hfr and other strains with integrated mob vectors as recipients of conjugal transfer can be improved by several means The relatively low recipient efficiency of natural HFR strains is attributable to the products of *traS* and *traT* genes of F (Clewell, 1993, *supra;* Firth et al., 1996, *supra*, Frost et al , 1994, *supra*; Achtman et al., *J. Mol. Biol.* 138, 779-795 (1980). These products are localized to the inner and outer membranes of F^- strains, respectively, where they serve to inhibit redundant matings between two strains which are both capable of donating DNA The effects of traS and traT, and cognate genes in other conjugal plasmids, can be eliminated by use of knockout cells incapable of expressing these enzymes or reduced by propagating cells on a carbon-limited source. (Peters et al., *J. Bacteriol.*, 178, 3037-3043 (1996)).

In some methods, the starting population of cells has a mobilizable vector integrated at different genomic sites. Directional transfer from *oriT* typically results in more frequent inheritance of traits proximal to *oriT*. This is because mating pairs are fragile and tend to dissociate (particularly when in liquid medium) resulting in the interruption of transfer In a population of cells having a mobilizable vector integrated at different sites, chromosomal exchange occurs in a more random fashion. Kits of Hfr strains are available from the *E. coli.* Genetic Stock Center and the *Salmonella* Genetic Stock Centre (Frost et al., 1994, *supra*). Alternatively, a library of strains with *oriT* at random sites and orientations can be produced by insertion mutagenesis using a transposon which bears *oriT*. The use of a transposon bearing an *oriT* [e.g., the Tn5-*oriT* described by Yakobson EA, et al. J. Bacteriol. 1984 Oct; 160(1): 451-453] provides a quick method of generating such a library. Transfer functions for mobilization from the transposon-borne *oriT* sites are provided by a helper vector in *trans*. It is possible to generate similar genetic constructs using other sequences known to one of skill as well.

In one aspect, a recursive scheme for genomic shuffling using Tn-*oriT* elements is provided. A prototrophic bacterial strain or set of related strains bearing a conjugal plasmid, such as the F fertility factor or a member of the *IncP* group of broad host range plasmids is mutagenized and screened for the desired properties. Individuals with the desired properties

are mutagenized with a Tn-*oriT* element and screened for acquisition of an auxotrophy (e.g., by replica-plating to a minimal and complete media) resulting from insertion of the Tn-*oriT* element in any one of many biosynthetic gene scattered across the genome. The resulting auxotrophs are pooled and allowed to mate under conditions promoting male-to-male matings, e.g., during growth in close proximity on a filter membrane Note that transfer functions are provided by the helper conjugal plasmid present in the original strain set. Recombinant transconjugants are selected on minimal medium and screened for further improvement

Optionally, strains bearing integrated mobilizable vectors are defective in mismatch repair gene(s) Inheritance of donor traits which arise from sequence heterologies increases in strains lacking the methyl-directed mismatch repair system. Optionally, the gene products which decrease recombination efficiency can be inhibited by small molecules.

Intergenic conjugal transfer between species such as *E. coli* and *Salmonella typhimurium*, which are 20% divergent at the DNA level, is also possible if the recipient strain is mutH, mutL or mutS (see Rayssiguier et al., *Nature* 342, 396-401 (1989)) Such transfer can be used to obtain recombination at several points as shown by the following example.

One example uses an *S. typhimurium* Hfr donor strain having markers thr557 at map position 0, pyrF2690 at 33 min, serA13 at 62 min and hfrK5 at 43 min MutS +/-, F- E. coli recipient strains had markers pyrD68 at 21 min aroC355 at 51 min, ilv3164 at 85 min and mutS215 at 59 min The triauxotrophic *S. typhimurium* Hfr donor and isogenic mutS+/- triauxotrophic E. coli recipient were inoculated into 3 ml of Lb broth and shaken at 37°C until fully grown. 100 µl of the donor and each recipient were mixed in 10 ml fresh LB broth, and then deposited to a sterile Millipore 0.45 µM HA filter using a Nalgene 250 ml reusable filtration device. The donor and recipients alone were similarly diluted and deposited to check for reversion. The filters with cells were placed cell-side-up on the surface of an LB agar plate which was incubated overnight at 37°C. The filters were removed with the aid of a sterile forceps and placed in a sterile 50 ml tube containing 5 ml of minimal salts broth. Vigorous vortexing was used to wash the cells from the filters. 100 µl of mating mixtures, as well as donor and recipient controls were spread to LB for viable cell counts and minimal glucose supplemented with either two of the three recipient requirements for single recombinant counts, one of the three requirements for double recombinant counts, or none of the three requirements for triple recombinant counts. The plates were incubated for 48 hr at 37° after which colonies were counted

Medium	Recombinant	Recombinant CFUs/Total CFUs		*mutS* / *mutS*⁻
Supplements	*Genotype*	*mutS*⁻	*mutS*	
Aro + Iiv	pyr^{+} aro^{-} ilv^{-}	-	-	-
Aro + Ura	pyr^{-} aro^{-} ilv^{+}	1.2×10^{-8}	2.5×10^{-6}	208
Ilv + Ura	pyr^{-} aro^{+} ilv^{-}	2.7×10^{-8}	3.0×10^{-6}	111
Aro	pyr^{+} aro^{-} ilv^{+}	-	-	-
Ilv	pyr^{+} aro^{+} ilv^{-}	-	-	-
Ura	pyr^{-} aro^{+} ilv^{+}	$<10^{-9}$	$<10^{-9}$	
nothing	pyr^{+} aro^{+} ilv^{+}			

Aro = aromatic amino acids and vitamins
Ilv = branched chain amino acids
Ura = uracil

The data indicate that recombinants can be generated at reasonable frequencies using Hfr matings. Intergeneric recombination is enhanced 100-200 fold in a recipient that is defective methyl-directed mismatch repair.

Frequencies are further enhanced by increasing the ratio of donor to recipient cells, or by repeatedly mating the original donor strains with the previously generated recombinant progeny.

U. INTRODUCTION OF FRAGMENTS BY CONJUGATION

Sobilizable vectors can also be used to transfer fragment libraries into cells to be evolved. This approach is particularly useful in situations in which the cells to be evolved cannot be efficiently transformed directly with the fragment library but can undergo conjugation with primary cells that can be transformed with the fragment library.

DNA fragments to be introduced into host cells encompasses diversity relative to the host cell genome. The diversity can be the result of natural diversity or mutagenesis. The DNA fragment library is cloned into a mobilizable vector having an origin of transfer. Some such vectors also contain mob genes although alternatively these functions can also be provided in trans. The vector should be capable of efficient conjugal transfer between primary cells and the intended host cells. The vector should also confer a selectable phenotype. This

phenotype can be the same as the phenotype being evolved or can be conferred by a marker, such as a drug resistance marker. The vector should preferably allow self-elimination in the intended host cells thereby allowing selection for cells in which a cloned fragment has undergone genetic exchange with a homologous host segment rather than duplication Such can be achieved by use of vector lacking an origin of replication functional in the intended host type or inclusion of a negative selection marker in the vector.

One suitable vector is the broad host range conjugation plasmid described by Simon et al., *Bio/Technology* 1, 784-791 (1983); TrieuCuot et al., *Gene* 102, 99-104 (1991); Bierman et al., *Gene* 116, 43-49 (1992). These plasmids can be transformed into E. coli and then force-mated into bacteria that are difficult or impossible to transform by chemical or electrical induction of competence. These plasmids contain the origin of the IncP plasmid, *oriT*. Mobilization functions are supplied in trans by chromosomally-integrated copies of the necessary genes. Conjugal transfer of DNA can in some cases be assisted by treatment of the recipient (if gram-positive) with sub-inhibitory concentrations of penicillins (Trieu-Cuot et al., 1993 *FEMS Microbiol. Lett.* 109, 19-23). To increase diversity in populations, recursive conjugal mating prior to screening is performed.

Cells that have undergone allelic exchange with library fragments can be screened or selected for evolution toward a desired phenotype. Subsequent rounds of recombination can be performed by repeating the conjugal transfer step. the library of fragments can be fresh or can be obtained from some (but not all) of the cells surviving a previous round of selection/screening. Conjugation-mediated shuffling can be combined with other methods of shuffling.

V. GENETIC EXCHANGE PROMOTED BY TRANSDUCING PHAGE

Phage transduction can include the transfer, from one cell to another, of nonviral genetic material within a viral coat (Masters, *in Escherichia coli and Salmonella Cellular and Molecular Biology* 2, 2421-2442 (1996). Perhaps the two best examples of generalized transducing phage are bacteriophages P1 and P22 of *E. coli* and *S. typhimurium*, respectively. Generalized transducing bacteriophage particles are formed at a low frequency during lytic infection when viral-genome-sized, doubled-stranded fragments of host (which serves as donor) chromosomal DNA are packaged into phage heads. Promiscuous high transducing (HT) mutants of bacteriophage P22 which efficiently package DNA with little sequence specificity have been isolated. Infection of a susceptible host results in a lysate in which up to 50% of the phage are transducing particles. Adsorption of the generalized

transducing particle to a susceptible recipient cell results in the injection of the donor chromosomal fragment. RecA-mediated homologous recombination following injection of the donor fragment can result in the inheritance of donor traits. Another type of phage which achieves quasi random insertion of DNA into the host chromosome is Mu. For an overview of Mu biology, *see*, Groisman (1991) in Methods in Enzymology v. 204. Mu can generate a variety of chromosomal rearrangements including deletions, inversions, duplications and transpositions. In addition, elements which combine the features of P22 and Mu are available, including Mud-P22, which contains the ends of the Mu genome in place of the P22 *att* site and *int* gene. *See*, Berg, *supra*

Generalized transducing phage can be used to exchange genetic material between a population of cells encompassing genetic diversity and susceptible to infection by the phage. Genetic diversity can be the result of natural variation between cells, induced mutation of cells or the introduction of fragment libraries into cells. DNA is then exchanged between cells by generalized transduction. If the phage does not cause lysis of cells, the entire population of cells can be propagated in the presence of phage. If the phage results in lytic infection, transduction is performed on a split pool basis. That is, the starting population of cells is divided into two. One subpopulation is used to prepare transducing phage. The transducing phage are then infected into the other subpopulation. Preferably, infection is performed at high multiplicity of phage per cell so that few cells remain uninfected. Cells surviving infection are propagated and screened or selected for evolution toward a desired property. The pool of cells surviving screening/selection can then be shuffled by a further round of generalized transduction or by other shuffling methods. Recursive split pool tranduction is optionally performed prior to selection to increase the diversity of any population to me screened.

The efficiency of the above methods can be increased by reducing infection of cells by infectious (nontransducing phage) and by reducing lysogen formation. The former can be achieved by inclusion of chelators of divalent cations, such as citrate and EGTA in culture media. Tail defective transducing phages can be used to allow only a single round of infection. Divalent cations are required for phage absorption and the inclusion of chelating agents therefore provides a means of preventing unwanted infection. Integration defective (*int*) derivatives of generalized transducing phage can be used to prevent lysogen formation. In a further variation, host cells with defects in mismatch repair gene(s) can be used to increase recombination between transduced DNA and genomic DNA.

1 Use of Locked in Prophages to Facilitate DNA Shuffling

The use of a hybrid, mobile genetic element (locked-in prophages) as a means to facilitate whole genome shuffling of organisms using phage transduction as a means to transfer DNA from donor to recipient is a preferred embodiment One such element (Mud-P22) based on the temperate Salmonella phage P22 has been described for use in genetic and physical mapping of mutations. *See,* Youderian et al. (1988) *Genetics* 118:581-592, and Benson and Goldman (1992) *J. Bacteriol.* 174(5):1673-1681 Individual Mud-P22 insertions package specific regions of the Salmonella chromosome into phage P22 particles. Libraries of random Mud-P22 insertions can be readily isolated and induced to create pools of phage particles packaging random chromosomal DNA fragments. These phage particles can be used to infect new cells and transfer the DNA from the host into the recipient in the process of transduction. Alternatively, the packaged chromosomal DNA can be isolated and manipulated further by techniques such as DNA shuffling or any other mutagenesis technique prior to being reintroduced into cells (especially *recD* cells for linear DNA) by transformation or electroporation, where they integrate into the chromosome.

Either the intact transducing phage particles or isolated DNA can be subjected to a variety of mutagens prior to reintroduction into cells to enhance the mutation rate. Mutator cell lines such as *mutD* can also be used for phage growth. Either method can be used recursively in a process to create genes or strains with desired properties. *E. coli* cells carrying a cosmid clone of Salmonella LPS genes are infectable by P22 phage. It is possible to develop similar genetic elements using other combinations of transposable elements and bacteriophages or viruses as well.

P22 is a lambdoid phage that packages its DNA into preassembled phage particles (heads) by a "headful" mechanism. Packaging of phage DNA is initiated at a specific site (*pac*) and proceeds unidirectionally along a linear, double stranded normally concatameric molecule. When the phage head is full (~43 kb), the DNA strand is cleaved, and packaging of the next phage head is initiated. Locked-in or excision-defective P22 prophages, however, initiate packaging at their *pac* site, and then proceed unidirectionally along the chromosome, packaging successive headfuls of chromosomal DNA (rather than phage DNA). When these transducing phages infect new Salmonella cells they inject the chromosomal DNA from the original host into the recipient cell, where it can recombine into the chromosome by homologous recombination creating a chimeric chromosome. Upon infection of recipient cells at a high multiplicity of infection, recombination can also occur between incoming transducing fragments prior to recombination into the chromosome.

Integration of such locked-in P22 prophages at various sites in the chromosome allows flanking regions to be amplified and packaged into phage particles The Mud-P22 mobile genetic element contains an excision-defective P22 prophage flanked by the ends of phage/transposon Mu The entire Mud-P22 element can transpose to virtually any location in the chromosome or other episome (eg. F', BAC clone) when the Mu A and B proteins are provided in *trans*

A number of embodiments for this type of genetic element are available. In one example, the locked in prophage are used as generalized transducing phage to transfer random fragments of a donor chromosome into a recipient. The Mud-P22 element acts as a transposon when Mu A and B transposase proteins are provided in *trans* and integrate copies of itself at random locations in the chromosome. In this way, a library of random chromosomal Mud-P22 insertions can be generated in a suitable host. When the Mud-P22 prophages in this library are induced, random fragments of chromosomal DNA will be packaged into phage particles. When these phages infect recipient cells, the chromosomal DNA is injected and can recombine into the chromosome of the recipient. These recipient cells are screened for a desired property and cells showing improvement are then propagated. The process can be repeated, since the Mud-P22 genetic element is not transferred to the recipient in this process. Infection at a high multiplicity allows for multiple chromosomal fragments to be injected and recombined into the recipient chromosome.

Locked in prophages can also be used as specialized transducing phage. Individual insertions near a gene of interest can be isolated from a random insertion library by a variety of methods. Induction of these specific prophages results in packaging of flanking chromosomal DNA including the gene(s) of interest into phage particles. Infection of recipient cells with these phages and recombination of the packaged DNA into the chromosome creates chimeric genes that can be screened for desired properties. Infection at a high multiplicity of infection can allow recombination between incoming transducing fragments prior to recombination into the chromosome.

These specialized transducing phage can also be used to isolate large quantities of high quality DNA containing specific genes of interest without any prior knowledge of the DNA sequence. Cloning of specific genes is not required. Insertion of such an element nearby a biosynthetic operon for example allows for large amounts of DNA from that operon to be isolated for use in DNA shuffling (*in vitro* and/or *in vivo*), cloning, sequencing, or other uses as set forth herein. DNA isolated from similar insertions in other organisms containing

homologous operons are optionally mixed for use in family shuffling formats as described herein, in which homologous genes from different organisms (or different chromosomal locations within a single species, or both) Alternatively, the transduced population is recursively transduced with pooled transducing phage or new transducing phage generated from the previously transduced cells. This can be carried out recursively to optimize the diversity of the genes prior to shuffling.

Phage isolated from insertions in a variety of strains or organisms containing homologous operons are optionally mixed and used to coinfect cells at a high MOI allowing for recombination between incoming transducing fragments prior to recombination into the chromosome

Locked in prophage are useful for mapping of genes, operons, and/or specific mutations with either desirable or undesirable phenotypes. Locked-in prophages can also provide a means to separate and map multiple mutations in a given host. If one is looking for beneficial mutations outside a gene or operon of interest, then an unmodified gene or operon can be transduced into a mutagenized or shuffled host then screened for the presence of desired secondary mutations. Alternatively, the gene/operon of interest can be readily moved from a mutagenized/shuffled host into a different background to screen/select for modifications in the gene/operon itself.

It is also possible to develop similar genetic elements using other combinations of transposable elements and bacteriophages or viruses as well. Similar systems are set up in other organisms, e.g., that do not allow replication of P22 or P1. Broad host range phages and transposable elements are especially useful. Similar genetic elements are derived from other temperate phages that also package by a headful mechanism. In general, these are the phages that are capable of generalized transduction. Viruses infecting eukaryotic cells may be adapted for similar purposes. Examples of generalized transducing phages that are useful are described in: Green *et al.*, "Isolation and preliminary characterization of lytic and lysogenic phages with wide host range within the streptomycetes", *J. Gen Microbiol* 131(9):2459-2465 (1985); Studdard *et al.*, "Genome structure in Streptomyces spp.: adjacent genes on the *S. coelicolor* A3(2) linkage map have cotransducible analogs in *S. venezuelae*", *J. Bacteriol* 169(8):3814-3816 (1987); Wang *et al.*, "High frequency generalized transduction by miniMu plasmid phage", *Genetics* 116(2):201-206, (1987); Welker, N. E., "Transduction in *Bacillus stearothermophilus*", *J. Bacteriol*, 176(11):3354-3359, (1988); Darzins *et al.*, "Mini-D3112 bacteriophage transposable elements for genetic analysis of *Pseudomonas aeruginosa*, *J.*

Bacteriol 171(7):3909-3916 (1989); Hugouvieux-Cotte-Pattat *et al.*, "Expanded linkage map of *Erwinia chrysanthemi* strain 3937", *Mol Microbiol* 3(5):573-581, (1989), Ichige *et al.*, "Establishment of gene transfer systems for and construction of the genetic map of a marine Vibrio strain", *J. Bacteriol* 171(4):1825-1834 (1989); Muramatsu *et al.*, "Two generalized transducing phages in *Vibrio parahaemolyticus* and *Vibrio alginolyticus*", *Microbiol Immunol* 35(12):1073-1084 (1991); Regue *et al.*, "A generalized transducing bacteriophage for *Serratia marcescens*", *Res Microbiol* 42(1):23-27, (1991); Kiesel *et al.*, "Phage Acm1-mediated transduction in the facultatively methanol-utilizing *Acetobacter methanolicus* MB 58/4", *J. Gen Virol* 74(9):1741-1745 (1993); Blahova *et al.*, "Transduction of imipenem resistance by the phage F-116 from a nosocomial strain of *Pseudomonas aeruginosa* isolated in Slovakia", *Acta Virol* 38(5):247-250 (1994); Kidambi *et al.*, "Evidence for phage-mediated gene transfer among Pseudomonas aeruginosa strains on the phylloplane", *Appl Environ Microbiol* 60:(2)496-500 (1994); Weiss *et al.*, "Isolation and characterization of a generalized transducing phage for *Xanthomonas campestris* pv. *campestris*", *J. Bacteriol* 176(11):3354-3359 (1994); Matsumoto *et al.*, "Clustering of the trp genes in Burkholderia (formerly Pseudomonas) cepacia", *FEMS Microbiol Lett* 134(2-3):265-271 (1995); Schicklmaier *et al.*, "Frequency of generalized transducing phages in natural isolates of the *Salmonella typhimurium* complex", *Appl Environ Microbiol* 61(4): 61(4):1637-1640 (1995); Humphrey *et al.*, "Purification and characterization of VSH-1, a generalized transducing bacteriophage of *Serpulina hyodysenteriae*", *J Bacteriol* 179(2):323-329 (1997); Willi *et al.*, "Transduction of antibiotic resistance markers among *Actinobacillus actinomycetemcomitans* strains by temperate bacteriophages Aa phi 23", *Cell Mol Life Sci* 53(11-12):904-910 (1997); Jensen *et al.*, "Prevalence of broad-host-range lytic bacteriophages of *Sphaerotilus natans, Escherichia coli*, and *Pseudomonas aeruginosa*", *Appl Environ Microbiol* 64(2):575-580 (1998), and Nedelmann *et al.*, "Generalized transduction for genetic linkage analysis and transfer of transposon insertions in different *Staphylococcus epidermidis* strains", *Zentiviralalbl Bakteriol* 287(1-2):85-92 (1998).

A Mud-P1/Tn-P1 system comparable to Mud-P22 is developed using phage P1. Phage P1 has an advantage of packaging much larger (~110 kb) fragments per headful. Phage P1 is currently used to create bacterial artificial chromosomes or BAC's. P1-based BAC vectors are designed along these principles so that cloned DNA is packaged into phage particles, rather than the current system, which requires DNA preparation from single-copy episomes. This combines the advantages of both systems in having the genes cloned in a

stable single-copy format, whilst allowing for amplification and specific packaging of cloned DNA upon induction of the prophage.

W RANDOM PLACEMENT OF GENES OR IMPROVED GENES THROUGHOUT THE GENOME FOR OPTIMIZATION OF GENE CONTEXT

The placement and orientation of genes in a host chromosome (the "context" of the gene in a chromosome) or episome has large effects on gene expression and activity. Random integration of plasmid or other episomal sequences into a host chromosome by non-homologous recombination, followed by selection or screening for the desired phenotype, is a preferred way of identifing optimal chromosomal positions for expression of a target This strategy is illustrated in Fig. 18

A variety of transposon mediated delivery systems can be employed to deliver genes of interest, either individual genes, genomic libraries, or a library of shuffled gene(s) randomly throughout the genome of a host. Thus, in one preferred embodiment, the improvement of a cellular function is achieved by cloning a gene of interest, for example a gene encoding a desired metabolic pathway, within a transposon delivery vehicle.

Such transposon vehicles are available for both Gram-negative and Gram-positive bacteria. De Lorenzo and Timis (1994) Methods in Enzymology 235:385-404 describe the analysis and construction of stable phenotypes in gram-negative Bacteria with Tn5- and Tn 10-derived minitransposons. Kleckner et al. (1991) Methods in Enzymology 204, chapter 7 describe uses of transposons such as Tn10, including for use in gram positive bacteria. Petit *et al.* (1990) *Journal of Bacteriology* 172(12):6736-6740 describe Tn10 derived transposons active in *Bacillus Subtilis*. The transposon delivery vehicle is introduced into a cell population, which is then selected for recombinant cells that have incorporated the transposon into the genome.

The selection is typically by any of a variety of drug resistant markers also carried within the transposon. The selected subpopulation is screened for cells having improved expression of the gene(s) of interest. Once cells harboring the genes of interest in the optimal location are isolated, the genes are amplified from within the genome using PCR, shuffled, and cloned back into a similar transposon delivery vehicle which contains a different selection marker within the transposon and lacks the transposon integrase gene.

This shuffled library is then transformed back into the strain harboring the original transposon, and the cells are selected for the presence of the new resistance marker and the loss of the previous selection marker. Selected cells are enriched for those that have

0 9 JAN 2003

exchanged by homologous recombination the original transposon for the new transposon carrying members of the shuffled library The surviving cells are then screened for further improvements in the expression of the desired phenotype. The genes from the improved cells are then amplified by the PCR and shuffled again This process is carried out recursively, oscillating each cycle between the different selection markers Once the gene(s) of interest are optimized to a desired level, the fragment can be amplified and again randomly distributed throughout the genome as described above to identify the optimal location of the improved genes.

Alternatively, the gene(s) conferring a desired property may not be known. In this case the DNA fragments cloned within the transposon delivery vehicle could be a library of genomic fragments originating from a population of cells derived from one or more strains having the desired property(ies). The library is delivered to a population of cells derived from one or more strains having or lacking the desired property(ies) and cells incorporating the transposon are selected. The surviving cells are then screened for acquisition or improvement of the desired property. The fragments contained within the surviving cells are amplified by PCR and then cloned as a pool into a similar transposon delivery vector harboring a different selection marker from the first delivery vector. This library is then delivered to the pool of surviving cells, and the population having acquired the new selective marker is selected. The selected cells are then screened for further acquisition or improvement of the desired property. In this way the different possible combinations of genes conferring or improving a desired phenotype are explored in a combinatorial fashion. This process is carried out repetitively with each new cycle employing an additional selection marker. Alternatively, PCR fragments are cloned into a pool of transposon vectors having different selective markers. These are delivered to cells and selected for 1, 2, 3, or more markers.

Alternatively, the amplified fragments from each improved cell are shuffled independently. The shuffled libraries are then cloned back into a transposon delivery vehicle similar to the original vector but containing a different selection marker and lacking the transposase gene. Selection is then for acquisition of the new marker and loss of the previous marker. Selected cells are enriched for those incorporating the shuffled variants of the amplified genes by homologous recombination. This process is carried out recursively, oscillating each cycle between the two selective markers.

X. IMPROVEMENT OF OVEREXPRESSED GENES FOR A DESIRED PHENOTYPE

The improvement of a cellular property or phenotype is often enhanced by increasing the copy number or expression of gene(s) participating in the expression of that property. Genes that have such an effect on a desired property can also be improved by DNA shuffling to have a similar effect A genomic DNA library is cloned into an overexpression vector and transformed into a target cell population such that the genomic fragments are highly expressed in cells selected for the presence of the overexpression vector The selected cells are then screened for improvement of a desired property The overexpression vector from the improved cells are isolated and the cloned genomic fragments shuffled. The genomic fragment carried in the vector from each improved isolate is shuffled independently or with identified homologous genes (family shuffling) The shuffled libraries are then delivered back to a population of cells and the selected transformants rescreened for further improvements in the desired property. This shuffling/screening process is cycled recursively until the desired property has been optimized to the desired level.

As stated above, gene dosage can greatly enhance a desired cellular property. One method of increasing gene copy number of unknown genes is using a method of random amplification (*see also*, Mavingui et al. (1997) *Nature Biotech*, 15, 564) In this method, a genomic library is cloned into a suicide vector containing a selective marker that also at higher dosage provides an enhanced phenotype An example of such a marker is the kanamycin resistance gene. At successively higher copy number, resistance to successively higher levels of kanamycin is achieved The genomic library is delivered to a target cell by any of a variety of methods including transformation, transduction, conjugation, etc. Cells that have incorporated the vector into the chromosome by homologous recombination between the vector and chromosomal copies of the cloned genes can be selected by requiring expression of the selection marker under conditions where the vector does not replicate. This recombination event results in the duplication of the cloned DNA fragment in the host chromosome with a copy of the vector and selection marker separating the two copies The population of surviving cells are screened for improvement of a desired cellular property resulting form the gene duplication event Further gene duplication events resulting in additional copies of the original cloned DNA fragments can be generated by further propagating the cells under successively more stringent selective conditions i.e. increased concentrations of kanamycin. In this case selection requires increased copies of the selective marker, but increased copies of the desired gene fragment is also concomitant. Surviving cells are further screened for an

improvement in the desired phenotype The resulting population of cells likely resulted in the amplification of different genes since often many genes effect a given phenotype. To generate a library of the possible combinations of these genes, the original selected library showing phenotypic improvements are recombined, using the methods described herein, e.g., protoplast fusion, split pool transduction, transformation, conjugation, etc

The recombined cells are selected for increased expression of the selective marker. Survivors are enriched for cells having incorporated additional copies of the vector sequence by homologous recombination, and these cells will be enriched for those having combined duplications of different genes. In other words, the duplication from one cell of enhanced phenotype becomes combined with the duplication of another cell of enhanced phenotype. These survivors are screened for further improvements in the desired phenotype This procedure is repeated recursively until the desired level of phenotypic expression is achieved

Alternatively, genes that have been identified or are suspected as being beneficial in increased copy number are cloned in tandem into appropriate plasmid vectors. These vectors are then transformed and propagated in an appropriate host organism. Plasmid-plasmid recombination between the cloned gene fragments result in further duplication of the genes. Resolution of the plasmid doublet can result in the uneven distribution of the gene copies, with some plasmids having additional gene copies and others having fewer gene copies. Cells carrying this distribution of plasmids are then screened for an improvement in the phenotype effected by the gene duplications.

In summary, a method of selecting for increased copy number of a nucleic acid sequence by the above procedure is provided. In the method, a genomic library in a suicide vector comprising a dose-sensitive selectable marker is provided, as noted above. The genomic library is transduced into a population of target cells. The target cells are selected in a population of target cells for increasing doses of the selectable marker under conditions in which the suicide vector does not replicate episomally. A plurality of target cells are selected for the desired phenotype, recombined and reselected. The process is recursively repeated, if desired, until the desired phenotype is obtained.

Y. STRATEGIES FOR IMPROVING GENOMIC SHUFFLING VIA TRANSFORMATION OF LINEAR DNA FRAGMENTS

Wild-type members of the Enterobacteriaceae (e.g., *Escherichia coli*) are typically resistant to genetic exchange following transformation of linear DNA molecules. This is due, at least in part, to the Exonuclease V (Exo V) activity of the RecBCD holoenzyme

which rapidly degrades linear DNA molecules following transformation Production of ExoV has been traced to the *recD* gene, which encodes the D subunit of the holoenzyme. As demonstrated by Russel et al (1989) Journal of Bacteriology 2609-2613, homologous recombination between a transformed linear donor DNA molecule and the chromosome of recipient is readily detected in a strains bearing a loss of function mutation in a *recD* mutant The use of *recD* strains provides a simple means for genomic shuffling of the *Enterobacteriaceae*. For example, a bacterial strain or set of related strains bearing a *recD* null mutation (e.g , the *E. coli recD1903*::mini-Tet allele) is mutagenized and screened for the desired properties. In a split-pool fashion, Chromosomal DNA prepared on one aliquot could be used to transform (e.g., via electroporation or chemically induced competence) the second aliquot. The resulting transformants are then screened for improvement, or recursively transformed prior to screening

The use of RecE/ recT as described *supra*, can improve homologous recombination of linear DNA fragments.

The RecBCD holoezyme plays an important role in initiation of RecA-dependent homologous recombination. Upon recognizing a dsDNA end, the RecBCD enzyme unwinds and degrades the DNA asymmetrically in a 5' to 3' direction until it encounters a chi (or "X")-site (consensus 5'-GCTGGTGG-3') which attenuates the nuclease activity. This results in the generation of a ssDNA terminating near the c site with a 3'-ssDNA tail that is preferred for RecA loading and subsequent invasion of dsDNA for homologous recombination. Accordingly, preprocessing of transforming fragments with a 5' to 3' specific ssDNA Exonuclease, such as Lamda (λ) exonuclease (available, e.g., from Boeringer Mannheim) prior to transformation may serve to stimulate homologous recombination in *recD*⁻ strain by providing ssDNA invasive end for RecA loading and subsequent strand invasion

The addition of DNA sequence encoding chi-sites (consensus 5'-GCTGGTGG-3') to DNA fragments can serve to both attenuate Exonuclease V activity and stimulate homologous recombination, thereby obviating the need for a *recD* mutation (see also, Kowalczykowski, *et al.* (1994) "Biochemistry of homologous recombination in *Escherichia coli*," *Microbiol. Rev.* 58:401-465 and Jessen, *et al.* (1998) "Modification of bacterial artificial chromosomes through Chi-stimulated homologous recombination and its application in zebrafish transgenesis." *Proc. Natl. Acad. Sci.* 95:5121-5126).

Chi sites are optionally included in linkers ligated to the ends of transforming fragments or incorporated into the external primers used to generate DNA fragments to be

transformed The use of recombination-stimulatory sequences such as chi is a generally useful approach for evolution of a broad range of cell types by fragment transformation

Methods to inhibit or mutate analogs of Exo V or other nucleases (such as, Exonucleases I (*endA1*), III (*nth*), IV (*nfo*), VII, and VIII of *E. coli*) is similarly useful. Inhibition or elimination of nucleases, or modification of ends of transforming DNA fragments to render them resistant to exonuclease activity has applications in evolution of a broad range of cell types.

Z SHUFFLING TO OPTIMIZE UNKNOWN INTERACTIONS

Many observed traits are the result of complex interactions of multiple genes or gene products Most such interactions are still uncharacterized Accordingly, it is often unclear which genes need to be optimized to achieve a desired trait, even if some of the genes contributing to the trait are known.

This lack of characterization is not an issue during DNA shuffling, which produces solutions that optimize whatever is selected for An alternative approach, which has the potential to solve not only this problem, but also anticipated future rate limiting factors, is complementation by overexpression of unknown genomic sequences.

A library of genomic DNA is first made as described, *supra*. This is transformed into the cell to be optimized and transformants are screened for increases in a desired property. Genomic fragments which result in an improved property are evolved by DNA shuffling to further increase their beneficial effect. This approach requires no sequence information, nor any knowledge or assumptions about the nature of protein or pathway interactions, or even of what steps are rate -limiting; it relies only on detection of the desired phenotype. This sort of random cloning and subsequent evolution by DNA shuffling of positively interacting genomic sequences is extremely powerful and generic. A variety of sources of genomic DNA are used, from isogenic strains to more distantly related species with potentially desirable properties. In addition, the technique is applicable to any cell for which the molecular biology basics of transformation and cloning vectors are available, and for any property which can be assayed (preferably in a high-throughput format). Alternatively, once optimized, the evolved DNA can be returned to the chromosome by homologous recombination or randomly by phage mediated site-specific recombination.

AA. HOMOLOGOUS RECOMBINATION WITHIN THE CHROMOSOME

Homologous recombination within the chromosome is used to circumvent the limitations of plasmid based evolution and size restrictions. The strategy is similar to that

described above for shuffling genes within their chromosomal context, except that no *in vitro* shuffling occurs Instead, the parent strain is treated with mutagens such as ultraviolet light or nitrosoguanidine, and improved mutants are selected. The improved mutants are pooled and split. Half of the pool is used to generate random genomic fragments for cloning into a homologous recombination vector. Additional genomic fragments are optionally derived from related species with desirable properties. The cloned genomic fragments are homologously recombined into the genomes of the remaining half of the mutant pool, and variants with improved properties are selected These are subjected to a further round of mutagenesis, selection and recombination Again this process is entirely generic for the improvement of any whole cell biocatalyst for which a recombination vector and an assay can be developed. Here again, it should be noted that recombination can be performed recursively prior to screening.

BB METHODS FOR RECURSIVE SEQUENCE RECOMBINATION

Some formats and examples for recursive sequence recombination, sometimes referred to as DNA shuffling or molecular breeding, have been described by the present inventors and co-workers in copending application, attorney docket no. 16528A-014612, filed March 25, 1996, PCT/US95/02126 filed February 17, 1995 (published as WO 95/22625); Stemmer, Science 270, 1510 (1995); Stemmer et al., *Gene,* 164, 49-53 (1995); Stemmer, *Bio/Technology,* 13, 549-553 (1995); Stemmer, *Proc. Natl. Acad. Sci. USA* 91, 10747-10751 (1994); Stemmer, *Nature* 370, 389-391 (1994); Crameri et al., *Nature Medicine,* 2(1):1-3, (1996), and Crameri et al., *Nature Biotechnology* 14, 315-319 (1996) (each of which is incorporated by reference in its entirety for all purposes)

As shown in Figs. 16 and 17, DNA Shuffling provides most rapid technology for evolution of complex new functions. As shown in Fig 16, panel (A), recombination in DNA shuffling achieves accumulation of multiple beneficial mutations in a few cycles. In contrast, because of the high frequency of deleterious mutations relative to beneficial ones, iterative point mutation must build beneficial mutations one at a time, and consequently requires many cycles to reach the same point. As shown in Fig. 16 panel B, rather than a simple linear sequence of mutation accumulation, DNA shuffling is a parallel process where multiple problems may be solved independently, and then combined.

1. *In Vitro* Formats

One format for shuffling *in vitro* is illustrated in Fig. 1. The initial substrates for recombination are a pool of related sequences. The X's in Fig. 1, panel A, show where the sequences diverge The sequences can be DNA or RNA and can be of various lengths

depending on the size of the gene or DNA fragment to be recombined or reassembled Preferably the sequences are from 50 bp to 50 kb

The pool of related substrates are converted into overlapping fragments, e.g., from about 5 bp to 5 kb or more, as shown in Fig 1, panel B Often, the size of the fragments is from about 10 bp to 1000 bp, and sometimes the size of the DNA fragments is from about 100 bp to 500 bp. The conversion can be effected by a number of different methods, such as DNaseI or RNase digestion, random shearing or partial restriction enzyme digestion. Alternatively, the conversion of substrates to fragments can be effected by incomplete PCR amplification of substrates or PCR primed from a single primer Alternatively, appropriate single-stranded fragments can be generated on a nucleic acid synthesizer The concentration of nucleic acid fragments of a particular length and sequence is often less than 0.1 % or 1% by weight of the total nucleic acid. The number of different specific nucleic acid fragments in the mixture is usually at least about 100, 500 or 1000

The mixed population of nucleic acid fragments are converted to at least partially single-stranded form. Conversion can be effected by heating to about 80 °C to 100 °C, more preferably from 90 °C to 96 °C, to form single-stranded nucleic acid fragments and then reannealing. Conversion can also be effected by treatment with single-stranded DNA binding protein or recA protein. Single-stranded nucleic acid fragments having regions of sequence identity with other single-stranded nucleic acid fragments can then be reannealed by cooling to 4°C to 75°C, and preferably from 40 °C to 65 °C Renaturation can be accelerated by the addition of polyethylene glycol (PEG), other volume-excluding reagents or salt. The salt concentration is preferably from 0 mM to 200 mM, more preferably the salt concentration is from 10 mM to 100 mM. The salt may be KCl or NaCl. The concentration of PEG is preferably from 0% to 20%, more preferably from 5% to 10%. The fragments that reanneal can be from different substrates as shown in Fig. 1, panel C. The annealed nucleic acid fragments are incubated in the presence of a nucleic acid polymerase, such as Taq or Klenow, or proofreading polymerases, such as pfu or pwo, and dNTP's (i.e. dATP, dCTP, dGTP and dTTP). If regions of sequence identity are large, Taq polymerase can be used with an annealing temperature of between 45-65°C. If the areas of identity are small, Klenow polymerase can be used with an annealing temperature of between 20-30°C (Stemmer, *Proc. Natl. Acad. Sci. USA* (1994), *supra*). The polymerase can be added to the random nucleic acid fragments prior to annealing, simultaneously with annealing or after annealing.

The process of denaturation, renaturation and incubation in the presence of polymerase of overlapping fragments to generate a collection of polynucleotides containing different permutations of fragments is sometimes referred to as shuffling of the nucleic acid *in vitro*. This cycle is repeated for a desired number of times Preferably the cycle is repeated from 2 to 100 times, more preferably the sequence is repeated from 10 to 40 times The resulting nucleic acids are a family of double-stranded polynucleotides of from about 50 bp to about 100 kb, preferably from 500 bp to 50 kb, as shown in Fig. 1, panel D The population represents variants of the starting substrates showing substantial sequence identity thereto but also diverging at several positions. The population has many more members than the starting substrates. The population of fragments resulting from shuffling is used to transform host cells, optionally after cloning into a vector.

In a variation of *in vitro* shuffling, subsequences of recombination substrates can be generated by amplifying the full-length sequences under conditions which produce a substantial fraction, typically at least 20 percent or more, of incompletely extended amplification products. The amplification products, including the incompletely extended amplification products are denatured and subjected to at least one additional cycle of reannealing and amplification. This variation, in which at least one cycle of reannealing and amplification provides a substantial fraction of incompletely extended products, is termed "stuttering." In the subsequent amplification round, the incompletely extended products reanneal to and prime extension on different sequence-related template species.

In a further variation, a mixture of fragments is spiked with one or more oligonucleotides. The oligonucleotides can be designed to include precharacterized mutations of a wildtype sequence, or sites of natural variations between individuals or species. The oligonucleotides also include sufficient sequence or structural homology flanking such mutations or variations to allow annealing with the wildtype fragments. Some oligonucleotides may be random sequences. Annealing temperatures can be adjusted depending on the length of homology.

In a further variation, recombination occurs in at least one cycle by template switching, such as when a DNA fragment derived from one template primes on the homologous position of a related but different template. Template switching can be induced by addition of recA, rad51, rad55, rad57 or other polymerases (e.g., viral polymerases, reverse transcriptase) to the amplification mixture. Template switching can also be increased by increasing the DNA template concentration.

In a further variation, at least one cycle of amplification can be conducted using a collection of overlapping single-stranded DNA fragments of related sequence, and different lengths. Fragments can be prepared using a single stranded DNA phage, such as M13. Each fragment can hybridize to and prime polynucleotide chain extension of a second fragment from the collection, thus forming sequence-recombined polynucleotides. In a further variation, ssDNA fragments of variable length can be generated from a single primer by Vent or other DNA polymerase on a first DNA template. The single stranded DNA fragments are used as primers for a second, Kunkel-type template, consisting of a uracil-containing circular ssDNA This results in multiple substitutions of the first template into the second *See* Levichkin et al., *Mol. Biology* 29, 572-577 (1995).

2. *In Vivo* Formats

(a). Plasmid-Plasmid Recombination

The initial substrates for recombination are a collection of polynucleotides comprising variant forms of a gene. The variant forms often show substantial sequence identity to each other sufficient to allow homologous recombination between substrates. The diversity between the polynucleotides can be natural (e.g., allelic or species variants), induced (e.g., error-prone PCR), or the result of *in vitro* recombination. Diversity can also result from resynthesizing genes encoding natural proteins with alternative and/or mixed codon usage. There should be at least sufficient diversity between substrates that recombination can generate more diverse products than there are starting materials. There must be at least two substrates differing in at least two positions. However, commonly a library of substrates of 10^3-10^8 members is employed. The degree of diversity depends on the length of the substrate being recombined and the extent of the functional change to be evolved. Diversity at between 0.1-50% of positions is typical. The diverse substrates are incorporated into plasmids. The plasmids are often standard cloning vectors, e.g., bacterial multicopy plasmids. However, in some methods to be described below, the plasmids include mobilization functions. The substrates can be incorporated into the same or different plasmids Often at least two different types of plasmid having different types of selection marker are used to allow selection for cells containing at least two types of vector. Also, where different types of plasmid are employed, the different plasmids can come from two distinct incompatibility groups to allow stable co-existence of two different plasmids within the cell Nevertheless, plasmids from the same incompatibility group can still co-exist within the same cell for sufficient time to allow homologous recombination to occur.

Plasmids containing diverse substrates are initially introduced into prokaryotic or eukaryotic cells by any transfection methods (e.g , chemical transformation, natural competence, electroporation, viral transduction or biolistics) Often, the plasmids are present at or near saturating concentration (with respect to maximum transfection capacity) to increase the probability of more than one plasmid entering the same cell The plasmids containing the various substrates can be transfected simultaneously or in multiple rounds For example, in the latter approach cells can be transfected with a first aliquot of plasmid, transfectants selected and propagated, and then infected with a second aliquot of plasmid.

Having introduced the plasmids into cells, recombination between substrates to generate recombinant genes occurs within cells containing multiple different plasmids merely by propagating in the cells. However, cells that receive only one plasmid are unable to participate in recombination and the potential contribution of substrates on such plasmids to evolution is not fully exploited (although these plasmids may contribute to some extent if they are propagated in mutator cells or otherwise accumulate point mutations (i e., by ultraviolet radiation treatment). The rate of evolution can be increased by allowing all substrates to participate in recombination Such can be achieved by subjecting transfected cells to electroporation. The conditions for electroporation are the same as those conventionally used for introducing exogenous DNA into cells (e.g., 1,000-2,500 volts, 400 μF and a 1-2 mM gap). Under these conditions, plasmids are exchanged between cells allowing all substrates to participate in recombination. In addition the products of recombination can undergo further rounds of recombination with each other or with the original substrate. The rate of evolution can also be increased by use of conjugative transfer. Conjugative transfer systems are known in many bacteria (*E. coli, P. aeruginosa, S. pneumoniae,* and *H. influenzae*) and can also be used to transfer DNA between bacteria and yeast or between bacteria and mammalian cells.

To exploit conjugative transfer, substrates are cloned into plasmids having MOB genes, and *tra* genes are also provided in cis or in trans to the MOB genes. The effect of conjugative transfer is very similar to electroporation in that it allows plasmids to move between cells and allows recombination between any substrate and the products of previous recombination to occur merely by propagating the culture The details of how conjugative transfer is exploited in these vectors are discussed in more detail below. The rate of evolution can also be increased by fusing protoplasts of cells to induce exchange of plasmids or chromosomes. Fusion can be induced by chemical agents, such as PEG, or viruses or viral proteins, such as influenza virus hemagglutinin, HSV-1 gB and gD. The rate of evolution can

also be increased by use of mutator host cells (e.g., Mut L, S, D, T, H and *Ataxia telangiectasia* human cell lines)

Alternatively, plasmids can be propagated together to encourage recombination , then isolated, pooled, and reintroduced into cells The combination of plasmids is different in each cell and recombination further increases the sequence diversity within the population This is optionally carried out recursively until the desired level of diversity is achieved. The population is then screened and selected and this process optionally repeated with any selected cells/plasmids.

The time for which cells are propagated and recombination is allowed to occur, of course, varies with the cell type but is generally not critical, because even a small degree of recombination can substantially increase diversity relative to the starting materials. Cells bearing plasmids containing recombined genes are subject to screening or selection for a desired function. For example, if the substrate being evolved contains a drug resistance gene, one selects for drug resistance Cells surviving screening or selection can be subjected to one or more rounds of screening/selection followed by recombination or can be subjected directly to an additional round of recombination.

The next round of recombination can be achieved by several different formats independently of the previous round For example, a further round of recombination can be effected simply by resuming the electroporation or conjugation-mediated intercellular transfer of plasmids described above. Alternatively, a fresh substrate or substrates, the same or different from previous substrates, can be transfected into cells surviving selection/screening Optionally, the new substrates are included in plasmid vectors bearing a different selective marker and/or from a different incompatibility group than the original plasmids. As a further alternative, cells surviving selection/screening can be subdivided into two subpopulations, and plasmid DNA from one subpopulation transfected into the other, where the substrates from the plasmids from the two subpopulations undergo a further round of recombination. In either of the latter two options, the rate of evolution can be increased by employing DNA extraction, electroporation, conjugation or mutator cells, as described above. In a still further variation, DNA from cells surviving screening/selection can be extracted and subjected to *in vitro* DNA shuffling.

After the second round of recombination, a second round of screening/selection is performed, preferably under conditions of increased stringency. If desired, further rounds of recombination and selection/screening can be performed using the same strategy as for the

second round With successive rounds of recombination and selection/screening, the surviving recombined substrates evolve toward acquisition of a desired phenotype Typically, in this and other methods of recursive recombination, the final product of recombination that has acquired the desired phenotype differs from starting substrates at 0.1%-25% of positions and has evolved at a rate orders of magnitude in excess (e.g., by at least 10-fold, 100-fold, 1000-fold, or 10,000 fold) of the rate of naturally acquired mutation of about 1 mutation per 10^{-9} positions per generation (*see* Anderson & Hughes, *Proc. Natl. Acad. Sci. USA* 93, 906-907 (1996)). As with other techniques herein, recombination steps can be performed recursively to enhance diversity prior to screening. In addition, the entire process can be performed in a recursive manner to generate desired organisms, clones or nucleic acids

3 Virus-Plasmid Recombination

The strategy used for plasmid-plasmid recombination can also be used for virus-plasmid recombination; usually, phage-plasmid recombination However, some additional comments particular to the use of viruses are appropriate. The initial substrates for recombination are cloned into both plasmid and viral vectors It is usually not critical which substrate(s) are inserted into the viral vector and which into the plasmid, although usually the viral vector should contain different substrate(s) from the plasmid. As before, the plasmid (and the virus) typically contains a selective marker. The plasmid and viral vectors can both be introduced into cells by transfection as described above. However, a more efficient procedure is to transform the cells with plasmid, select transformants and infect the transformants with a virus. Because the efficiency of infection of many viruses approaches 100% of cells, most cells transformed and infected by this route contain both a plasmid and virus bearing different substrates.

Homologous recombination occurs between plasmid and virus generating both recombined plasmids and recombined virus. For some viruses, such as filamentous phage, in which intracellular DNA exists in both double-stranded and single-stranded forms, both can participate in recombination Provided that the virus is not one that rapidly kills cells, recombination can be augmented by use of electroporation or conjugation to transfer plasmids between cells. Recombination can also be augmented for some types of virus by allowing the progeny virus from one cell to reinfect other cells. For some types of virus, virus infected-cells show resistance to superinfection. However, such resistance can be overcome by infecting at high multiplicity and/or using mutant strains of the virus in which resistance to

superinfection is reduced. Recursive infection and transformation prior to screening can be performed to enhance diversity

The result of infecting plasmid-containing cells with virus depends on the nature of the virus. Some viruses, such as filamentous phage, stably exist with a plasmid in the cell and also extrude progeny phage from the cell. Other viruses, such as lambda having a cosmid genome, stably exist in a cell like plasmids without producing progeny virions Other viruses, such as the T-phage and lytic lambda, undergo recombination with the plasmid but ultimately kill the host cell and destroy plasmid DNA. For viruses that infect cells without killing the host, cells containing recombinant plasmids and virus can be screened/selected using the same approach as for plasmid-plasmid recombination. Progeny virus extruded by cells surviving selection/screening can also be collected and used as substrates in subsequent rounds of recombination. For viruses that kill their host cells, recombinant genes resulting from recombination reside only in the progeny virus. If the screening or selective assay requires expression of recombinant genes in a cell, the recombinant genes should be transferred from the progeny virus to another vector, e.g., a plasmid vector, and retransfected into cells before selection/screening is performed.

For filamentous phage, the products of recombination are present in both cells surviving recombination and in phage extruded from these cells. The dual source of recombinant products provides some additional options relative to the plasmid-plasmid recombination. For example, DNA can be isolated from phage particles for use in a round of *in vitro* recombination. Alternatively, the progeny phage can be used to transfect or infect cells surviving a previous round of screening/selection, or fresh cells transfected with fresh substrates for recombination.

4. Virus-Virus Recombination

The principles described for plasmid-plasmid and plasmid-viral recombination can be applied to virus-virus recombination with a few modifications. The initial substrates for recombination are cloned into a viral vector. Usually, the same vector is used for all substrates. Preferably, the virus is one that, naturally or as a result of mutation, does not kill cells. After insertion, some viral genomes can be packaged *in vitro*. The packaged viruses are used to infect cells at high multiplicity such that there is a high probability that a cell receives multiple viruses bearing different substrates.

After the initial round of infection, subsequent steps depend on the nature of infection as discussed in the previous section. For example, if the viruses have phagemid

genomes such as lambda cosmids or M13, F1 or Fd phagemids, the phagemids behave as plasmids within the cell and undergo recombination simply by propagating in the cells Recombination and sequence diversity can be enhanced by electroporation of cells Following selection/screening, cosmids containing recombinant genes can be recovered from surviving cells (e.g., by heat induction of a cos⁻ lysogenic host cell), repackaged *in vitro*, and used to infect fresh cells at high multiplicity for a further round of recombination

If the viruses are filamentous phage, recombination of replicating form DNA occurs by propagating the culture of infected cells. Selection/screening identifies colonies of cells containing viral vectors having recombinant genes with improved properties, together with phage extruded from such cells. Subsequent options are essentially the same as for plasmid-viral recombination

5. Chromosome-Plasmid Recombination

This format can be used to evolve both the chromosomal and plasmid-borne substrates The format is particularly useful in situations in which many chromosomal genes contribute to a phenotype or one does not know the exact location of the chromosomal gene(s) to be evolved. The initial substrates for recombination are cloned into a plasmid vector. If the chromosomal gene(s) to be evolved are known, the substrates constitute a family of sequences showing a high degree of sequence identity but some divergence from the chromosomal gene. If the chromosomal genes to be evolved have not been located, the initial substrates usually constitute a library of DNA segments of which only a small number show sequence identity to the gene or gene(s) to be evolved. Divergence between plasmid-borne substrate and the chromosomal gene(s) can be induced by mutagenesis or by obtaining the plasmid-borne substrates from a different species than that of the cells bearing the chromosome.

The plasmids bearing substrates for recombination are transfected into cells having chromosomal gene(s) to be evolved. Evolution can occur simply by propagating the culture, and can be accelerated by transferring plasmids between cells by conjugation, electroporation or protoplast fusion. Evolution can be further accelerated by use of mutator host cells or by seeding a culture of nonmutator host cells being evolved with mutator host cells and inducing intercellular transfer of plasmids by electroporation, conjugation or protoplast fusion. Alternatively, recursive isolation and transformation can be used. Preferably, mutator host cells used for seeding contain a negative selection marker to facilitate isolation of a pure culture of the nonmutator cells being evolved. Selection/screening

identifies cells bearing chromosomes and/or plasmids that have evolved toward acquisition of a desired function.

Subsequent rounds of recombination and selection/screening proceed in similar fashion to those described for plasmid-plasmid recombination. For example, further recombination can be effected by propagating cells surviving recombination in combination with electroporation, conjugative transfer of plasmids, or protoplast fusion. Alternatively, plasmids bearing additional substrates for recombination can be introduced into the surviving cells. Preferably, such plasmids are from a different incompatibility group and bear a different selective marker than the original plasmids to allow selection for cells containing at least two different plasmids. As a further alternative, plasmid and/or chromosomal DNA can be isolated from a subpopulation of surviving cells and transfected into a second subpopulation. Chromosomal DNA can be cloned into a plasmid vector before transfection

6. Virus-Chromosome Recombination

As in the other methods described above, the virus is usually one that does not kill the cells, and is often a phage or phagemid. The procedure is substantially the same as for plasmid-chromosome recombination. Substrates for recombination are cloned into the vector. Vectors including the substrates can then be transfected into cells or *in vitro* packaged and introduced into cells by infection. Viral genomes recombine with host chromosomes merely by propagating a culture. Evolution can be accelerated by allowing intercellular transfer of viral genomes by electroporation, or reinfection of cells by progeny virions Screening/selection identifies cells having chromosomes and/or viral genomes that have evolved toward acquisition of a desired function.

There are several options for subsequent rounds of recombination. For example, viral genomes can be transferred between cells surviving selection/recombination by recursive isolation and transfection and electroporation. Alternatively, viruses extruded from cells surviving selection/screening can be pooled and used to superinfect the cells at high multiplicity. Alternatively, fresh substrates for recombination can be introduced into the cells, either on plasmid or viral vectors.

CC. POOLWISE WHOLE GENOME RECOMBINATION

Asexual evolution is a slow and inefficient process. Populations move as individuals rather than as a group. A diverse population is generated by mutagenesis of a single parent, resulting in a distribution of fit and unfit individuals. In the absence of a sexual cycle, each piece of genetic information for the surviving population remains in the individual

mutants. Selection of the fittest results in many fit individuals being discarded, along with the genetically useful information they carry. Asexual evolution proceeds one genetic event at a time, and is thus limited by the intrinsic value of a single genetic event Sexual evolution moves more quickly and efficiently Mating within a population consolidates genetic information within the population and results in useful information being combined together. The combining of useful genetic information results in progeny that are much more fit than their parents. Sexual evolution thus proceeds much faster by multiple genetic events. These differences are further illustrated in Fig. 17 In contrast to sexual evolution, DNA shuffling is the recursive mutagenesis, recombination, and selection of DNA sequences (*see also*, Fig. 25.).

Sexual recombination in nature effects pairwise recombination and results in progeny that are genetic hybrids of two parents. In contrast, DNA shuffling *in vitro* effects poolwise recombination, in which progeny are hybrids of multiple parental molecules. This is because DNA shuffling effects many individual pairwise recombination events with each thermal cycle. After many cycles the result is a repetitively inbred population, with the "progeny" being the F_X (for X cycles of reassembly) of the original parental molecules. These progeny are potentially descendants of many or all of the original parents. The graph shown in Fig. 25 shows a plot of the potential number of mutations an individual can accumulate by sequential, pairwise and poolwise recombination.

Poolwise recombination is an important feature to DNA shuffling in that it provides a means of generating a greater proportion of the possible combinations of mutations from a single "breeding" experiment. In this way, the "genetic potential" of a population can be readily assessed by screening the progeny of a single DNA shuffling experiment.

For example, if a population consists of 10 single mutant parents, there are 2^{10} = 1024 possible combinations of those mutations ranging from progeny having 0-10 mutations. Of these 1024, only 56 will result from a single pairwise cross (Fig. 14) (i.e those having 0, 1, and 2 mutations). In nature the multiparent combinations will eventually arise after multiple random sexual matings, assuming no selection is imparted to remove some mutations from the population. In this way, sex effects the consolidation and sampling of all useful mutant combinations possible within a population. For the purposes of directed evolution, having the greatest number of mutant combinations entering a screen or selection is desirable so that the best progeny (i.e., according to the selection criteria used in the selection screen) is identified in the shortest possible time.

One challenge to *in vivo* and whole genome shuffling is devising methods for effecting poolwise recombination or multiple repetitive pairwise recombination events. In crosses with a single pairwise cross per cycle before screening, the ability to screen the "genetic potential" of the starting population is limited For this reason, the rate of *in vivo* and whole genome shuffling mediated cellular evolution would be facilitated by effecting poolwise recombination. Two strategies for poolwise recombination are described below (protoplast fusion and transduction)

1 Protoplast Fusion:

Protoplast fusion (discussed *supra*) mediated whole genome shuffling (WGS) is one format that can directly effect poolwise recombination. Whole gene shuffling is the recursive recombination of whole genomes, in the form of one or more nucleic acid molecule(s) (fragments, chromosomes, episomes, etc), from a population of organisms, resulting in the production of new organisms having distributed genetic information from at least two of the starting population of organisms. The process of protoplast fusion is further illustrated in Fig. 26.

Progeny resulting from the fusion of multiple parent protoplasts have been observed (Hopwood & Wright, 1978), however, these progeny are rare (10^{-4}-10^{-6}). The low frequency is attributed to the distribution of fusants arising from two, three, four, etc parents and the likelihood of the multiple recombination events (6 crossovers for a four parent cross) that would have to occur for multiparent progeny to arise. Thus, it is useful to enrich for the multiparent progeny. This can be accomplished, e.g., by repetitive fusion or enrichment for multiply fused protoplasts. The process of poolwise fusion and recombination is further illustrated in Fig. 27.

2. Repetitive Fusion:

Protoplasts of identified parental cells are prepared, fused and regenerated. Protoplasts of the regenerated progeny are then, without screening or enrichment, formed, fused and regenerated. This can be carried out for two, three, or more cycles before screening to increase the representation of multiparent progeny. The number of possible mutations/progeny doubles for each cycle. For example, if one cross produces predominantly progeny with 0, 1, and 2 mutations, a breeding of this population with itself will produce progeny with 0, 1, 2, 3, and 4 mutations (Fig. 15), the third cross up to eight, etc. The representation of the multiparent progeny from these subsequent crosses will not be as high as the single and double parent progeny, but it will be detectable and much higher than from a

single cross. The repetitive fusion prior to screening is analogous to many sexual crosses within a population, and the individual thermal cycles of *in vitro* DNA shuffling described *supra*. A factor effecting the value of this approach is the starting size of the parental population. As the population grows, it becomes more likely that a multiparent fusion will arise from repetitive fusions. For example, if 4 parents are fused twice, the 4 parent progeny will make up approximately 0.2% of the total progeny This is sufficient to find in a population of 3000 (95% confidence), but better representation is preferable. If ten parents are fused twice >20% of the progeny will be four parent offspring.

3. Enrichment for multiply fused protoplasts:

After the fusion of a population of protoplasts, the fusants are typically diluted into hypotonic medium, to dilute out the fusing agent (e.g., 50% PEG). The fused cells can be grown for a short period to regenerate cell walls or separated directly and are then separated on the basis of size This is carried out, e g., by cell sorting, using light dispersion as an estimate of size, to isolate the largest fusants. Alternatively the fusants can be sorted by FACS on the basis of DNA content. The large fusants or those containing more DNA result from the fusion of multiple parents and are more likely to segregate to multiparent progeny. The enriched fusants are regenerated and screened directly or the progeny are fused recursively as above to further enrich the population for diverse mutant combinations.

4. Transduction:

Transduction can theoretically effect poolwise recombination, if the transducing phage particles contain predominantly host genomic DNA rather than phage DNA. If phage DNA is overly represented, then most cells will receive at least one undesired phage genome. Phage particles generated from locked-in-prophage (*supra*) are useful for this purpose. A population of cells is infected with an appropriate transducing phage, and the lysate is collected and used to infect the same starting population. A high multiplicity of infection is employed to deliver multiple genomic fragments to each infected cell, thereby increasing the chance of producing recombinants containing mutations from more than two parent genomes. The resulting transductants are recovered under conditions where phage can not propagate e.g., in the presence of citrate. This population is then screened directly or infected again with phage, with the resulting transducing particles being used to transduce the first progeny. This would mimic recursive protoplast fusion, multiple sexual recombination, and *in vitro* DNA shuffling.

DD. METHODS FOR WHOLE GENOME SHUFFLING BY BLIND FAMILY SHUFFLING OF PARSED GENOMES AND RECURSIVE CYCLES OF FORCED INTEGRATION AND EXCISION BY HOMOLOGOUS RECOMBINATION, AND SCREENING FOR IMPROVED PHENOTYPES.

In vitro methods have been developed to shuffle single genes and operons, as set forth, e.g., herein. "Family" shuffling of homologous genes within species and from different species is also an effective methods for accelerating molecular evolution This section describes additional methods for extending these methods such that they can be applied to whole genomes.

In some cases, the genes that encode rate limiting steps in a biochemical process, or that contribute to a phenotype of interest are known. This method can be used to target family shuffled libraries to such loci, generating libraries of organisms with high quality family shuffled libraries of alleles at the locus of interest. An example of such a gene would be the evolution of a host chaperonin to more efficiently chaperone the folding of an overexpressed protein in E. coli.

The goals of this process are to shuffle homologous genes from two or more species and to then integrate the shuffled genes into the chromosome of a target organism Integration of multiple shuffled genes at multiple loci can be achieved using recursive cycles of integration (generating duplications), excision (leaving the improved allele in the chromosome) and transfer of additional evolved genes by serially applying the same procedure.

In the first step, genes to be shuffled into suitable bacterial vectors are subcloned. These vectors can be plasmids, cosmids, BACS or the like. Thus, fragments from 100 bp to 100 kb can be handled. Homologous fragments are then "family shuffled" together (i.e. homologous fragments from different species or chromosomal locations are homologously recombined). As a simple case, homologs from two species (say, E. coli and Salmonella) are cloned, family shuffled in vitro and cloned into an allele replacement vector (e.g., a vector with a positively selectable marker, a negatively selectable marker and conditionally active origin of replication). The basic strategy for whole genome family shuffling of parsed (subcloned) genomes is additionally set forth in Fig. 22.

The vectors are transfected into E. coli and selected, e.g., for drug resistance. Most drug resistant cells should arise by homologous recombination between a family shuffled insert and a chromosomal copy of the cloned insert. Colonies with improved phenotype are screened (e.g., by mass spectroscopy for enzyme activity or small molecule production, or a chromogenic screen, or the like, depending on the phenotype to be assayed). Negative

selection (i.e. suc selection) is imposed to force excision of tandem duplication. Roughly half of the colonies should retain the improved phenotype. Importantly, this process regenerates a 'clean' chromosome in which the wild type locus is replaced with a family shuffled fragment that encodes a beneficial allele. Since the chromosome is "clean" (i.e., has no vector sequences), other improved alleles can also be moved into this point on the chromosome by homologous recombination.

Selection or screening for improved phenotype can occur either after step 3 or step 4 in Figure 22. If selection or screening takes place after step 3, then the improved allele can be conveniently moved to other strains by, for example, P1 transduction. One can then regenerate a strain containing the improved allele but lacking vector sequences by "negative selection" against the suc marker. In subsequent rounds, independently identified improved variants of the gene can be sequentially moved into the improved strain (e.g., by P1 transduction of the drug marked tandem duplication above). Transductants are screened for further improvement in phenotype by virtue of receiving the transduced tandem duplication, which itself contains the family shuffled genetic material. Negative selection is again imposed and the process of shuffling the improved strain is recursively repeated as desired.

Although this process was described with reference to targeting a gene or genes of interest, it can be used "blindly," making no assumptions about which locus is to be targeted. This procedure is set forth in Fig. 23. For example, the whole genome of an organism of interest is cloned into manageable fragments (e.g., 10 kb for plasmid-based methods). Homologous fragments are then isolated from related species by the method shown in Figure 23. Forced recombination with chromosomal homologs creates chimeras (Fig. 22).

EE. METHODS FOR HIGH THROUGHPUT FAMILY SHUFFLING OF GENES

For *E. coli*., cloning the genome in 10 kb fragments requires about 300 clones. The homologous fragments are isolated, e.g., from *Salmonella*. This gives roughly three hundred pairs of homologous fragments. Each pair is family shuffled and the shuffled fragments are cloned into an allele replacement vector. The inserts are integrated into the *E. coli* genome as described above. A global screen is made to identify variants with an improved phenotype. This serves as the basis collection of improvements that are to be shuffled to produce a desired strain. The shuffling of these independently identified variants into one super strain is done as described above

Family shuffling has been shown to be an efficient method for creating high quality libraries of genetic variants. Given a cloned gene from one species, it is of interest to

quickly and rapidly isolate homologs from other species, and this process can be rate limiting For example, if one wants to perform family shuffling on an entire genome, one may need to construct hundreds to thousands of individual family shuffled libraries.

In this embodiment, a gene of interest is optionally cloned into a vector in which ssDNA can be made. An example of such a vector is a phagemid vector with an M13 origin of replication. Genomic DNA or cDNA from a species of interest is isolated, denatured, annealed to the phagemid, and then enzymatically manipulated to clone it The cloned DNA is then used to family shuffle with the original gene of interest. PCR based formats are also available as outlined in Figure 24. These formats require no intermediate cloning steps, and are, therefore, of particular interest for high throughput applications.

Alternatively, the gene of interest can be fished out using purified RecA protein. The gene of interest is PCR amplified using primers that are tagged with an affinity tag such as biotin, denatured, then coated with RecA protein (or an improved variant thereof). The coated ssDNA is then mixed with a gDNA plasmid library. Under the appropriate conditions, such as in the presence of non-hydrolyzable rATP analogs, RecA will catalyze the hybridization of the RecA coated gene (ssDNA) in the plasmid library. The heteroduplex is then affinity purified from the non-hybridizing plasmids of the gene library by adsorbtion of the labeled PCR products and its associated homologous DNA to an appropriate affinity matrix. The homologous DNA is used in a family shuffling reaction for improvement of the desired function.

Shuffling the *E. coli* chaperonin gene *DnaJ* with other homologs is described below as an example. The example can be generalized to any other gene, including eukaryotic genes such as plant or animal genes (including mammalian genes), by following the format described. Fig. 24 provides a schematic outline of the steps to high throughput family shuffling.

As a first step, the E. coli *DnaJ* gene is cloned into an M13 phagemid vector. ssDNA is then produced, preferably in a dut(-) ung(-) strain so that Kunkel site directed mutagenesis protocols can be applied. Genomic DNA is then isolated from a non- *E. coil* source, such as *Salmonella* and *Yersinia Pestis*. The bacterial genomic DNAs are denatured and reannealed to the phagemid ssDNA (e.g., about 1 microgram of ssDNA). The reannealed product is treated with an enzyme such as Mung Bean nuclease that degrades ssDNA as an exonuclease but not as an endonuclease (the nuclease does not degrade mismatched DNA that is embedded in a larger annealed fragment). The standard Kunkel site directed mutagenesis

protocol is used to extend the fragment and the target cells are transformed with the resulting mutagenized DNA.

In a first variation on the above, the procedure is adapted to the situation where the target gene or genes of interest are unknown In this variation, the whole genome of the organism of interest is cloned in fragments (e.g., of about 10 kb each) into a phagemid. Single stranded phagemid DNA is then produced. Genomic DNA from the related species is denatured and annealed to the phagemids. Mung bean nuclease is used to trim away unhybridized DNA ends. Polymerase plus ligase is used to fill in the resulting gapped circles. These clones are transformed into a mismatch repair deficient strain. When the mismatched molecules are replicated in the bacteria, most colonies contain both the E. coli and the homologous fragment. The two homologous genes are then isolated from the colonies (e.g., either by standard plasmid purification or colony PCR) and shuffled.

Another approach to generating chimeras that requires no in vitro shuffling is simply to clone the *Salmonella* genome into an allele replacement vector, transform *E. coli*, and select for chromosomal integrants. Homologous recombination between Salmonella genes and E. coli homologs generate shuffled chimeras. A global screen is done to screen for improved phenotypes. Alternately, recursive transformation and recombination is performed to increase diversity prior to screening. If colonies with improved phenotypes are obtained, it is verified that the improvement is due to allele replacement by P1 transduction into a fresh strain and counterscreening for improved phenotype. A collection of such improved alleles can then be combined into one strain using the methods for whole genome shuffling by blind family shuffling of parsed genomes as set forth herein. Additionally, once these loci are identified, it is likely that further rounds of shuffling and screening will yield further improvements. This could be done by cloning the chimeric gene and then using the methods described in this disclosure to breed the gene with homologs from many different strains of bacteria.

In general, the transformants contain clones of the homologue of the target gene (e.g., E. coli DnaJ in the example above). Mismatch repair in vivo results in a decrease in diversity of the gene. There are at least two solutions to this. First, transduction can be performed into a mismatch repair deficient strain. Alternatively or in addition, the M13 template DNA can be selectively degraded, leaving the cloned homologue. This can be done using methods similar to the standard Eckstein site directed mutagenesis technique (General texts which describe general molecular biological techniques useful herein, including mutagenesis, include Sambrook et al., Molecular Cloning - A Laboratory Manual (2nd Ed.),

Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook") and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 1998) ("Ausubel")).

This method relies on incorporation of alpha thiol modified dNTP's during synthesis of the new strand followed by selective degradation of the template and resynthesis of the template strand. In one embodiment, the template strand is grown in a dut(-) ung(-) strain so that uracil is incorporated into the phagemid DNA. After extension as noted above (and before transformation) the DNA is treated with uracil glycosylate and an apurinic site endonuclease such as Endo III or Endo IV. The treated DNA is then treated with a processive exonuclease that resects from the resulting gaps while leaving the other strand intact (as in Eckstein mutagenesis). The DNA is polymerized and ligated. Target cells are then transformed. This process enriches for clones encoding the homologue which is not derived from the target (i.e., in the example above, the non- *E. coli*. homologue).

An analogous procedure is optionally performed in a PCR format. As applied to the DnaJ illustration above, DnaJ DNA is amplified by PCR with primers that build 30-mer priming sites on each end. The PCR is denatured and annealed with an excess of *Salmonella* genomic DNA. The *Salmonella* DnaJ gene hybribidizes with the *E. coli* homologue. After treatment with Mung Bean nuclease, the resulting mismatched hybrid is PCR amplified with the flanking 30-mer primers. This PCR product can be used directly for family shuffling. See, e.g., Fig. 24.

As genomics provides an increasing amount of sequence information, it is increasingly possible to directly PCR amplify homologs with designed primers. For example, given the sequence of the *E. coli* genome and of a related genome (i.e. *Salmonella*), each genome can be PCR amplified with designed primers in, e.g., 5 kb fragments. The homologous fragments can be put together in a pairwise fashion for shuffling. For genome shuffling, the shuffled products are cloned into the allele replacement vector and bred into the genome as described *supra*.

FF. HYPER-RECOMBINOGENIC RECA CLONES

The invention further provides hyper-recombinogenic RecA proteins (*see*, the examples below). Examples of such proteins are from clones 2, 4, 5, 6 and 13 shown in Fig. 13. It is fully expected that one of skill can make a variety of related recombinogenic proteins given the disclosed sequences.

Clones comprising the sequences in Figs. 12 and 13 are optionally used as the starting point for any of the shuffling methods herein, providing a starting point for mutation and recombination to improve the clones which are shown.

Standard molecular biological techniques can be used to make nucleic acids which comprise the given nucleic acids, e.g., by cloning the nucleic acids into any known vector. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds , Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

It will be appreciated that conservative substitutions of the given sequences can be used to produce nucleic acids which encode hyperrecombinogenic clones. "Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation. One of skill will

recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in any described sequence. Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). *See also,* Creighton (1984) *Proteins* W.H. Freeman and Company Finally, the addition of sequences which do not alter the encoded activity of a nucleic acid molecule, such as a non-functional sequence is a conservative modification of the basic nucleic acid.

One of skill will appreciate that many conservative variations of the nucleic acid constructs disclosed yield a functionally identical construct. For example, due to the degeneracy of the genetic code, "silent substitutions" (*i.e.,* substitutions of a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of *every* nucleic acid sequence which encodes an amino acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence of a packaging or packageable construct are substituted with different amino acids with highly similar properties, are also readily identified as being highly similar to a disclosed construct. Such conservatively substituted variations of each explicitly disclosed sequence are a feature of the present invention.

Nucleic acids which hybridize under stringent conditions to the nucleic acids in the figures are a feature of the invention. "Stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe

assays", Elsevier, New York Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and ph. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe Very stringent conditions are selected to be equal to the T_m for a particular probe. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

Finally, preferred nucleic acids encode hyper-recombinogenic RecA proteins which are at least one order of magnitude (10 times) as active as a wild-type RecA protein in a standard assay for Rec A activity.

GG. recE / recT MEDIATED SHUFFLING *IN VIVO*

Like recA, recE and recT (or their homologues, for example the lambda recombination proteins redα and redβ) can stimulate homologous recombination *in vivo*. *See,* Muyrers et al. (1999) *Nucleic Acids Res* 27(6):1555-7 and Zhang et al. (1998) *Nat Genet* 20(2):123-8

Hyper-recombinogenic recE and recT are evolved by the same method as described for recA. Alternatively, variants with increased recombinogenicity are selected by their ability to cause recombination between a suicide vector (lacking an origin of replication) carrying a selectable marker, and a homologous region in either the chromosome or a stably-maintained episome.

A plasmid containing recA and recE genes is shuffled (either using these genes as single starting points, or by family shuffling (with for example redα and redβ, or other homologous genes identified from available sequence databases). This shuffled library is then cloned into a vector with a selectable marker and transformed into an appropriate recombination-deficient strain. The library of cells would then be transformed with a second selectable marker, either borne on a suicide vector or as a linear DNA fragment with regions at its ends that are homologous to a target sequence (either in the plasmid or in the host chromosome). Integration of this marker by homologous recombination is a selectable event, dependent on the activity of the recE and recT gene products. The recE / recT genes are

isolated from cells in which homologous recombination has occurred The process is repeated several times to enrich for the most efficient variants before the next round of shuffling is performed In addition, cycles of recombination without selection can be performed to increase the diversity of a cell population prior to selection

Once hyper-recombinogenic recE / recT genes are isolated they are used as described for hyper-recombinogenic recA. For example they are expressed (constitutively or conditionally) in a host cell to facilitate homologous recombination between variant gene fragments and homologues within the host cell. They are alternatively introduced by microinjection, biolistics, lipofection or other means into a host cell at the same time as the variant genes.

Hyper-recombinogenic recE/ recT (either of bacterial / phage origin, or from plant homologues) are useful for facilitating homologous recombination in plants. They are, for example, cloned into the Agrobacterium cloning vector, where they are expressed upon entry into the plant, thereby stimulating homologous recombination in the recipient cell

In a preferred embodiment, recE/ recT are used and or generated in mutS strains.

HH. MULTI-CYCLIC RECOMBINATION

As noted, protoplast fusion is an efficient means of recombining two microbial genomes. The process reproducibly results in about 10% of a non-selected population being recombinant chimeric organisms.

Protoplasts are cells that have been stripped of their cell walls by treatment in hypotonic medium with cell wall degrading enzymes. Protoplast fusion is the induced fusion of the membranes of two or more of these protoplasts by fusogenic agents such as polyethylene glycol. Fusion results in cytoplasmic mixing and places the genomes of the fused cells within the same membrane. Under these conditions recombination between the genomes is frequent.

The fused protoplasts are regenerated, and, during cell division, single genomes segregate into each daughter cell. Typically, 10% of these daughter cells have genomes that originate partially from more than one of the original parental protoplast genomes.

This result is similar to that of the crossing over of sister chromatids in eukaryotic cells during prophase of meiosis II. The percentage of daughter cells that are recombinant is just lower after protoplast fusion. While protoplast fusion does result in

efficient recombination, the recombination predominantly occurs between two cells as in sexual recombination

In order to efficiently generate libraries of whole genome shuffled libraries, daughter cells having genetic information originating from multiple parents are made

In vitro DNA shuffling results in the efficient poolwise recombination of multiple homologous DNA sequences The reassembly of full length genes from a mixed pool of small gene fragments requires multiple annealing and elongation cycles, the thermal cycles of the primerless PCR reaction. During each thermal cycle, many pairs of fragments anneal and are extended to form a combinatorial population of larger chimeric DNA fragments. After the first cycle of reassembly, chimeric fragments contain sequences originating from two different parent genes. This is similar to the result of a single sexual cycle within a population, pairwise cross, or protoplast fusion. During the second cycle, these chimeric fragments can anneal with each other, or with other small fragments, resulting in chimeras originating from up to four different parental sequences.

This second cycle is analogous to the entire progeny from a single sexual cross inbreeding with itself. Further cycles will result in chimeras originating from 8, 16, 32, etc parental sequences and are analogous to further inbreedings of the progeny population. The power of *in vitro* DNA shuffling is that a large combinatorial library can be generated from a single pool of DNA fragments reassembled by these recursive pairwise "matings." As described above, *in vivo* shuffling strategies, such as protoplast fusion, result in a single pairwise mating reaction. Thus, to generate the level of diversity obtained by *in vitro* methods, *in vivo* methods are carried out recursively. That is, a pool of organisms is recombined and the progeny pooled, without selection, and then recombined again. This process is repeated for sufficient cycles to result in progeny having multiple parental sequences.

Described below is a method used to shuffle four strains of *Streptomyces coelicolor*. From the initial four strains each containing a unique nutritional marker, three to four rounds of recursive pooled protoplast fusion was sufficient to generate a population of shuffled organisms containing all 16 possible combinations of the four markers. This represents a 10^6 fold improvement in the generation of four parent progeny as compared to a single pooled fusion of the four strains.

As set forth in Figure 31, protoplasts were generated from several strains of *S. coelicolor*, pooled and fused. Mycelia were regenerated and allowed to sporulate. The spores

were collected, allowed to grow into Mycelia, formed into protoplasts, pooled and fused and the process repeated for three to four rounds. the resulting spores were then subject to screening.

The basic protocol for generating a whole genome shuffled library from four *S. coelicolor* strains, each having one of four distinct markers, was as follows Four mycelial cultures, each of a strain having one of four different markers, were grown to early stationary phase. The mycelia from each were harvested by centrifugation and washed. Protoplasts from each culture were prepared as follows.

Approximately 10^9 *S. coelicolor* spores were inoculated into 50ml YEME with .0.5% Glycine in a 250ml baffled flask The spores were incubated at 30°C for 36-40 hours in an orbital shaker. Mycelium were verified using a microscope. Some strains needed an additional day of growth. The culture was transferred into a 50ml tube and centrifuged at 4,000 rpm for 10 min. The mycelium were twice washed with 10.3% sucrose and centrifuged at 4,000 rpm for 10 min. (mycelium can be stored at -80°C after wash). 5ml of lysozyme was added to the ~0.5g of mycelium pellet. The pellet was suspended and incubated at 30°C for 20-60 min., with gentle shaking every 10 min. The microscope was checked for protoplasting every 20 min. Once the majority were protoplasts, protoplasting was stopped by adding 10ml of P buffer. The protoplasts were filtered through cotton and the protoplast spun down at 3,000rpm for 7 min at room temperature. The supernatant was discarded and the protoplast gently resuspended, adding a suitable amount of P buffer according to the pellet size (usually about 500µl). Ten-fold serial dilutions were made in P buffer, and the protoplasts counted at a 10^{-2} dilution. Protoplasts were adjusted to 10^{10} protoplasts per ml.

The protoplasts from each culture were quantitated by microscopy. 10^8 protoplast from each culture were mixed in the same tube, washed, and then fused by the addition of 50% PEG. The fused protoplasts were diluted and plated regeneration medium and incubated until the colonies were sporulating (four days). Spores were harvested and washed. These spores represent a pool of all the recombinants and parents form the fusion. A sample of the pooled spores was then used to inoculate a single liquid culture. The culture was grown to early stationary phase, the myclelia harvested, and protoplasts prepared. 10^8 protoplasts from this "mycelial library" were then fused with themselves by the addition of 50%PEG. The protoplast fusion/regeneration/harvesting/protoplast preparation steps were repeated two times. The spores resulting from the fourth round of fusion were considered the "whole genome shuffled library" and they were screened for the frequency of the 16 possible

combinations of the four markers The results from each round of fusion are shown figure 33 and in the following table

The results of the shuffling procedure are set forth in Figure 33 In particular, adding rounds of recombination prior to selection produced significant increases in the number of clones which incorporated all four of the relevant selectable markers, indicating that the population became increasingly diverse be recursive pooling and sporulation Additional results are set forth in the following table.

Table: Fusion #1, 1st, 2nd, 3rd, and 4th round

	1st round fusion			2nd round fusion			3rd round fusion			4th round fusion		
MM+	Percent	% w/o background	Total %	Percent	% w/o background	Total %	Percent	% w/o background	Total %	Percent	% w/o background	Total %
MM	0	0	0	0.016	0.016	0.016	0.295	0.295	0.295	1.42	1.42	1.42
A	0.024	0.024		0.72	0.704		1	0.705		1.68	0.26	
C	0.12	0.12		2.2	2.184		4.38	4.085		7.01	5.59	
P	0.21	0.21	0.386	2.3	2.284	5.446	3.4	3.105	8.1	7.77	6.35	12.03
U	0.032	0.032		0.29	0.274		0.5	0.205		1.25	-0.17	
AC	0.85	0.706		4.7	1.796		8.9	3.815		15.3	8.03	
AP	0.8	0.566		7.4	4.396		6.6	2.495		7.92	-0.11	
AU	0.7	0.644		2.5	1.506		1.75	0.545		1.73	0.22	
CP	5	4.67	11.492	17.7	13.216	25.066	48	40.515	54.28	76.5	63.14	73.82
CU	2.5	2.348		6	3.526		9.4	4.815		11.28	4.44	
PU	2.8	2.558		3.2	0.626		5.7	2.095		5.7	-1.9	
ACP	6	-0.296		37	12.404		53	-2.015		71.3	-13.38	
ACU	32	28.126		17.7	7.694		17.7	3.235		15.57	-4.22	
APU	12	7.966	103.858	9.2	-0.606	27.366	8.1	-1.345	17.76	7.77	1.7	-14.07
CPU	78	68.062		30	7.874		73	17.885		80.7	1.83	
ACPU	100	100	115.736	100	100	57.894	100	100	80.435	100	100	73.2

The four strains of the four parent shuffling were each auxotrophic for three and prototrophic for one of four possible nutritional markers: arginine (A), cystine (C), proling (P), and/or uracil (U) Spores from each fusion were plated in each of the 16 possible combinations of these four nutrients, and the percent of the population growing on a particulate medium was calculated as the ration of those colonies form a selective plate to those growing on a plate having all four nutrients (all variants grow on the medium having all four nutrients, thus the colonies from this plate tus represent the total viable population) The corrected percentages for each of the no, one, two, and three marker phenotypes were determined by subtracting the percentage of cells having additional markers that might grow on the medium having "unnecessary" nutrients. For example, the number of colonies growing on no additional nutrients (the prototroph) was subtracted from the number of colonies growing on any plate requiring nutrients.

II. WHOLE GENOME SHUFFLING THROUGH ORGANIZED HETERODUPLEX SHUFFLING

A new procedure to optimize phenotypes of interests by heteroduplex shuffling of cosmids libraries of the organism of choice, is provided. This procedure does not require protoplast fusion and is applicable to bacteria for which well-established genetic systems are available, including cosmid cloning, transformation, in vitro packaging/transfection and plasmid transfer/mobilization. Microorganism that can be improved by these methods include *Escherichia coli, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas spp., Rhizobium spp., Xanthomonas spp.*, and other gram-negative organisms This method is also applicable to Gram-positive microorganisms.

A basic procedure for whole genome shuffling through organized heteroduplex shuffling is set forth in Figure 34.

In step A, Chromosomal DNA of the organism to be improved is digested with suitable restriction enzymes and ligated into a cosmid. The cosmid used for cosmid-based heteroduplex guided WGS has at least two rare restriction enzyme recognition sites (e.g. Sfr and NotI) to be used for linearization in subsequent steps. Sufficient cosmids to represent the complete chromosome are purified and stored in 96-well microtiter dishes. In step B, small samples of the library are mutagenized in vitro using hydroxylamine or other mutagenic chemicals. In step C, a sample from each well of the mutagenized collection is used to transfect the target cells. In step D, the transfectants are assayed (as a pool from each mutagenized sample-well) for phenotypic improvements. Positives from this assay indicate that a cosmid from a particular well can confer phenotypic improvements and thus contain

large genomic fragments that are suitable targets for heteroduplex mediated shuffling In step E, the transfected cells harboring a mutant library of the identified cosmid(s) are separated by plating on solid media and screened for independent mutants conferring an improved phenotype. In step F, DNA from positive cells is isolated and pooled by origin. In step G, the selected cosmid pools are divided so that one sample can be digested with Sfr and the other with NotI These samples are pooled, denatured, reannealed, and religated.

In step H, target cells are transfected with the resulting heteroduplexes and propagated to allow "recombination" to occur between the strands of the heteroduplexes in vivo. The transfectants can be screened (the population will represent the pairwise recombinants) or, commonly, as represented by step I, the recombined cosmids are further shuffled by recursive in vitro heteroduplex formation and in vivo recombination (to generate a complete combinatorial library of the possible mutations) prior to screening. An additional mutagenesis step could also be added for increased diversity during the shuffling process

In step J, once several cosmids harboring different distributed loci have been improved, they are combined into the same host by chromosome integration. This organism can be used directly or subjected to a new round of heteroduplex guided whole genome shuffling.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention. Essentially equivalent variations upon the exact procedures set forth will be apparent to one of skill upon review of the present disclosure.

A. EXAMPLE 1: EVOLVING HYPER-RECOMBINOGENIC RECA

RecA protein is implicated in most *E. coli* homologous recombination pathways. Most mutations in recA inhibit recombination, but some have been reported to increase recombination (Kowalczykowski et al., *Microbiol. Rev.*, 58, 401-465 (1994)). The following example describes evolution of RecA to acquire hyper-recombinogenic activity useful in *in* vivo shuffling formats.

Hyperrecombinogenic RecA was selected using a modification of a system developed by Shen et al., *Genetics* 112, 441-457 (1986); Shen et al., *Mol. Gen. Genet.* 218, 358-360 (1989)) to measure the effect of substrate length and homology on recombination frequency. Shen & Huang's system used plasmids and bacteriophages with small (31-430 bp) regions of homology at which the two could recombine. In a restrictive host, only phage that had incorporated the plasmid sequence were able to form plaques.

For shuffling of recA, endogenous recA and mutS were deleted from host strain MC1061 In this strain, no recombination was seen between plasmid and phage *E. coli* recA was then cloned into two of the recombination vectors (Bp221 and πMT631c18) Plasmids containing cloned RecA were able to recombine with homologous phage:λV3 (430 bp identity with Bp221),λV13 (430 bp stretch of 89% identity with Bp221) and λlink H (31bp identity with πMt631c18, except for 1 mismatch at position 18).

The cloned RecA was then shuffled *in vitro* using the standard DNase-treatment followed by PCR-based reassembly. Shuffled plasmids were transformed into the non-recombining host strain These cells were grown up overnight, infected with phage λVc, λV13 or λlink H, and plated onto NZCYM plates in the presence of a 10-fold excess of MC1061 lacking plasmid The more efficiently a recA allele promotes recombination between plasmid and phage, the more highly the allele is represented in the bacteriophage DNA. Consequently, harvesting all the phage from the plates and recovering the recA genes selects for the most recombinogenic recA alleles.

Recombination frequencies for wild type and a pool of hyper-recombinogenic RecA after 3 rounds of shuffling were as follows:

Cross	Wild Type	Hyper Recom
BP221 x V3	6.5×10^{-4}	3.3×10^{-2}
BP221 x V13	2.2×10^{-5}	1.0×10^{-3}
πMT631c18 x link H	8.7×10^{-6}	4.7×10^{-5}

These results indicate a 50-fold increase in recombination for the 430 bp substrate, and a 5-fold increase for the 31 bp substrate.

The recombination frequency between BP221 and V3 for five individual clonal isolates are shown below, and the DNA and protein sequences and alignments thereof are included in Figs. 12 and 13.

Wildtype: 1.6×10^{-4}

Clone 2: 9.8×10^{-3} (61 x increase)

Clone 4: 9.9×10^{-3} (62 x increase)

Clone 5: 6.2×10^{-3} (39 x increase)

Clone 6: 8.5×10^{-3} (53 x increase)

Clone 13: 0.019 (116 x increase)

Clones 2, 4, 5, 6 and 13 can be used as the substrates in subsequent rounds of shuffling, if further improvement in recA is desired. Not all of the variations from the wildtype recA

sequence necessarily contribute to the hyperrecombinogenic phenotype Silent variations can be eliminated by backcrossing. Alternatively, variants of recA incorporating individual points of variation from wildtype at codons 5, 18, 156, 190, 236, 268, 271, 283, 304, 312, 317, 345 and 353 can be tested for activity.

B EXAMPLE 2: WHOLE ORGANISM EVOLUTION FOR HYPER-RECOMBINATION

The possibility of selection for an *E. coli* strain with an increased level of recombination was indicated from phenotypes of wild-type, Δ*recA*, *mutS* and Δ*recA mutS* strains following exposure to mitomycin C, an inter-strand cross-linking agent of DNA

Exposure of *E. coli* to mitomycin C causes inter-strand cross-linking of DNA thereby blocking DNA replication Repair of the inter-strand DNA cross links in *E. coli* occurs via a RecA-dependent recombinational repair pathway (Friedberg et al., *in DNA Repair and Mutagenesis* (1995) pp. 191-232). Processing of cross-links during repair results in occasional double-strand DNA breaks, which too are repaired by a RecA-dependent recombinational route Accordingly, recA$^-$ strains are significantly more sensitive than wildtype strains to mitomycin C exposure. In fact, mitomycin C is used in simple disk-sensitivity assays to differentiate between RecA$^+$ and RecA$^-$ strains.

In addition to its recombinogenic properties, mitomycin C is a mutagen. Exposure to DNA damaging agents, such as mitomycin C, typically results in the induction of the *E. coli* SOS regulon which includes products involved in error-prone repair of DNA damage (Friedberg et al., 1995, *supra,* at pp. 465-522).

Following phage P1-mediated generalized transduction of the Δ(*recA-srl*)::Tn10 allele (a nonfunctional allele) into wild-type and *mutS E. coli*, tetracycline-resistant transductants were screened for a *recA*$^-$phenotype using the mitomycin C-sensitivity assay. It was observed in LB overlays with a 1/4 inch filter disk saturated with 10 μg of mitomycin C following 48 hours at 37°C, growth of the wild-type and *mutS* strains was inhibited within a region with a radius of about 10 mm from the center of the disk. DNA cross-linking at high levels of mitomycin C saturates recombinational repair resulting in lethal blockage of DNA replication. Both strains gave rise to occasional colony forming units within the zone of inhibition, although, the frequency of colonies was ~10-20-fold higher in the *mutS* strain. This is presumably due to the increased rate of spontaneous mutation of *mutS* backgrounds. A side-by-side comparison demonstrated that the Δ*recA* and Δ*recA mutS* strains were significantly more sensitive to mitomycin C with growth inhibited in a region extending about 15 mm from the center of the disk. However, in contrast to the recA$^+$ strains, no Mit^r

individuals were seen within the region of growth inhibition-not even in the *mutS* background The appearance of Mit^r individuals in *recA*$^+$ backgrounds, but not in Δ*recA* backgrounds indicates the Mit^r is dependent upon a functional RecA protein and suggests that Mit^r may result from an increased capacity for recombinational repair of mitomycin C-induced damage

Mutations which lead to increased capacity for RecA-mediated recombinational repair may be diverse, unexpected, unlinked, and potentially synergistic. A recursive protocol alternating selection for Mit^r and chromosomal shuffling evolves individual cells with a dramatically increased capacity for recombination.

The recursive protocol is as follows. Following exposure of a *mutS* strain to mitomycin C, Mit^r individuals are pooled and cross-bread [e.g., via Hfr-mediated chromosomal shuffling or split-pool generalized transduction, or protoplast fusion). Alleles which result in Mit^r and presumably result in an increased capacity for recombinational repair are shuffled among the population in the absence of mismatch repair. In addition, error-prone repair following exposure to mitomycin C can introduce new mutations for the next round of shuffling. The process is repeated using increasingly more stringent exposures to mitomycin C. A number of parallel selections in the first round as a means of generating a variety of alleles. Optionally, recombinogencity of isolates can be monitored for hyper-recombination using a plasmid x plasmid assay or a chromosome x chromosome assay (e.g., that of Konrad, *J. Bacteriol.* 130, 167-172 (1977)).

C. EXAMPLE 3: WHOLE GENOME SHUFFLING OF *STREPTOMYCES COELICOLOR* TO IMPROVE THE PRODUCTION OF γ -ACTINORHODIN

To improve the production of the secondary metabolite γ-actinorhodin from *S. coelicolor*, the entire genome of this organism is shuffled either alone or with its close relative *S. lividans*. In the first procedure described below, genetic diversity arises from random mutations generated by chemical or physical means. In the second procedure, genetic diversity arises from the natural diversity existing between the genomes of *S. coelicolor* and *S. lividans*

Spore suspensions of *S. coelicolor* are resuspended in sterile water and subjected to UV mutagenesis such that 1% of the spores survive (~600 "energy" units using a Stratalinker, Stratagene), and the resulting mutants are "grown out" on sporulation agar. Individual spores represent uninucleate cells harboring different mutations within their genome. Spores are collected, washed, and plated on solid medium, preferably soy agar, R5, or other rich medium that results in sporulating colonies. Colonies are then imaged and picked

randomly using an automated colony picker, for example the Q-bot (Genetix). Alternatively colonies producing larger or darker halos of blue pigment are picked in addition or preferentially.

The colonies are inoculated into 96 well microtitre plates containing 1/3 x YEME medium (170µl /well) Two sterile 3mm glass beads are added to each well, and the plates are shaken at 150-250 rpm at 30 ^{O}C in a humidified incubator. The plates are incubated up to 7 days and the cell supernatents are assayed for γ-actinorhodin production

To assay, 50µL of supernatant is added to 100µL of distilled water in a 96 well polypropylene microtitre plate, and the plate is centrifuged at 4000 rpm to pellet the mycelia 50 µL of the cleared supernatant is then removed and added to a flat bottom polystyrene 96 well microtitre plate containing 150 µL 1M KOH in each well. The resulting plates are then read in a microtitre plate reader measuring the absorbance at 654 nm of the individual sample. as a measure of the content γ-actinorhodin

Mycelia from cultures producing γ-actinorhodin at levels significantly higher than that of wildtype *S. coelicolor* are then isolated These are propagated on solid sporulation medium, and spore preparations of each improved mutant are made. From these preparations protoplasts of each of the improved mutants are generated, pooled together, and fused (as described in Genetic Manipulation of Streptomyces -A laboratory Manual, Hopwood, D.A., et al). The fused protoplasts are regenerated and allowed to sporulate. Spores are collected and either plated on solid medium for further picking and screening, or, to increase the representation of multiparent progeny, are used to generate protoplasts and fused again (or several times as described previously for methods to effect poolwise recombination) before further picking and screening.

Further improved mutants result from the combination of two or more mutations that have additive or synergistic effects on g-actinorhodin production. Further improved mutants can be again mated by protoplast poolwise fusion, or they can be exposed to random mutagenesis to create a new population of cells to be screened and mated for further improvements.

As an alternative to random mutagenesis a source of genetic diversity, natural diversity can be employed. In this case, protoplasts generated from wildtype *S. coelicolor and S. lividans* are fused together. Spores from the regenerated progeny of this mating are then either repetitively fused and regenerated to create additional diversity, or they are separated on solid medium, picked, and screened for enhanced production of g-actinorhodin. As before, the

improved subpopulation are mated together to identify further improved family shuffled organisms

D. EXAMPLE 4: A HIGH THROUGHPUT ACTINORHODIN ASSAY

Additional Details on a high-throughput shuffling actinorhodin assay used to select mycelia are set forth in Figure 32. In brief, shufflants were picked by standard automated procedures using a Q-bot robotic system and transferred to standard 96 well plates After incubation at 30°C for 7 days, the resulting mycelia were centrifuged, and a sample of cell supernatant was removed and mixed with 0.1 M KOH in a 96 well plate and the absorbance read at 654nm. The best positive clones were selected and grown in shake flasks.

Approximately 10^9 protoplasts were centrifuged at 3,000rpm for 7 min When more than one strain was used, equal number of protoplasts were obtained from each strain. Most of the buffer was removed and the pellet suspended in the remaining buffer (~25µl total volume) by gentle flicking 0.5ml of 50% PEG1000 was added and mixed with the protoplasts by gently pipetting in and out 2 times. The mixture was then incubated for 2 minutes 0.5ml of P buffer was added and gently mixed. (This is the fusion at a dilution of 10^{-1}). A ten-fold serial dilution was performed in P buffer After 2 minutes, dilutions were plated at 10^{-1}, 10^{-2} and 10^{-3} onto R5 plates with 50µl of each, 2^{-3} plates each dilution. (for plating, -20 of 3mm glass beads were used, gentle shaking). As a first control, for regeneration of protoplasts, the same number of protoplasts were used as above, adding P buffer to a total of 1ml (this is the regeneration at dilution 10^{-1}) The mixture was further diluted (10X) in P buffer. The dilutions were plated at 10^{-3}, 10^{-4} and 10^{-5} onto R5 plates with 50µl of each. As a second control, (as a non-protoplasting mycelia background check) the same number of protoplasts as above were used adding 0.1% SDS to a total of 1ml (this is the background at dilution 10^{-1}) After further 10X dilution in 0.1% SDS, the dilution was plated at 10^{-1}, 10^{-2} and 10^{-3} onto R5 plates with 50µl of each. The plates were air dried and Incubated at 30°C for 3 days

The number of colonies was counted from each plate (those that were countable), using the number of regenerated protoplast as 100% and calculating the percentage of background (usually less than one) and fusion survival (usually greater than 10). The fusion plates were incubated at 30°C for 2 more days until all colonies were well sporulated. Spores were harvested from those plates having less than 5,000 colonies. Spores were filtered through cotton and washed once with water, suspended in 20% Glycerol and counted. Those spores are used for further study, culture inoculation or simply stored at -20°C.

E EXAMPLE 4: WHOLE GENOME SHUFFLING OF RHODOCOCCUS FOR TWO-PHASE REACTION CATALYSIS

This example provides an example of how to apply the techniques described herein to technologies that allow the generic improvement of biotransformations catalyzed by whole cells. *Rhodococcus* was selected as an initial target because it is both representative of systems in which molecular biology is rudimentary (as is common in whole cell catalysts which are generally selected by screening environmental isolates), and because it is an organism that can catalyze two-phase reactions

The goal of whole genome shuffling of *Rhodococcus* is to obtain an increase in flux through any chosen pathway The substrate specificity of the pathway can be altered to accept molecules which are not currently substrates. Each of these features can be selected for during whole genome shuffling.

During whole genome shuffling, libraries of shuffled enzymes and pathways are made and transformed into *Rhodococcus* and screened, preferably by high-throughput assays for improvements in the target phenotype, e.g., by mass spectroscopy for measuring the product.

As noted above, the chromosomal context of genes can have dramatic effects on their activities. Cloning of the target genes onto a small plasmid in *Rhodococcus* can dramatically reduce the overall pathway activity (by a factor of 5- to 10-fold or more). Thus, the starting point for DNA shuffling of a pathway (on a plasmid) can be 10-fold lower than the activity of wild-type strain. By contrast, integration of the genes into random sites in the *Rhodococcus* chromosome can result in a significant (5- to 10-fold) increase in activity. A similar phenomenon was observed in the recent directed evolution in *E coli* of an arsenate resistance operon (originally from *Staphylococcus aureus*) by DNA shuffling Shuffling of this plasmid produced sequence changes that led to efficient integration of the operon into the *E coli* chromosome. Of the total 50-fold increase in arsenate resistance obtained by directed evolution of the three gene pathway, approximately 10-fold resulted from this integration into the chromosome. The position within the chromosome is also likely to be important: for example sequences close to the replication origin have an effectively higher gene dosage and therefore greater expression level

In order to fully exploit unpredictable chromosomal position effects, and to incorporate them into a directed evolution strategy which utilizes multiple cycles of mutation, recombination and selection, genes are manipulated *in vitro* and then transferred to an optimal chromosomal position. Recombination between plasmid and chromosome occurs in two

different ways. Integration takes place at a position where there is significant sequence homology between plasmid and chromosome, i e., by homologous recombination. Integration also takes place where there is no apparent sequence identity, i.e , by non-homologous recombination. These two recombination mechanisms are effected by different cellular machineries and have different potential applications in directed evolution

To combine the increase in activity that resulted from gene duplication and chromosomal integration of the target pathway with the powerful technique of DNA shuffling, libraries of shuffled genes are made *in vitro*, and integrated into the chromosome in place of the wild-type genes by homologous recombination. Recombinants are then be screened for increased activity. This process is optionally made recursive as discussed herein. The best *Rhodococcus* variants are pooled, and the pool divided in two. Genes are cloned out of the pool by PCR, shuffled together and re-integrated into the chromosomes of the other half of the pool by homologous recombination. Recombinants are once again be screened, the best taken and pooled and the process optionally repeated

Sometimes there are complex interactions between enzymes catalyzing successive reactions in a pathway. Sometimes the presence of one enzyme can adversely affect the activities of others in the pathway. This can be the result of protein-protein interactions, or inhibition of one enzyme by the product of another, or an imbalance of primary or secondary metabolism.

This problem is overcome by DNA shuffling, which produces solutions in the target gene cluster that bring about improvements in whatever trait is screened. An alternative approach, which can solve not only this problem, but also anticipated future rate limiting steps such as supply of reducing power and substrate transportation, is complementation by overexpression of other as yet unknown genomic sequences

A library of *Rhodococcus* genomic DNA in a multicopy *Rhodococcus* vector such as pRC1 is first made. This is transformed into *Rhodococcus* and transformants are screened for increases in the desired phenotype. Genomic fragments which result in increased pathway activity are evolved by DNA shuffling to further increase their beneficial effect on a selected property. This approach requires no sequence information, nor any knowledge or assumptions about the nature of protein or pathway interactions, or even of the rate -limiting step; it relies only on detection of the desired phenotype. This sort of random cloning and subsequent evolution by DNA shuffling of positively interacting genomic sequences is extremely powerful and generic A variety of sources of genomic DNA are used, from

isogenic strains to more distantly related species with potentially desirable properties. In addition, the technique is, in principle, applicable to any microorganism for which the molecular biology basics of transformation and cloning vectors are available, and for any property which can be assayed, preferably in a high-throughput format

Homologous recombination within the chromosome is used to circumvent the limitations of plasmid-evolution and size restrictions, and is optionally used to alter central metabolism The strategy is similar to that described above for shuffling genes within their chromosomal context, except that no *in vitro* shuffling occurs. Instead, the parent strain is treated with mutagens such as ultraviolet light or nitrosoguanidine, and improved mutants are selected The improved mutants are pooled and split. Half of the pool is used to generate random genomic fragments for cloning into a homologous recombination vector. Additional genomic fragments are derived from related species with desirable properties (in this case higher metabolic rates and the ability to grow on cheaper carbon sources). The cloned genomic fragments are homologously recombined into the genomes of the remaining half of the mutant pool, and variants with improved phenotypes are selected. These are subjected to a further round of mutagenesis, selection and recombination. Again this process is entirely generic for the improvement of any whole cell biocatalyst for which a recombination vector and an assay can be developed. Recursive recombination can be performed to increase the diversity of the pool at any step in the process.

Efficient homologous recombination is important for the recursivity of the chromosomal evolution strategies outlined above. Non-homologous recombination results in a futile integration (upon selection) followed by excision (following counterselection) of the entire plasmid. Alternatively, if no counter-selection were used, there is integration of more and more copies of plasmid / genomic sequences which is both unstable and also requires an additional selectable marker for each cycle. Furthermore, additional non-homologous recombination will occur at random positions and may or may not lead to good expression of the integrated sequence.

F. EXAMPLE 5: INCREASING THE RATE OF HOMOLOGOUS RECOMBINATION IN *RHODOCOCCUS*

A genetic approach is used to increase the rate of homologous recombination in *Rhodococcus*. Both targeted and non-targeted strategies to evolve increases in homologous recombination are used. *Rhodococcus recA* is evolved by DNA shuffling to increase its ability to promote homologous recombination within the chromosome. The *recA* gene was chosen

because there are variants of *recA* known to result in increased rates of homologous recombination in *E coli.* as discussed above

The *recA* gene from *Rhodococcus* is DNA shuffled and cloned into a plasmid that carries a selectable marker and a disrupted copy of the *Rhodococcus* homolog of the *S cerevisiae URA3* gene (a gene which also confers sensitivity to the uracil precursor analogue 5-fluoroorotic acid). Homologous integration of the plasmid into the chromosome disrupts the host uracil synthesis pathway leading to a strain that carries the selectable marker and is also resistant to 5-fluoroorotic acid The shuffled *recA* genes is integrated, and can be amplified from the chromosome, shuffled again and cloned back into the integration-selection vector. At each cycle, the *recA* genes promoting the greatest degree of homologous recombination are those that are the best represented as integrants in the genome. Thus a *Rhodococcus recA* with enhanced homologous recombination-promoting activity is evolved

Many other genes are involved in several different homologous recombination pathways, and mutations in some of these proteins may also lead to cells with an increased level of homologous recombination. For example mutations in *E coli* DNA polymerase III have recently been shown to increase RecA-independent homologous recombination. Resistance to DNA cross-linking agents such as nitrous acid, mitomycin and ultraviolet are dependent on homologous recombination. Thus, increases in the activity of this pathway result in increased resistance to these agents. *Rhodococcus* cells are mutagenized and selected for increased tolerance to DNA cross-linking agents. These mutants are tested for the rate at which a plasmid will integrate homologously into the chromosome. Genomic libraries are prepared from these mutants, combined as described above, and used to evolve a strain with even higher levels of homologous recombination.

The foregoing description of the preferred embodiments of the present invention has been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and many modifications and variations are possible in light of the above teaching. Such modifications and variations which may be apparent to a person skilled in the art are intended to be within the scope of this invention. All patent documents and publications cited above are incorporated by reference in their entirety for all purposes to the same extent as if each item were so individually denoted.

WHAT IS CLAIMED IS

1 . A method of evolving a cell to acquire a desired property, comprising:

(i.) forming protoplasts of a population of different cells,

(ii) fusing the protoplasts to form hybrid protoplasts, in which genomes from the protoplasts recombine to form hybrid genomes;

(iii.) incubating the hybrid protoplasts under conditions promoting regeneration of cells, thereby producing regenerated cells;

(iv.) repeatedly forming protoplasts from the regenerated cells, fusing the protoplasts to form hybrid protoplasts, in which genomes from the protoplasts recombine to form additional hybrid genomes; incubating the additional hybrid protoplasts under conditions promoting regeneration of cells, thereby producing additional regenerated cells; and,

(v.) selecting or screening to isolate regenerated cells or additionally regenerated cells that have evolved toward acquisition of the desired property.

2. The method of claim 1 ., wherein the desired property is selected from: heat tolerance, ethanol production, ethanol tolerance, acid, improved production and maintanance of enzyme cofactors, improved production and maintanance of NAD(P)H, and improved glucose transport.

3. The method of claim 1 ., further comprising repeating steps (i.)-(v.) with regenerated cells in step (iii.) or additional regenerated cells in step (iv.) being used to form the protoplasts in step (i.) until the regenerated cells have acquired the desired property

4. The method of claim 1 ., comprising step (iv), wherein step (iv). is performed prior to step (v.).

5. The method of claim 1 , wherein the hybrid protoplasts comprise cells having more than two parental genomes

6. The method of claim 1 , wherein the different cells are fungal cells, and the regenerated cells are fungi mycelia

7. The method of claim 6 , wherein protoplasts are provided by treating mycelia or spores with an enzyme

8. The method of claim 6 , wherein the fungal cells are from a fragile strain, lacking capacity for intact cell wall synthesis, whereby protoplast form spontaneously.

9. The method of claim 6 , further comprising treating the mycelia with an inhibitor of cell wall formation to generate protoplasts

10. The method of claim 1 , further comprising selecting or screening to isolate regenerated cells with hybrid genomes free from cells with parental genomes.

11. The method of claim 1 , wherein a first subpopulation of cells contain a first marker and the second subpopulation of cells contain a second marker, and the method further comprising selecting or screening to identify regenerated cells expressing both the first and second marker.

12. The method of claim 1 , wherein the first marker is a membrane marker and the second marker is a genetic marker.

13. The method of claim 1 , wherein the first marker is a first subunit of a heteromeric enzyme and the second marker is a second subunit of the heteromeric enzyme.

14. The method of claim 1 , further comprising transforming protoplasts with a library of DNA fragments in at least one cycle.

15. The method of claim 14, wherein the DNA fragments are accompanied by a restriction enzyme

16. The method of claim 1 , further comprising exposing the protoplasts to ultraviolet irradiation in at least one cycle

17. The method of claim 1 , wherein the desired property is the expression of a protein, primary metabolite, or secondary metabolite

18. The method of claim 1 , wherein the desired property is the secretion of a protein or secondary metabolite.

19. The method of claim 18, wherein the secondary metabolite is selected from taxol, cyclosporin A, and erythromycin

20. The method of claim 1 , wherein the desired property is capacity for meiosis.

21. The method of claim 1 , wherein the desired property is compatibility to form a heterokaryon with another strain

22. The method of claim 1 , further comprising exposing the protoplasts or mycelia to a mutagenic agent in at least one cycle

23. A method for whole genome shuffling through organized heteroduplex shuffling, the method comprising:

(a). providing chromosomal DNA of an organism which is targeted for shuffling, digesting the chromosomal DNA with one or more restriction enzymes, ligating the chromosomal DNA into a cosmid, the cosmid comprising at least two rare restriction enzyme recognition sites, aliquoting, purifying, and storing sufficient cosmids to represent a complete chromosome;

(b). mutagenizing aliquots of the library in vitro using a mutagen;

(c) transfecting a sample from a plurality of the mutagenized aliquots into a population of target cells;

(d). assaying resulting transfectants for phenotypic improvements;

(e). growing transfected cells harboring a mutant library of the identified cosmid(s) on media and screening the resulting cell colonies for independent mutants conferring an desired phenotype;

(f). isolating and pooling DNA from cells identified in the screening;

(g) dividing the selected pools and digesting at least one sample with a rare-cutting restriction enzyme, pooling the cleaved samples, denaturing the samples, reannealing the samples and religating the samples; and,

(h). transfecting target cells with the resulting heteroduplexes and propagating the cells to allow recombination to occur between the strands of the heteroduplexes in vivo

24. The method of claim 23, further comprising additionally screening the transfectants.

25. The method of claim 23, further comprising further shuffling the heteroduplexes by recursive in vitro heteroduplex formation and in vivo recombination prior to additionally screening the transfectants.

26. The method of claim 24, further comprising performing an additional mutagenesis step to increase diversity during the shuffling process

27. The method of claim 23, further comprising combining one or more heteroduplexes into a host chromosome by chromosome integration.

28. The method of claim 27, further comprising repeating steps (a).-(h), using the organism resulting from chromosome integration as the source for chromosomal DNA in step (a).

29. The method of claim 23, wherein the cosmid comprises restriction sites for Sfr or NotI.

30. The method of claim 23, wherein the transfectants are assayed as a pool from each mutagenized aliquot.

31. The method of claim 23, wherein a positive assay result indicates that a cosmid from a particular aliquot can confer phenotypic improvements and contains large genomic fragments that are suitable targets for heteroduplex mediated shuffling

32. The method of claim 23, wherein the mutagen is a chemical mutagen.

33. The method of claim 23, wherein growing transfected cells harboring a mutant library of the identified cosmid(s) on media comprises plating the transfected cells on solid media