



- (51) **International Patent Classification:**  
*C12N 1/08* (2006.01) *C12N 15/10* (2006.01)
- (21) **International Application Number:**  
PCT/US2012/061027
- (22) **International Filing Date:**  
19 October 2012 (19.10.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
61/549,375 20 October 2011 (20.10.2011) US
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- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,

HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

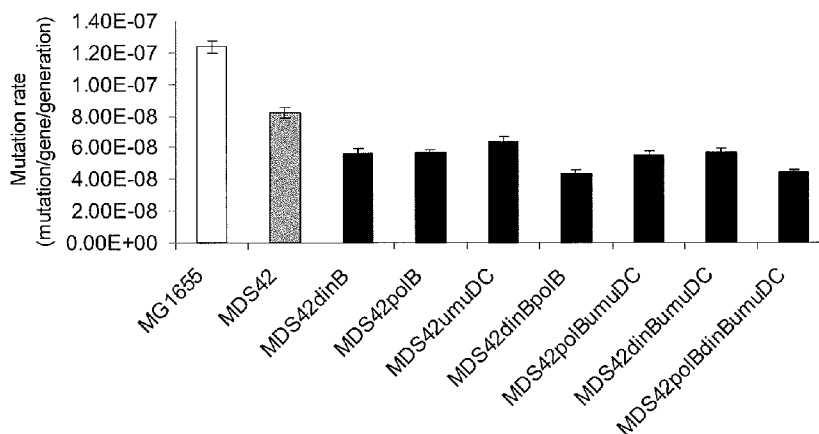
- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *of inventorship (Rule 4.17(iv))*

**Published:**

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) **Title:** REDUCED GENOME BACTERIA WITH IMPROVED GENETIC STABILITY

(57) **Abstract:** Reduced genome bacteria with improved genetic stability are provided. Also provided are methods of producing polypeptides using the reduced genome bacteria with improved genetic stability.

**REDUCED GENOME BACTERIA WITH IMPROVED GENETIC  
STABILITY**

[0001] This application claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application No. 61/549,375 filed October 20, 2011, the entire contents of which are hereby incorporated by reference.

**Field of the Invention**

[0002] The present invention is directed to reduced genome bacteria having a very low mutation rate and methods of using the reduced genome bacteria. The reduced genome bacteria are particularly useful for high fidelity maintenance of nucleic acids and stable expression of genes that have proved difficult to clone in bacterial hosts.

**Background of the Invention**

[0003] Intrinsic mechanisms for generating diversity are important for survival of bacterial populations in the dynamically changing environmental conditions present in nature. However, in the controlled environment of the laboratory, these mechanisms can lead to unwanted genotypic and phenotypic alterations and the spontaneous genetic modification of an established production strain or a clone library is generally highly undesirable.

[0004] *Escherichia coli* (*E. coli*) is a universal cloning host and is the most common organism used in the production of proteins, metabolites and secondary metabolites in both research and industry. Several modifications have been made to improve the performance of *E. coli* hosts in these settings all of which follow the basic principle of streamlining metabolic pathways for the increased production of a given biomaterial coupled with reduction of unwanted byproducts. Along these lines, a variety of nonessential genes have been removed from an *E. coli* background to form viable reduced genome *E. coli* strains with little or no significant reduction in growth.

[0005] Although these reduced genome bacteria have proved beneficial in many respects, some genes, in their functional forms, remain difficult or impossible to clone in bacterial vectors even in reduced genome bacteria. Accordingly, there is a need for stable bacterial hosts with very low mutation rates in which such genes could be cloned.

### **Summary of the Invention**

[0006] The present invention provides a reduced genome bacterium wherein the gene(s) encoding at least one of the three error-prone DNA polymerases, Pol II, PolIV and PolV, are non-functional. In a preferred embodiment, the genes encoding Pol II and PolIV are non-functional and the gene encoding PolV is functional or non-functional. In a particularly preferred embodiment, none of these genes is functional in the reduced genome bacteria. The genes may be rendered non-functional by deletion of the genes in part or in whole from the genome of the bacteria or may be rendered non-functional by disrupting the genes.

[0007] In one embodiment, the genome of the reduced genome bacterium has a genome that is genetically engineered to be from about 5% to about 30% smaller than the genome of its native parent strain and lacks all insertion sequences. Reduced genome bacteria may be produced by deleting selected genes from a native parental strain of a bacterium or may, for example, be entirely synthesized as an assembly of preselected genes. As is readily apparent from the discussion herein, a reduced genome bacterium has fewer than the full complement of genes found in a native parent strain to which it is compared, and with which it shares certain essential genes.

[0008] Methods for producing a polypeptide employing the reduced genome bacteria are also provided. In one embodiment, the polypeptide is produced by culturing reduced genome bacteria having one or more non-functional genes selected from the group consisting of the genes encoding Pol II, PolIV and PolV and further comprising a nucleic acid encoding the polypeptide operatively linked to an expression control sequence, under conditions suitable for expression of the polypeptide. In a related embodiment, the nucleic acid encodes a "toxic" polypeptide

which is difficult or impossible to clone in bacteria having functional PolII, PolIV and PolV genes.

[0009] These and other embodiments of the present invention are described in more detail herein below.

### **Description of the Drawings**

[0010] Figure 1 illustrates the spontaneous mutation rates of reduced genome bacteria (MDS42) with non-functional (deletions in this case) *dinB*, *polB*, and *umuDC* genes, separately and in every possible combination as compared with MDS42 and MG1655 controls. Mutation rates were determined by a fluctuation analysis on mutations occurring in *cycA*. The decrease in mutation rate between wild-type MG1655 to MDS42 is due to the absence of insertion events, while the further decrease from MDS42 to the different error-prone polymerase mutants is due to a lower point-mutation rate. Values are averages of 4 independent measurements

[0011] Figure 2 illustrates the effect of different stresses on the mutation rate of various strains. The effect of stress imposed by overproduction of green fluorescent protein (GFP), overproduction of a toxic peptide from pSG-ORF238, and treatment with mitomycin C are shown. All measurements were made using the *cycA* fluctuation assay, values are averages of 3 independent measurements each. BL21(DE3) and MDS42*recA* failed to grow in the presence of 0.1 µg/ml mitomycin C.

[0012] Figure 3 illustrates a comparison of the mutational spectra of various strains. The bar graph shows the distribution of *cycA* mutation types, detected by polymerase chain reaction (PCR) analysis. The share of deletions in MG1655, MDS42, and MDS42*polBdinBumuDC* is too low to be visible, and no deletions were detected in BL21(DE3).

[0013] Figure 4 illustrates the mutation rate of MDS42 and MDS42*polBdinBumuDC* measured by a rifampicin resistance assay. Values are averages of 3 independent measurements each.

[0014] Figure 5 illustrates the toxic effect of the overproduction of the SinI enzyme on the growth of MDS42-T7 (mcrBC<sup>-</sup>) host. SinI was overproduced from the IPTG-inducible pSin32 plasmid. Both measurements are averages of the O.D.<sub>540</sub> values of 25 independent colonies each, measured every 5 minutes using the Bioscreen C automated instrument.

[0015] Figure 6 illustrates the effect of overproduction of SinI methyltransferase on the mutation rate. The SinI methyltransferase enzyme was overproduced from the pSin32 plasmid. Mutation rates were measured using the *cycA* fluctuation assay. Values are an average of 3 independent measurements each.

[0016] Figure 7 illustrates the accumulation of plasmids with mutated *sinI* in various hosts. SinI methyltransferase was expressed from pSin32. Plasmids were isolated at various intervals and screened (by transformation in McrBC<sup>+</sup> and McrBC<sup>-</sup> hosts) for mutations resulting in a loss of function of the enzyme. Values are averages of 3 independent measurements each.

[0017] Figure 8 illustrates the time required to grow to OD=0.7 for cultures of various strains expressing SinI. Growth curves of strains harboring pSin32, induced with IPTG at OD=0.2 (0 min), were measured. OD=0.7 was selected as a cutoff to indicate that a sample had overcome the toxic effect of the overproduced SinI methyltransferase. Values shown are averages of measurements on 50 independent samples of each strain.

[0018] Figure 9 illustrates mutations in *sinI*, carried on pSin32 plasmids able to transform MG1655. Eight pSin32 plasmid samples, isolated from McrBC<sup>+</sup> host, were sequenced. Seven of them carried frameshift mutations that created new stop codons in *sinI* (positions 182, 183, 194, 195, 196, 208 and 862). One of the mutations was a A→C transition leading to an Asn→Thr change (position 880). Nucleotide position of each mutation is shown relative to the first nucleotide of *sinI*.

### **Detailed Description of the Invention**

**[0019]** While the present invention is capable of being embodied in various forms, the description below of several embodiments is made with the understanding that the present disclosure is to be considered as an exemplification of the invention, and is not intended to limit the invention to the specific embodiments illustrated. Headings are provided for convenience only and are not to be construed to limit the invention in any manner. Embodiments illustrated under any heading may be combined with embodiments illustrated under any other heading.

**[0020]** The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word “about.” In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. As used herein, the terms “about” and “approximately” when referring to a numerical value shall have their plain and ordinary meanings to one skilled in the pertinent art at issue. Also, the disclosure of ranges is intended as a continuous range including every value between the minimum and maximum values recited as well as any ranges that can be formed by such values. This includes ranges that can be formed that do or do not include a finite upper and/or lower boundary. This also includes ratios that are derivable by dividing a given disclosed numeral into another disclosed numeral. Accordingly, the skilled person will appreciate that many such ratios, ranges, and ranges of ratios can be unambiguously derived from the data and numbers presented herein and all represent various embodiments of the present invention.

**[0021]** The term “reduced genome bacterium” herein means a bacterium having about 1% to about 75% of its genome (e.g. protein coding genes) deleted, for example about 5%, about 10%, about 20%, about 30% about 40%, about 50% or about 60% of the genome deleted. In one embodiment, the reduced genome bacteria used in the practice of the present invention have a genome that is preferably genetically engineered to be at least two percent (2%) and up to twenty percent (20%) (including any number therebetween) smaller than the genome of a native parent strain.

Preferably, the genome is at least five percent (5%) and up to thirty percent (30%) smaller than the genome of a native parent strain. More preferably, the genome is eight percent (8%) to fourteen percent (14%) to twenty percent (20%) (including any number therebetween) or more smaller than the genome of the native parent strain. Alternatively, the genome may be engineered to be less than 20%, less than 30%, less than 40% or less than 50% smaller than the genome of a native parental strain. The term “native parental strain” means a bacterial strain found in a natural or native environment as commonly understood by the scientific community and on whose genome a series of deletions can be made to generate a bacterial strain with a smaller genome. Native parent strain also refers to a strain against which the engineered strain is compared and wherein the engineered strain has less than the full complement of the native parent strain. The percentage by which a genome has become smaller after a series of deletions is calculated by dividing “the total number of base pairs deleted after all of the deletions” by “the total number of base pairs in the genome before all of the deletions” and then multiplying by 100. Similarly, the percentage by which the genome is smaller than the native parent strain is calculated by dividing the total number of nucleotides in the strain with the smaller genome (regardless of the process by which it was produced) by the total number of nucleotides in a native parent strain and then multiplying by 100.

**[0022]** In one embodiment, the term “reduced genome bacteria” refers to bacteria for which removal of the above amounts of genome does not unacceptably affect the ability of the organism to grow on minimal medium. Whether removal of two or more genes “unacceptably affects” the ability of the organism to grow on minimal medium in the present context depends on the specific application. For example, a 30% reduction in proliferation rate may be acceptable for one application but not another. In addition, adverse effect of deleting a DNA sequence from the genome may be reduced by measures such as changing culture conditions. Such measures may turn an otherwise unacceptable adverse effect to an acceptable one. In one embodiment, the proliferation rate is approximately the same as the parental strain. However, proliferation rates ranging from about 5%, 10%, 15%, 20%, 30%, 40% to about 50% lower than that of the parental strain are within the scope of the invention. More particularly, doubling times of bacteria of the present invention may range from

about five minutes to about three hours. Non-limiting examples of suitable reduced genome bacteria, as well as methods for deleting DNA from a bacterium such as *E. coli*, are disclosed in U.S. Pat Nos. 6,989,265 and 7,303,906, U.S. Pat. Pub. Nos. 20060270043, 2006/0199257 and 2007/0054358 and WIPO Pub. No. WO 2003/070880, each of which is hereby incorporated by reference herein.

**[0023]** In several embodiments, a reduced genome bacterium is provided having at least one non-functional gene selected from the group consisting of the gene(s) encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V. Reduced genome bacteria in which one or more of these genes are non-functional exhibit a substantial improvement in genetic stability compared to bacteria having the same genetic background but in which these genes are functional. In one aspect, the gene(s) are rendered non-functional by deletion, for example by the “scarless” deletion methods described at column 8, line 45 to column 14, line 41 of U.S. Patent No. 6,989,265. These methods result in a precise deletion of the target gene with no inserted DNA resulting from the deletion process (i.e. “scarless” deletions) and are therefore the preferred deletion methods. It is to be understood, however, that any method of deleting target genes, in whole or in part, known in the art may be employed to render one or more of the genes encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V non-functional. Alternatively, one or more of the genes encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V may be rendered non-functional by disrupting the gene(s) by using any technique known in the art. For example, the target gene(s) may be disrupted by replacing the gene with a non-functional allele by homologous recombination. Disruption and deletion may be used in combination to produce any combination of non-functional genes encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V in the bacterium.

**[0024]** In one embodiment, any one of the genes encoding DNA Polymerase II, IV and V may be rendered non-functional and the remaining two genes may be functional. In other embodiments, any combination of two of the genes encoding DNA Polymerase II, IV and V may be rendered non-functional in the reduced genome bacteria and the remaining gene may be functional. For example, the genes encoding



DNA Polymerase II and IV may be rendered non-functional and the gene encoding DNA Polymerase V may be functional. Alternatively, the genes encoding DNA Polymerase II and V may be rendered non-functional and the gene encoding DNA Polymerase IV may be functional. Alternatively, the genes encoding DNA polymerase IV and V may be rendered non-functional and the gene encoding DNA Polymerase II may be functional. In a preferred embodiment, the genes encoding DNA Polymerase II and DNA Polymerase IV are non-functional in the reduced genome bacterium and the gene encoding DNA Polymerase V is either functional or non-functional. In a particularly preferred embodiment, the genes encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V are all non-functional in the reduced genome bacterium.

**[0025]** In another preferred embodiment, the reduced genome bacterium with one or more non-functional gene selected from the group consisting of the gene(s) encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V has a genome that is genetically engineered to be at least five percent (5%) and up to thirty percent (30%) (including any number therebetween) smaller than the genome of a native parent strain. In another preferred embodiment, the reduced genome bacterium has a genome that is between 4.41 Mb and 3.71 Mb, between 4.41 Mb and 3.25 Mb or between 4.41 Mb and 2.78 Mb.

**[0026]** The parent of the reduced genome bacterium of the invention may be any bacterial strain. In a preferred embodiment, the parent of the reduced genome bacterium of the invention is an *E. coli* strain, such as an *E. coli* K-12 or B strain. *E. coli* K12 strains include derivative strains such as MG1655, W3110, DH1, DH10B, DH5 $\alpha$ , Inv $\alpha$ , Top10, Top10F, JM103, JM105, JM109, MC1061, MC4100, XL1-Blue, EC100, BW2952, or EC300. *E. coli* B strains include REL606, BL/R and BL21(DE3).

**[0027]** The nucleotide sequence of the genome of the parental strain may be partially or completely known. The complete genomic sequence of several *E. coli* and other commonly used laboratory microorganisms is known (see e.g. Blattner et al., Science, 277:1453-74 (1997); GenBank Accession No. U00096; NCBI database,

Accession No. AP009048, Perna et al., Nature, 409, 529-533 (2001); Hayashi et al., DNA Res., 8, 11-22 (2001); Welch et al., Proc. Natl. Acad. Sci., USA 99:17020-17024 (2002), GenBank Accession No. AE014075, EMBL Accession No. CP000948, EMBL Accession No. CP001637, EMBL Accession No. CP001396, EMBL Accession No. CP000819, and EMBL Accession No. CP001509, each of which is incorporated herein by reference).

**[0028]** In a preferred embodiment, the parent of the reduced genome bacterium of the invention is *E. coli* strain K12 MG1655 (annotated version m56), (NCBI accession no. U000961) with a genome having 4,639,674 base pairs. In another preferred embodiment, the parent of the reduced genome bacterium is *E. coli* strain BL21(DE3) (EMBL accession no. CP001509) with a genome having 4,557,508 base pairs. The coordinates of the genes encoding DNA Polymerase II (*polB*), DNA Polymerase IV (*dinB*) and DNA Polymerase V (*umuDC*) in the *E. coli* K12 MG1655 genome are provided at Table 1.

Table 1

Gene	Coordinates
<i>polB</i> (b0060)	63429-65780
<i>dinB</i> (b0231)	250898-251953
<i>umuDC</i> (b1183-b1184)	1229990-1231677

**[0029]** In a particularly preferred embodiment, a reduced genome *E. coli* bacterium is provided having a genome between five percent (5%) and thirty percent (30%) smaller than the genome of a native parent strain and lacking all insertion sequence (IS) elements and having at least one non-functional gene selected from the group consisting of the gene(s) encoding DNA Polymerase II, DNA Polymerase IV and DNA Polymerase V. Positions of the IS elements on a genome map of *E. coli* MG1655 (annotated version 54) are shown in Fig. 1 and Table 2 of U.S. Patent

Publication No. 2003/138937, the contents of which are incorporated herein by reference. Insertion sequence elements which commonly occur in *E. coli* and which may be removed, include without limitation, IS1, IS2, IS3, IS4, IS5, IS30, IS150, IS186, IS600, IS911 and IS10. In a particularly preferred embodiment, a reduced genome *E. coli* is provided lacking all insertion sequences and having non-functional *polB* and *dinB* genes and even more preferably having non-functional *polB*, *dinB* and *umuDC* genes.

**[0030]** In a related embodiment, the reduced genome bacterium is an *E. coli* bacterium lacking at least the following genes (identified by “b” numbers based on the designations set out in Blattner *et al.*, Science, 277:1453-74 and in GenBank Accession No. 400096): b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-b4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, b4455, b1786, b0150-b0153 and b2945 and also having one or more non-functional genes selected from *polB*, *dinB* and *umuDC*. In a particularly preferred embodiment, *polB* and *dinB* are non-functional and even more preferably *polB*, *dinB* and *umuDC* are all non-functional. The reduced genome *E. coli* bacterium may be strain MDS42, the genome of which lacks all insertion sequences, with one or more non-functional *polB*, *dinB* and *umuDC* genes, preferably with non-functional *polB* and *dinB* genes and even more preferably with all three genes non-functional. The reduced genome may also be strain MDS43 or MDS66 (or any derivative strain), with one or more non-functional *polB*, *dinB* and *umuDC* genes, preferably with all three genes non-functional.

**[0031]** Various protein coding genes can be deleted to form reduced genome bacteria. In *E. coli* and other bacteria, a type of DNA sequence that can be deleted

includes those that in general will adversely affect the stability of the organism or of the gene products of that organism. Such elements that give rise to instability include without limitation transposable elements, insertion sequences, and other “selfish DNA” elements which may play a role in genome instability. For example, insertion sequence (IS) elements and their associated transposes are often found in bacterial genomes, and thus are targets for deletion. IS sequences are common in *E. coli*, and all of them may be deleted. For purposes of clarity in this document, we use the term IS element and transposable element generically to refer to DNA elements, whether intact or defective, that can move from one point to another in the genome. An example of the detrimental effects of IS elements in science and technology is the fact that they can hop from the genome of the host *E. coli* into a BAC plasmid during propagation for sequencing. This artifact can be prevented by deletion from the host cells of all IS elements. For a specific application, other specific genes associated with genomic instability, such as active and inactive prophages may also be deleted.

**[0032]** Reduced genome bacteria of the invention may also be engineered to lack, for example, without limitation, certain genes unnecessary for growth and metabolism of the bacteria, pseudogenes, prophage, undesirable endogenous restriction-modification genes, pathogenicity genes, toxin genes, fimbrial genes, periplasmic protein genes, invasin genes, lipopolysaccharide genes, class III secretion systems, phage virulence determinants, phage receptors, pathogenicity islands, RHS elements, sequences of unknown function and sequences not found in common between two strains of the same native parental species of bacterium. Other DNA sequences that are not required for cell survival can also be deleted or omitted.

**[0033]** The reduced genome bacteria of the invention may comprise a heterologous nucleic acid encoding a polypeptide. The polypeptide may be a therapeutic protein such as insulin, an interleukin, a cytokine, a growth hormone, a growth factor, erythropoietin, a colony stimulating factor, interferon, or an antibody. The heterologous nucleic acid may be placed within a vector such as a plasmid and operatively linked to a promoter and optionally additional regulatory sequences.

[0034] Reduced genome bacteria having one or more non-functional *polB*, *dinB* and *umuDC* genes, preferably with at least non-functional *polB* and *dinB* genes, and further lacking all insertion sequences exhibit surprising genetic stability that enables the cloning of toxic nucleic acids which are difficult or impossible to isolate or maintain even in reduced genome bacteria lacking all insertion sequences. A “toxic” nucleic acid may be a nucleic acid which, when propagated in a host strain, results in an elevated mutation rate. A toxic nucleic acid may also result in an elevated rate of IS element transposition. An elevated rate of mutation of a toxic nucleic acid may be determined by comparison to a host strain propagating a control nucleic acid.

[0035] The reduced genome bacteria comprising one or more non-functional *polB*, *dinB* and *umuDC* genes may be used to produce polypeptides. Briefly a bacterium of the invention comprising a heterologous nucleic acid encoding a polypeptide operatively linked to an expression control sequence, as described above, may be incubated under conditions sufficient to allow expression of the polypeptide product.

[0036] Overexpression of even a well-tolerated protein of interest may lead to elevated IS transposition rates and activate the stress response of the cell leading to significantly increased mutation rates. Plasmids encoding the protein of interest rapidly acquire loss-of-function mutations under these conditions and bacteria carrying these mutated plasmids quickly take become dominant in the culture due at least in part to the growth inhibitory effect of intact plasmids encoding the overexpressed protein. Bacteria of the invention, which exhibit a surprising genomic stability and fidelity, delay the appearance of such mutant plasmids and the cells can produce the functional toxic protein for an extended period of time.

[0037] Recombinant proteins may be expressed in the periplasm or cytoplasm. The expression of proteins in the periplasm is routinely used for industrial use and has been reviewed in Hanahan, J. Mol. Biol., 166:557-580 (1983); Hockney, Trends Biotechnol., 12:456-632 (1994); and Hannig *et al.*, Trends Biotechnol., 16:54-60 (1998), each of which is incorporated herein by reference. Recombinant proteins may be produced in the periplasm by expressing fusion proteins in which they are attached to a signal peptide that causes secretion into the periplasmic space. There, the signal

peptide may be cleaved off by specific signal peptidases. The protein transported into the periplasmic space may be biologically active.

[0038] The recombinant protein may be co-expressed with chaperones/disulfide-bond forming enzymes, which may provide proper folding of the recombinant protein. Nucleic acid sequences of such proteins useful for periplasmic expression of recombinant protein include, without limitation, those described in U.S. Pat. Nos. 5,747,662; 5,578,464 and 6,022,952, each of which is incorporated herein by reference.

### **Example 1**

#### **Production of Reduced Genome *E. coli***

[0039] Reduced genome strain MDS39 was produced as described in International Patent Publication No. WO 2003/070880, which is incorporated herein by reference. Briefly, a series of reduced genome strains (MDS01-MDS39) were produced by making a series of 39 cumulative deletions (approximately 14.1% of the genome) of nucleic acid sequences from the parental strain *E. coli* MG1655.

[0040] Hybridization to genome scanning chips (NimbleGen Systems, Madison, WI) containing the K-12 sequence and all sequences in the IS database revealed that MDS39, the first strain designed to lack all IS elements, unexpectedly contained additional copies of an IS element that had hopped to new locations during its production. These IS elements were deleted to produce MDS40. The *fhuACDB* (the *tonA* locus) was deleted from MDS40 to produce MDS41. The location and function of each cumulative deletion made to produce MDS01-MDS41 can be found at Table 2 of U.S. Application Publication No. 2007/0054358, the entire content of which is incorporated herein by reference. The *endA* gene was then deleted from MDS41 to produce MDS42.

[0041] The genes coding for DNA polymerase II (*polB*), DNA polymerase IV (*dinB*) and DNA polymerase V (*umuDC*) were deleted from the genome of MDS42 in a scarless manner using a suicide plasmid-based method as described in U.S. Patent

No. 6,989,265 and Feher *et al.*, Methods Mol. Biol., 416:251-259 (2008) with plasmids pST76-A and pSTKST. Gene deletions were made individually and also joined in all possible combinations to produce the following strains: MDS42*polB*, MDS42*dinB*, MDS42*umuDC*, MDS42*polBdinB*, MDS42*polBumuDC*, MDS42*dinBumuDC* and MDS42*polBdinBumuDC*. Individual deletions were combined by P1 phage transduction of the marked (with integrated suicide-plasmids) intermediates of the deletion constructs, followed by endonuclease cleavage-stimulated out-recombination and loss of the plasmid. All deletions were verified by polymerase chain reaction (PCR) and sequencing using flanking primers. Primer sequences used in the study are listed at Table 2:

Table 2

Primer Name	Sequence (5'-3')	Application
polB-A	ccgaattcagtatccaggcgagt	deletion of <i>polB</i>
polB-BR	caggcaggtgtggcggagggaataact	deletion of <i>polB</i>
polB-BF	tccgccacacctgcctgcgccacgct	deletion of <i>polB</i>
polB-C	ccggatccattggcggcattgt	deletion of <i>polB</i>
polB-D	tgctgaacaccagtttgct	deletion of <i>polB</i>
polB-E	aaccggtgaagtgggtga	deletion of <i>polB</i>
dinB-A	ccggtaccgggcataccgatgcga	deletion of <i>dinB</i>
dinB-BR	cagaatatacattgctcacctctcaacact	deletion of <i>dinB</i>
dinB-BF	gaggtgagcaatgtatattctggtgtgca	deletion of <i>dinB</i>
dinB-C	ccggatccgcccgttaacgcataa	deletion of <i>dinB</i>
dinB-D	gtgttcgactcgtcgat	deletion of <i>dinB</i>
dinB-E	gagtcgtcgtagagtgcg	deletion of <i>dinB</i>
umuDC-A	ggaattcggatgagcgtcgtcgcca	deletion of <i>umuDC</i>
umuDC-BR	ttgagcgcacaacagcagcgatgacaa	deletion of <i>umuDC</i>
umuDC-BF	gctgctgtgttgctgctcaatgaacctt	deletion of <i>umuDC</i>
umuDC-C	gctgcagatcgcttacgtgattgtc	deletion of <i>umuDC</i>
umuDC-D	aatgtccatctgcggtt	deletion of <i>umuDC</i>
umuDC-E	gctctatccttcgcccgtt	deletion of <i>umuDC</i>
lexA-A	ggtatggctcgattttggata	modification of <i>lexA</i>
lexA-BR	gatatctttcatcgCcatcccgtgacgcga	modification of <i>lexA</i>
lexA-BF	ggatgGcgatgaaagatatcgga	modification of <i>lexA</i>
lexA-C	ccggatcccagcaacggaacggt	modification of <i>lexA</i>
lexA-D	cggtgctgattgccatta	modification of <i>lexA</i>
lexA-E	gggtatcaagatgacca	modification of <i>lexA</i>
recA-D	cggctagcgcagggatgttgattc	deletion of <i>recA</i>
recA-E	gtgctgattatgccgtgt	deletion of <i>recA</i>
BMD30-A	ccgaattcagtcgcacgcaactt	deletion of <i>mcrBC</i>
BMD30-BR	ctcgccttaattacatacttttggtgc	deletion of <i>mcrBC</i>
BMD30-BF	tatgtaaattaaggcgagattattaaa	deletion of <i>mcrBC</i>

BMD30-C	ccggatccacatggcgcgttaca	deletion of <i>mcrBC</i>
BMD30-D	tgataccgccgcacaaca	deletion of <i>mcrBC</i>
BMD30-E	actggtgtgtctcgcaag	deletion of <i>mcrBC</i>
cycA-D	ctgatgccggtagggtct	analysis of <i>cycA</i> mutations
cycA-E	gcgccatccagcatgata	analysis of <i>cycA</i> mutations
AK54-D	atgataatgaatgacatca	sequencing of <i>sinI</i>
AK55-E	ctcgagttagaccaactctcaaa	sequencing of <i>sinI</i>
Sce2	attaccctgttatcccta	pST76 sequencing primer
T7	taatacgactcactataggg	pST76 sequencing primer

Primers marked with A, C, BF, and BR were used to create homology regions by recombinant PCR for genomic integration of the suicide plasmids. Primers marked with D or E were homologous to flanking genomic regions, and were used for checking the deletions/allele replacements by PCR and sequencing. Capital letters in *lexA* primers indicate the point mutation introduced in the gene.

### **Example 2**

#### **Spontaneous Mutation Rates in Reduced Genome *E. coli***

[0042] The spontaneous mutation rate of each strain was then determined using a D-cycloserine resistance assay, detecting all types of mutations in the *cycA* gene, as described in Feher *et al.*, Mutat. Res. 595(1-2):184-190 (2006). Briefly, in a fluctuation assay, 20 tubes of 1 ml MS medium (as described in Hall, Mol. Biol. Evol., 15(1):1-5 (1998)) supplemented with 0.2% glucose were inoculated with approximately  $10^4$  cells each, and cultures were grown to early stationary phase. Aliquots of 50  $\mu$ l from each tube were then spread on MS plates containing D-cycloserine (0.04 mM). The estimated number of mutations per tube ( $m$ ) was calculated from the number of colonies by using the Ma-Sandri-Sarkar maximum likelihood method (Sarkar *et al.*, Genetica, 85(2):173-179 (1992)). Equation 41 from Stewart *et al.*, Genetics, 124(1):175-185 (1990) was used to extrapolate the obtained  $m$  value, valid for 50  $\mu$ l, to 1 ml. Statistical comparisons of  $m$  values were made only when the difference in total cell number was negligible ( $<3\%$ ,  $P \leq 0.6$ , with a two-tailed, unpaired  $t$  test). The total number of cells in a tube was calculated by spreading dilutions from three random tubes onto nonselective plates. Dividing the



number of mutations per tube by the average total number of cells in a tube gave the mutation rate (mutation/cell/generation).

**[0043]** The deletion of each gene by itself results in at least a 20% decrease in mutation rate measured by this method (all values significant,  $P < 0.05$ , two-tailed, unpaired t test). The results are graphically depicted at Figure 1. Combining the different deletions decreased the mutation rate decreased further, with the lowest mutation rates being that of MDS42*polBdinB* and the triple deletion strain MDS42*polBdinBumuDC*. The effect of combining the *polB* and *dinB* deletions is multiplicative, indicating an independent mode of action for these polymerases. The deletion of *umuDC* generated no additional decrease of the mutation rate when any of the other two error prone polymerases were missing, possibly marking an interaction among the genes or their products. Compared to the parent MDS42 strain, strains MDS42*polBdinB* and MDS42*polBdinBumuDC* showed a nearly 50% reduction in spontaneous mutation rate ( $8.2 \times 10^{-8}$  mutation/cell/generation decreased to  $4.34 \times 10^{-8}$  and  $4.45 \times 10^{-8}$  respectively).

**[0044]** To verify that the absence of genes encoding DNA polymerase II, IV and V has no adverse effect on fitness, growth rates of the different strains were measured in MOPS minimal medium. Ten parallel cultures originating from 10 individual colonies for each strain were picked and grown in a Bioscreen C instrument. Growth curves were measured by following the optical densities (O.D.) at 540 nm of each culture. None of the deletions had a significant effect on fitness in MOPS minimal medium, even when combined in the triple deletion strain MDS42*polBdinBumuDC*.

**[0045]** To determine whether upstream inactivation of the entire SOS response via regulator mutants would have the same effects on the spontaneous mutation rate as elimination of the genes encoding DNA polymerase II, IV and V, MDS42*recA* and MDS42*lexA* were created. MDS42*recA* comprises a scarless deletion of *recA* (coordinates 2820783-2821861 of MG1655), the product of which is required for induction of the autoproteolysis of LexA. MDS42*lexA* comprises a replacement of the *lexA* gene with a non-functional allele in which the serine at position 119 is replaced with alanine (S119A). Each of these genes (*recA* and *lexA*) is required in order to de-

repress the SOS regulon genes. Accordingly, neither MDS42*recA* nor MDS42*lexA* is able to induce the SOS pathway. None of these modifications had an adverse effect on the overall fitness of the strains, as measured by growth rates of the strains in MOPS minimal medium as described above. Surprisingly, neither strain showed a significant decrease in spontaneous mutation rate when compared to MDS42. In the case of MDS42*lexA*, a slight increase was actually observed ( $2.07 \times 10^{-7}$  compared to  $8.2 \times 10^{-8}$  of MDS42). The results are illustrated at Figure 2 (blank bars (unstressed)).

### Example 3

#### Stress-Induced Mutation Rates in Reduced Genome *E. coli*

[0046] The mutation rates of MDS42*recA*, MDS42*lexA* and MDS42*polb**dinBumuDC* under stressful conditions were then measured and compared.

[0047] Mitomycin-C, a DNA cross-linking agent that causes lesions in double stranded DNA, directly activates the SOS response, leading to up-regulation of DNA polymerases II, IV and V. A sub-inhibitory concentration (0.1  $\mu\text{g/ml}$ ) of mitomycin-C was used to stress the cells and the effect on mutation rates was analyzed. The results are illustrated at Figure 2 (hatched bars (mitomycin))

[0048] Protein overproduction imposes stress on the host cell. The effect of overproduction of a benign protein, Green Fluorescent Protein (GFP), on mutation rates was tested. The gene encoding GFP was cloned on a plasmid as an inducible construct controlled by a T7 promoter. To express the GFP, T7 RNA polymerase encoding variants of strains MDS42*polBdinBumuDC* (MDS42*polBdinBumuDC*-T7), MDS42*recA* (MDS42*recA*-T7), and MDS42*lexA* (MDS42*lexA*-T7) were constructed by replacing the *yahA-yaiL* genomic region with an IPTG-inducible *lac* operator/T7 polymerase cassette. T7 RNA polymerase encoding variants of MDS42 (MDS42-T7), MG1655 (MG1655-T7), and the widely used protein production strain BL21(DE3) were also constructed and the effects of overexpression of GFP on mutation rate in

each strain was measured and compared. The results are illustrated at Figure 2 (solid black bars (pET-GFP)).

[0049] Next, the effect of overproduction of a toxic protein (ORF238, a small, leucine-rich hydrophobic protein) on mutation rates in the bacteria was tested by transforming the strains with plasmid pSG-ORF238, an IPTG-inducible, pSG1144-based construct capable of overproducing the ORF238 protein. The results are illustrated at Figure 2 (shaded bars (pSG-ORF238)). Overproduction of ORF238 significantly increased the mutation rate of MDS42. The values for MDS42*polBdinBumuDC* remained stable under the same conditions.

[0050] The results demonstrate that, with the exception of MDS42*recA* and MDS42*polBdinBumuDC*, the various stresses increased the mutation rate of all strains including MDS42. Overproduction of the toxic ORF238 protein had the largest effect: a greater than 5-fold increase in mutation rate was measured. Sub-inhibitory concentration of mitomycin-C caused a greater than 2-3-fold increase in the mutation rate and BL21(DE3) and MDS42*recA* were unable to grow under these conditions. Overproduction of GFP had a relatively minor effect, resulting in a 1.5 to 2-fold increase in mutation rates.

[0051] In contrast, no significant increase in mutation rate in the presence of any of the stressors could be seen in either MDS42*recA* or MDS42*polBdinBumuDC*. Interestingly, MDS42*lexA* did not follow this behavior – the strain showed an increase in mutation rate in response to all of the stresses. MDS42*polBdinBumuDC* can be characterized as the genetically most stable strain, displaying the lowest spontaneous mutation rate and showing negligible response to stressful conditions.

[0052] The most commonly used protein production strain, BL21(DE3), displayed a mutation rate nearly two orders of magnitude higher than MDS42*polBdinBumuDC* when overproducing the toxic ORF238 protein. To analyze this difference, mutation spectra of BL21(DE3), MG1655, MDS42 and MDS42*polBdinBumuDC* were studied by PCR analysis of *cycA* in cycloserine-resistant mutants. Briefly, a 1,877-bp

genomic segment encompassing the entire gene was amplified from mutant cells using the primer pair *cycA1-D/cycA2-E*. A representative sample was obtained by analyzing 5 colonies from each parallel plate, yielding a total of 96 samples per experiment. The amplified fragments were resolved on an agarose gel and compared to a fragment generated from the wild-type template. Identical sizes indicated a mutation affecting only one or a few nucleotides, a decrease in size or failure of amplification indicated a deletion, and a detectable size increase indicated in IS insertion. The results are illustrated at Figure 3. In MG1655, 74% of the mutations proved to be point mutations, 24% were IS insertions, and 2% were deletions. In contrast, in BL21(DE3), 77% of *cycA* mutations were IS insertions. Although the proportion of point mutations in BL21(DE3) was much smaller (74% in MG1655 versus 23% in BL21(DE3)), the actual rate of point mutations was significantly higher in BL21(DE3) ( $2.28 \times 10^{-7}$  compared to  $9.2 \times 10^{-8}$  in MG1655). No deletions were found among the *cycA* alleles in BL21(DE3).

**[0053]** To confirm the data obtained using the *cycA* fluctuation assay, mutation rates of MDS42 and MDS42*polBdinBumuDC* under each of the different stress conditions were also measured using the rifampicin resistance assay. This assay detects point mutations in the essential *rpoB* gene, as described in Jin and Gross, J. Mol. Biol., 202(1):45-58 (1988). Briefly, twenty tubes of 1 ml LB were inoculated with  $10^4$  cells each, and cultures grown to early stationary phase. Appropriate dilutions were spread onto non-selective LB agar plates and LB agar plates containing rifampicin (100  $\mu\text{g/ml}$ ). Colony counts were performed after 24 or 48 hours, respectively. Mutation frequencies were reported as a proportion of the number of rifampicin-resistant colonies relative to the total viable count. The results correspond to the mean value obtained in three independent experiments for each strain and condition. When required, different stress conditions were provided in the same manner as in the *cycA* assay. The data obtained using the rifampicin resistance assay were consistent with the *cycA* fluctuation data, as illustrated at Figure 4. MDS42*polBdinBumuDC* had a significantly lower spontaneous mutation frequency compared to MDS42. In response to the overproduction of the toxic ORF238 protein, as well as in the presence of mitomycin-C, the mutation rate of MDS42

became significantly elevated, while the response of MDS42*polBdinBumuDC* was much less substantial.

#### Example 4

##### **MDS42*polBdinBumuDC* Provides Improved Stability to a Toxic Protein-Expressing Plasmid**

[0054] To demonstrate the surprising advantage of reduced genome bacteria comprising non-functional *polB*, *dinB* and/or *umuDC* genes, a plasmid-based mutation screen was designed. Plasmid pSin32 carries an inducible copy of *sinI*, coding for the SinI methyltransferase of *Salmonella enterica* serovar Infantis, cloned into the XhoI site of the pET3-His plasmid. SinI methylates the inner cytosines in DNA at GG(A/T)CC sites, producing 5-methylcytosine, thereby creating targets for the McrBC endonuclease, which cleaves DNA containing methylcytosine. A plasmid carrying methylated SinI sites (e.g. pSin32, self-methylated at its 8 SinI sites), therefore cannot establish itself in a mcrBC<sup>+</sup> host. When introduced into mcrBC<sup>-</sup> hosts, the plasmid is methylated when expression of *sinI* is induced, but can be maintained.

[0055] The *mcrBC* gene was deleted during production of MDS42 and accordingly all MDS42 strains are *mcrBC*<sup>-</sup>. The *mcrBC* gene was deleted from BL21(DE3) to create strain BL21(DE3)*mcrBC*<sup>-</sup>. Plasmid pSin32 was electroporated into MDS42-T7, MDS42*polBdinBumuDC*-T7 and BL21(DE3)*mcrBC*<sup>-</sup>. After 1 hour of recovery incubation at 37°C in 1 ml LB, 100µl of the transformed cultures were placed in 100 ml LB supplemented with ampicillin (Ap) and incubated at 37°C. From the remaining 900µl, plasmid DNA was isolated according to standard protocols. After 7 hours of incubation, the cultures reached O.D.<sub>540</sub> = ~0.2, at which point the samples were induced with IPTG (1 mM final concentration). Samples for plasmid preparation were also taken at this time (8-hour samples), followed by additional samples being taken every 2 hours, up to 18 hours, then at 24 and 36 hours of post-transformation growth. Purified pSin32 plasmid samples (9 from each strain) were then transformed into MDS42 (McrBC<sup>-</sup>) and MG1655 (McrBC<sup>+</sup>). By counting transformed MG1655

and MDS42 colonies for each plasmid sample, the relative number of mutated plasmids could be calculated. To obtain an absolute value for mutated plasmid numbers, each batch of electrocompetent MDS42 and MG1655 indicator strains was transformed with a control (pST76-A) plasmid carrying an Ap resistance cassette. The ratio of MG1655 and MDS42 transformants was then used as a correcting factor to calculate the absolute values for the number of mutated pSin32 plasmids for each sample.

**[0056]** Following transformation of BL21(DE3)*mcrBC*, MDS42-T7 and MDS42*polBdinBumuDC*-T7 with pSin32, it was found that, upon induction by IPTG, overproduction of the SinI enzyme had a moderate growth-inhibiting effect even in *McrBC*<sup>-</sup> strains (Figure 5). While this moderate toxicity leads to an elevation in the mutation rate of MDS42-T7, the effect is much weaker in MDS42*polBdinBumuDC*-T7 (Figure 6), supporting the findings discussed above.

**[0057]** Following IPTG –induction, plasmid samples were taken at regular intervals. The fraction of the plasmid sample that carried *sinI*-disabling mutations (unmethylated plasmids) was detected by transforming the plasmid samples back into MG1655 (*mcrBC*<sup>+</sup>). The total plasmid number per sample was determined by simultaneously transforming the samples into MDS42. After correcting each value with the transformant number from a control plasmid for each set of electrocompetent cells, the ratio of plasmids coding for functional/non-functional *sinI* was calculated. The results are illustrated at Figure 7.

**[0058]** Surprisingly, 96.7% of the starting (0 hour) plasmid sample, originating from MDS42, could not be established in MG1655. This indicated that, even in a host lacking T7 polymerase, spurious transcription of *sinI* had resulted in SinI expression, and consequently methylation of *sinI* sites. The methylated status of the SinI sites in the original plasmid sample was confirmed by their uncleavability by SinI.

**[0059]** Differences regarding clone stability in the different strains became evident after IPTG-induction of SinI expression. Thirty-six hours after transformation (28 hours after IPTG-induction), 51.7% of pSin32 harbored in BL21(DE3)*mcrBC* cells

carried mutations preventing the production of active SinI. This value was significantly lower in MDS42-T7 (25.8%). In MDS42*polBdinBumuDC*-T7, the fraction of mutated pSin32 plasmids was even lower (8.2%). The non-methylated status of the SinI sites on the plasmids carrying a mutated *sinI* gene was confirmed by their cleavability by SinI.

**[0060]** The accumulation of mutant plasmids in BL21(DE3)*mcrBC* and MDS42-T7 was due to a combined effect of stress-induced mutagenesis and growth inhibition by the SinI-expressing plasmid. Overproduction of the enzyme elevated mutation rates and reduced growth. In these slow-growing cultures, over time, SinI-inactivating mutations arose, which then, having resumed their normal growth rate, quickly outgrew the rest of the culture. In low-mutation-rate MDS42*polBdinBumuDC*-T7, SinI-inactivating mutations developed, on average, over a longer time period. Growth curve measurements of 50 independent colonies of MDS42-T7 and MDS42*polBdinBumuDC*-T7, all carrying the pSin32 plasmid, support this notion (Figure 8). An O.D.<sub>540</sub> value of 0.7 was used as a cutoff to indicate that a culture had overcome the growth-hindering effect of the induced plasmid. The average time taken for MDS42*polBdinBumuDC*-T7 to reach this level of density was significantly longer than for MDS42-T7 (727.8 and 571.8 minutes, respectively;  $P < 0.005$ , two-tailed, unpaired *t* test).

**[0061]** To verify that mutations had indeed taken place in the plasmids that allowed for growth in *McrBC*<sup>+</sup> cells, the *sinI* region of 8 different plasmid samples (taken from viable, pSin32-transformed MG1655 colonies) were sequenced (Figure 9). In seven out of the eight cases, a frameshift mutation had occurred in *sinI*, resulting in a new stop codon within the gene. The eighth case displayed an A to C transversion, resulting in the N880T mutation of the protein. Six out of the seven new stop codons caused by the frameshifts were located within the first 125 bp of the gene.

**[0062]** These results demonstrate a clear and unexpected practical advantage of reduced genome bacteria having non-functional genes encoding DNA polymerase II, IV and/or V particularly in an IS element-free genetic background. When SinI was overproduced, the *sinI* gene, carried on a plasmid, acquired loss-of-function mutations

approximately three times less frequently in MDS42*polBdinBumuDC* than in MDS42, and over five times less frequently than in BL21(DE3)*mcrBC*. Remarkably, after only 16 hours of overproduction in BL21(DE3)*mcrBC*, nearly half of all *sinI* genes encoded on the plasmids had suffered a disabling mutation.

[0063] The unexpectedly high ratio of mutated clones in the SinI-overexpressing culture cannot be explained solely by the stress-induced mutagenesis, the overall mutation rate of which is too low in absolute values (in the order of  $10^{-6}$  mutations/gene/generation) to cause such a dramatic effect. Rather, the phenomenon is in large part due to the growth inhibitory effect of the plasmid carrying the toxic gene. The chain of events is the following: Upon expression of a toxic gene, the growth rate of the cell is reduced. At the same time, mutation rate is increased by the stress. Once a mutant that no longer expresses the toxic function arises in the plasmid population, the cell harboring it can resume normal growth and become dominant in the culture. In reduced genome bacteria having one or more non-functional *polB*, *dinB* and/or *umuDC* genes, as exemplified by MDS42*polBdinBumuDC*, appearance of such mutants is delayed and the cells can produce the functional toxic product for an extended period of time. The advantage of strains such as MDS42*polBdinBumuDC* over parent strain MDS42 and the commonly used production strain BL21(DE3) is striking and increases as the severity of the stress of overproducing a product increases. Bacterial strains with high genomic stability as described herein are particularly valuable in therapeutic applications, where fidelity of the nucleic acid and/or protein product is of primary importance. Bacteria of the invention are also surprisingly useful where long-term continuous culture conditions are required.



What is claimed is:

1. A reduced genome *Escherichia coli* bacterium, having a genome that is genetically engineered to be from about 5% to about 30% smaller than the genome of its native parent strain, wherein said bacterium lacks all insertion sequences and comprises at least one non-functional gene selected from the group consisting of *polB*, *dinB*, and *umuDC*.

2. The bacterium of claim 1, wherein said bacterium has deleted therefrom at least the following DNA segments: b0245-b0301, b0303-b0310, b1336-b1411, b4426-b4427, b2441-b2450, b2622-b2654, b2657-b2660, b4462, b1994-b2008, b4435, b3322-b3338, b2349-b2363, b1539-b1579, b4269-b4320, b2968-b2972, b2975-b2977, b2979-b2987, b4466-4468, b1137-b1172, b0537-b0565, b0016-b0022, b4412-b4413, b0577-b0582, b4415, b2389-b2390, b2392-b2395, b0358-b0368, b0370-b0380, b2856-b2863, b3042-b3048, b0656, b1325-b1333, b2030-b2062, b2190-b2192, b3215-b3219, b3504-b3505, b1070-b1083, b1878-b1894, b1917-b1950, b4324-b4342, b4345-b4358, b4486, b0497-b0502, b0700-b0706, b1456-b1462, b3481-b3484, b3592-b3596, b0981-b0988, b1021-b1029, b2080-b2096, b4438, b3440-b3445, b4451, b3556-b3558, b4455, b1786, b0150-b0153 and b2945 of the *E. coli* K-12 strain MG1655.

3. The bacterium of claim 2, wherein the native parent strain of said bacterium is a B strain.

4. The bacterium of claim 3, wherein the native parent strain of said bacterium is strain BL21(DE3).

5. The bacterium of claim 2, wherein the native parent strain of said bacterium is a K12 strain.

6. The bacterium of claim 5, wherein the native parent strain of said bacterium is K12 strain MG1655.

7. The bacterium of claim 6, wherein said bacterium is MDS42.

8. The bacterium of claim 6, wherein said bacterium is MDS66.

9. The bacterium of any preceding claim having at least two non-functional genes selected from the group consisting of *polB*, *dinB*, and *umuDC*.

10. The bacterium of claim 9, having non-functional *polB* and *dinB* genes.

11. The bacterium of claim 10, having a functional *umuDC* gene.

12. The bacterium of claim 10, having a non-functional *umuDC* gene.

13. The bacterium of any preceding claim comprising a heterologous nucleic acid.
14. The bacterium of claim 12 wherein said heterologous nucleic acid comprises a nucleic acid encoding a polypeptide operatively linked to an expression control sequence.
15. A method for producing a polypeptide comprising incubating the bacterium of claim 14 under conditions suitable for expressing the polypeptide and collecting the polypeptide.

FIGURE 1

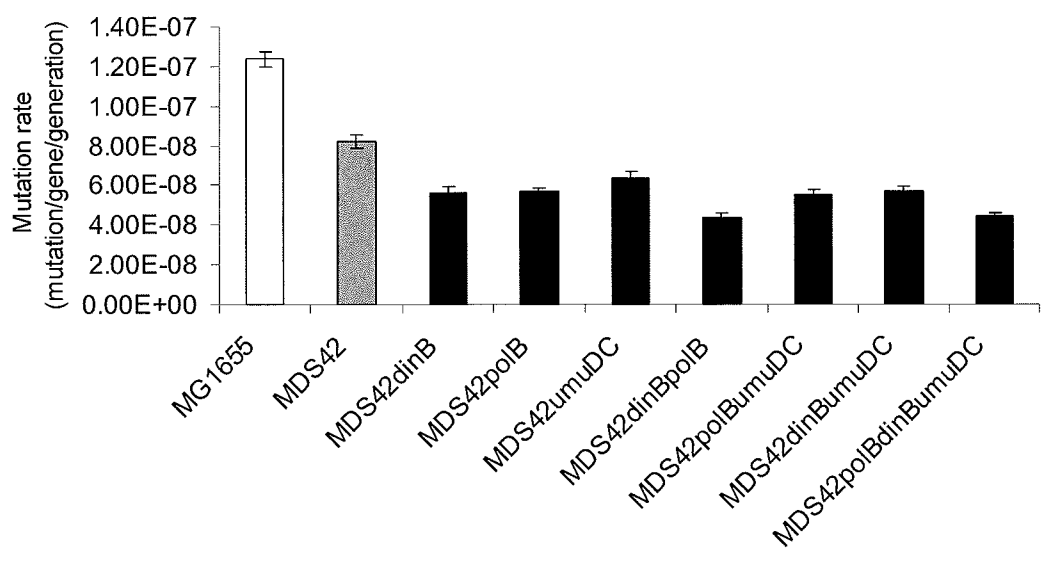


Figure 2

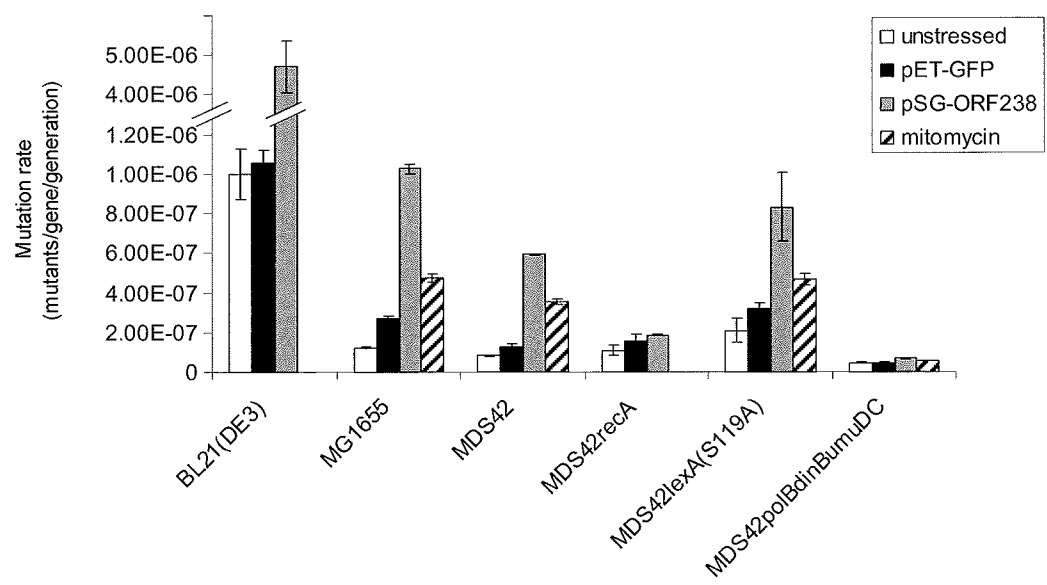


Figure 3

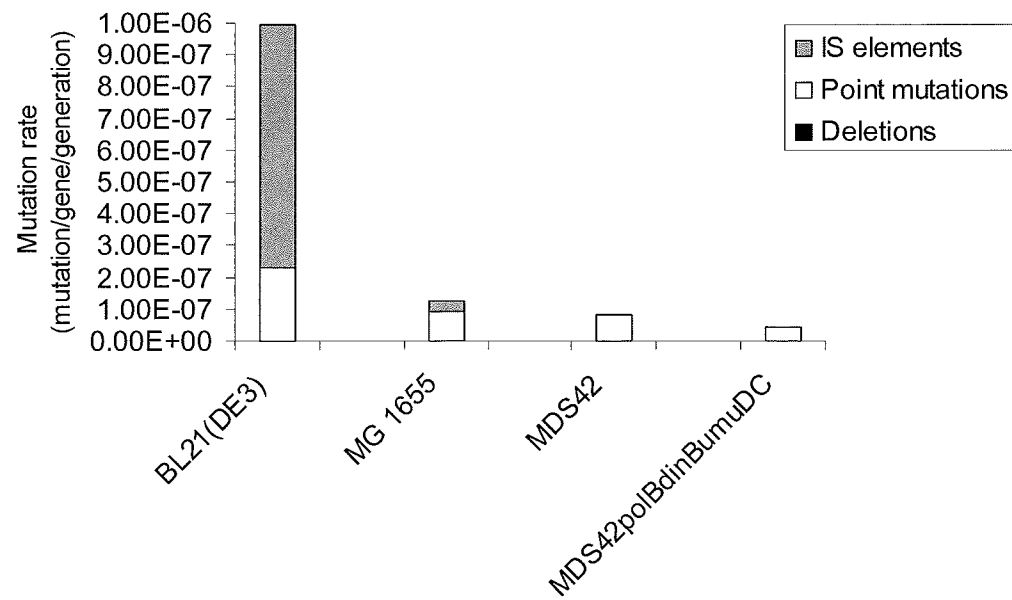


Figure 4

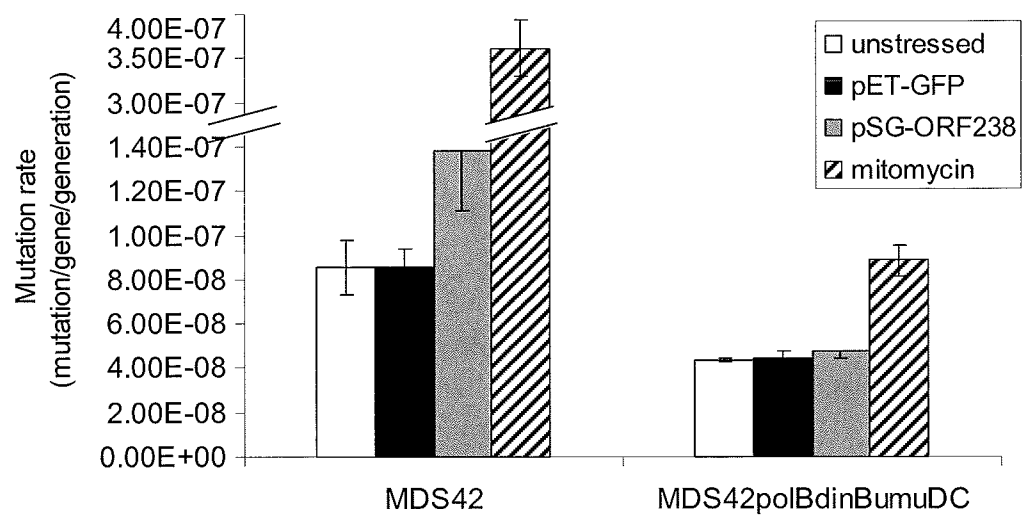


Figure 5

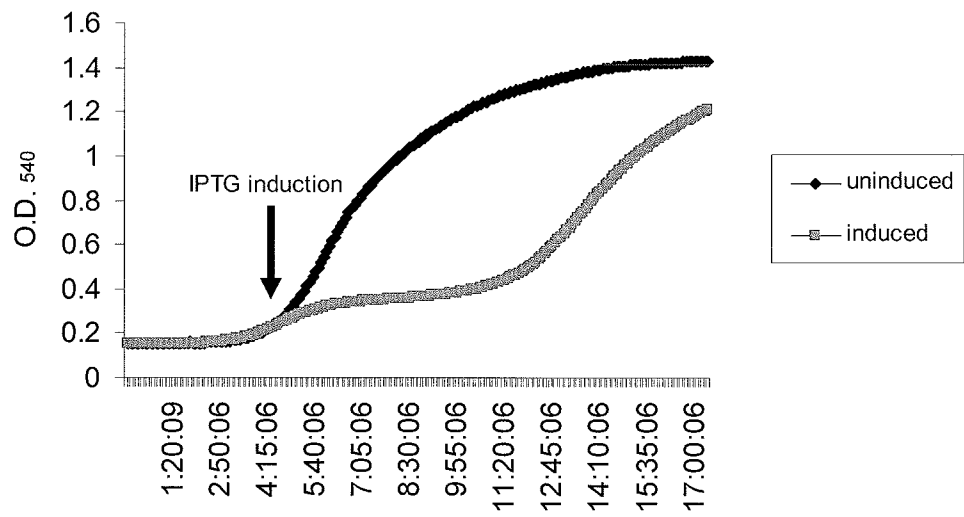


Figure 6

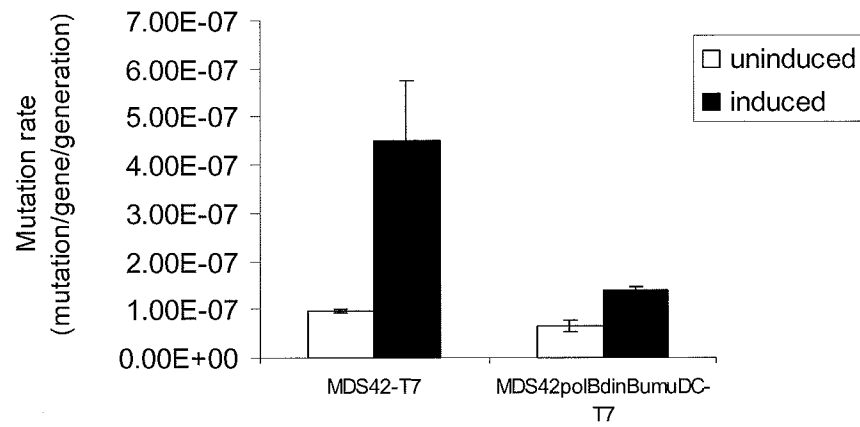




Figure 7

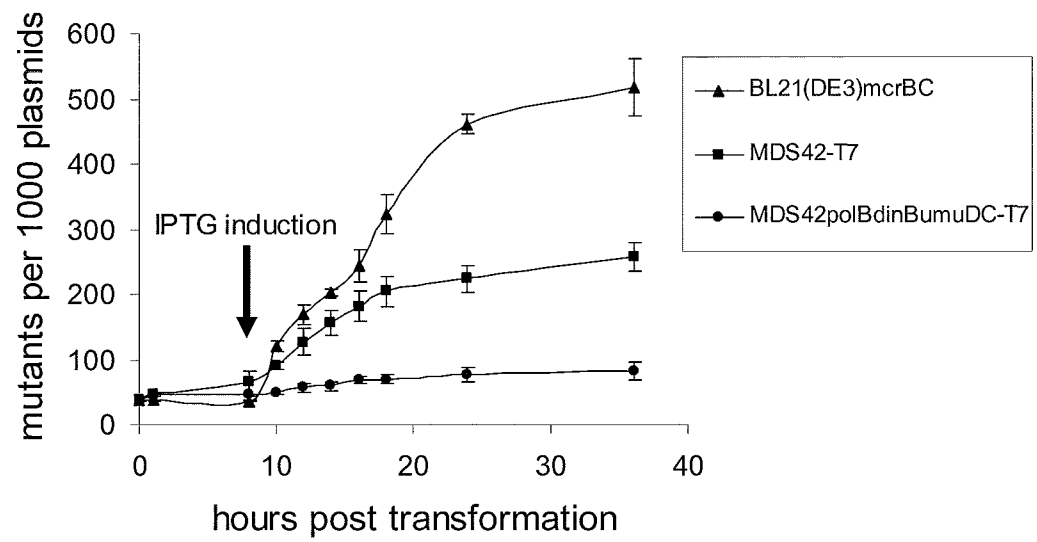


Figure 8

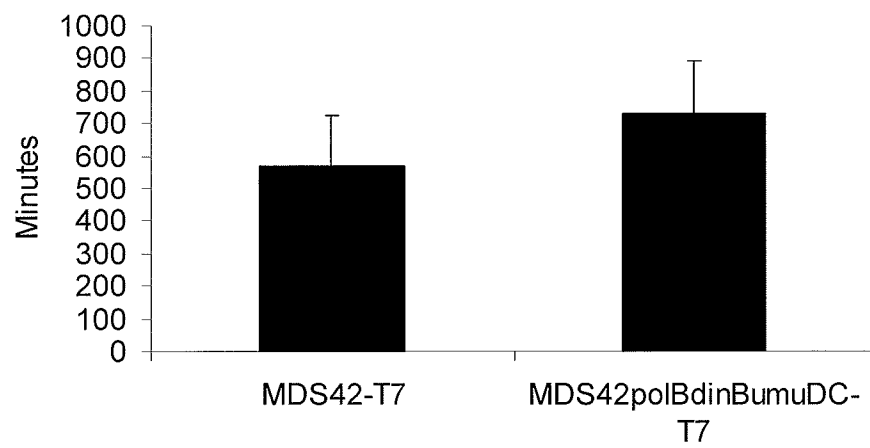
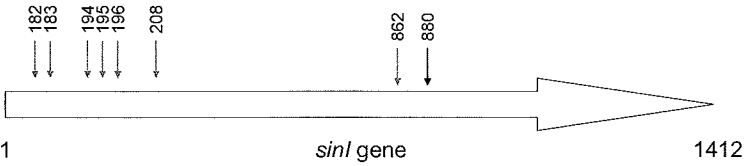


Figure 9



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2012/061027

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> INV. C12N1/08 C12N15/10 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/024756 A2 (SCARAB GENOMICS LLC [US]; CAMPBELL JOHN W [US]; BLATTNER FREDERICK R []) 1 March 2007 (2007-03-01) the whole document	1-15
A	----- US 2003/138937 A1 (BLATTNER FREDERICK R [US] ET AL) 24 July 2003 (2003-07-24) cited in the application the whole document -----	1-15
<div style="display: flex; justify-content: space-between; align-items: center;"> <div style="display: flex; align-items: center;"> <input type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div style="display: flex; align-items: center;"> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search  <div style="text-align: center; font-size: 1.2em;">15 February 2013</div>	Date of mailing of the international search report  <div style="text-align: center; font-size: 1.2em;">26/02/2013</div>	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  <div style="text-align: center; font-size: 1.2em;">Stoyanov, Borislav</div>	

# INTERNATIONAL SEARCH REPORT

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