Recent advances and versatility of MAGE towards industrial applications

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Received: 9 September 2015 / Revised: 31 October 2015 / Accepted: 4 November 2015 / Published online: 7 November 2015
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Abstract The genome engineering toolkit has expanded significantly in recent years, allowing us to study the functions of genes in cellular networks and assist in over-production of proteins, drugs, chemicals and biofuels. Multiplex automated genome engineering (MAGE) has been recently developed and gained more scientific interest towards strain engineering. MAGE is a simple, rapid and efficient tool for manipulating genes simultaneously in multiple loci, assigning genetic codes and integrating non-natural amino acids. MAGE can be further expanded towards the engineering of fast, robust and over-producing strains for chemicals, drugs and biofuels at industrial scales.

Keywords Multiplex automated genome engineering · Genome · Drugs · Chemicals · Biofuels

Abbreviations

aaRS	Aminoacyl-tRNA synthetase
CAGE	Conjugative assembly genome engineering
CoS-MAGE	Coselection MAGE
DXP	1-Deoxy-D-xylulose-5-phosphate
GRO	Genomically recoded organism
MAGE	Multiplex automated genomic engineering
MMR	Mismatch repair
NSAAs	Non-standard amino acids
ORF	Open reading frame
RBS	Ribosome binding site

RF1

Release factor 1

Introduction

Recent advances in synthetic biology have led to development of novel genetic parts, devices and circuits towards biotechnological applications. In the past decade, synthetic biology has gained more scientific interest towards the building of synthetic circuits in a controllable and predictive manner (Purnick and Weiss 2009; Khalil and Collins 2010; Singh 2014). A number of synthetic devices and circuits such as riboregulators (Callura et al. 2010; Klauser and Hartig 2013; Green et al. 2014), riboswitches (Bayer and Smolke 2005; Wang et al. 2008; Edwards et al. 2010), oscillators (Elowitz and Leibler 2000; Stricker et al. 2008; Tigges et al. 2009; Mondragón-Palomino et al. 2011) and biologic gates (Tamsir et al. 2011; Ausländer et al. 2012; Moon et al. 2012) have been designed and characterized in a wide range of hosts.

However, the ultimate goal of synthetic biology is to design and build biological systems that reprogramme cellular machinery, produce energy, provide food, and maintain and enhance health and environment. Globally research in the synthetic biology and its application are on high priority (Na et al. 2013; Ausländer and Fussenegger 2013; Soma et al. 2014). Synthetic circuits sense signals and process a coordinated therapeutic output such as control of cancer (Culler et al. 2010; Nissim and Bar-Ziv 2010), T cell population controllers (Chen et al. 2010), blue-light-triggered glucose homeostasis for cure of diabetes (Ye et al. 2011) and artificial insemination (Kemmer et al. 2011).

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Recently, applications of synthetic biology towards production of valuables such as drugs, fuels, antibiotics and therapeutics or novel circuits with desired functions have gained more scientific attention. At the same time, the increasing demand and supply of biologically produced valuables has become a great priority. However, natural strains do not typically possess fast and robust cellular machinery to produce sufficient quantities of these valuables. Thus, genome engineering is a path towards improving the capacity of cellular machinery. Genome engineering and synthetic genomics are currently among the most promising technologies in terms of applied science and industrial innovation, and they have gained further scientific interest because of increasing ease in how we may manipulate genomes by insertion, deletion and modification (Wang et al. 2009; Gibson et al. 2010).

There is a great range and accessibility to genome sequencing data and gene synthesis technologies that are ideal for the redesigning of genomes or pathways (Gibson et al. 2009; Kosuri et al. 2010; Kim et al. 2012) and the synthesis of genomes (Gibson et al. 2010; Lartigue et al. 2009). Recently, Wang et al. (2009) have developed multiplex automated genomic engineering (MAGE) which is a rapid, specific and efficient tool for genome modifications (insertion, deletion, mutation) simultaneously in multiple loci and generating billions of genome diversity. Wild type genome does not contain the capability to produce industrially useful products because they lack stronger promoters, ribosome binding sites or down regulating regulatory genes and some time incomplete pathways. Some researchers have addressed the problem using genome engineering and manipulation by gene synthesis as an attractive approach for producing a highly reprogrammed genome. MAGE was implemented in a range of applications such as changing the genetic code (Isaacs et al. 2011, incorporation of non-standard amino acids (Lajoie et al. 2013; Rovner et al. 2015) and insertion of His-tags (Wang et al. 2012b). This approach can be further expanded towards the designing of synthetic genomes (Haimovich et al. 2015) for the better understanding of molecular mechanism and for the creation of more economical cell factories. However, genome synthesis remains quite an expensive and laborious process. The purpose of this review is to describe and use of MAGE and its recent advances in the quick and efficient manipulation of *Escherichia coli* genome.

Basic design and procedure of MAGE

MAGE is a novel and versatile tool, demonstrating one of breakthroughs of synthetic biology towards targeted genome engineering. We provide here basic ideas presenting how MAGE can be designed and simultaneously target a

number of genetic loci on the chromosome for modification in cells towards the generation of combinatorial genomic diversity. The biological process of MAGE is based on the inactivation of mismatch repair (MMR) system by the *mutS* gene and the lambda (λ) red recombinase systems (Exo, Beta and Gam). As shown in Fig. 1, MAGE facilitates the rapid and continuous generation of sequence diversity at a number of targets on the chromosome across a large population of cells through the repeated introduction of single stranded oligos. The single stranded oligos should be 90 bases long in total with both the ends containing 20–35 bases of homology for the regions of choice for modification (Wang et al. 2009).

The technique of MAGE is practically begun by growing the cells at 30 °C until the cell density reaches mid-log phase. In this process, the λ -red proteins are expressed under the control of pL promoter which is regulated by temperature sensitive λ CI. The λ CI represses the expression of λ -red proteins. The cells are transferred to 42 °C for 15 min for heat shock induction where λ CI is inactivated due to high temperature and pL promoter expresses the λ -red proteins. The Exo protein degrades dsDNA in the 5'–3' direction. The Beta protein binds to ssDNA and generates recombination while Gam plays a key role in binding to the RecBCD protein complex and subsequently preventing this complex from binding to dsDNA ends (Karakousis et al. 1998; Datta et al. 2008). It also helps to induce the high efficiency of ssDNA recombination (Ellis et al. 2001; Costantino and Court 2003; Sharan et al. 2009). The cells are then moved to 4 °C to repress λ -red and prevent degradation, and then washed with chilled distilled water thrice. A pool of single stranded oligos is introduced into cells via electroporation and these oligos become incorporated into the lagging strand of the replication fork during DNA replication (Ellis et al. 2001).

Growth medium is added to the culture, which is then transferred to 30 °C for 2–3 h for the recovery of cells with different sequence diversity. MAGE cycling should be repeated many times as required by the experimental design. Each cell of the generated heterogeneous population contains a different set of mutations. There are a number of applications of MAGE which facilitates the rapid and continuous generation of a diverse set of genetic changes including for example, mismatches, insertions, and deletions. As depicted in Fig. 2, oligo mediated allelic replacement is the capability of introducing a number of genetic changes at high efficiency. With MAGE, up to 30 bp mismatch mutations and insertion could be possible (Fig. 2a, b) while up to the 45 kbp may be chromosomally sequences deleted (Fig. 2c) and two-state hybridization free energy delta G between oligo and the targeted complement region in the genome was also predicted. It indicates that a pool of oligos with generate sequences have

Fig. 1 Basic design and cycling of MAGE which begin with growing the cells at 30 °C until cell density reaches the mid-log phase and lambda (λ) red proteins are expressed under the control of pL promoter which is regulated by temperature sensitive CI. Then cells are moved to 42 °C for 15 min for the heat shock induction of λ -red proteins. Cells are moved to 4 °C to repress the λ -red and prevent degradation. Cells were subsequently washed thrice and resuspended in chilled distilled water. Single stranded oligos were introduced into cells via electroporation and incorporated into the lagging strand of the replication fork during DNA replication. The cells were kept at 30 °C for 2–3 h for recovery of generated sequences diversity before proceeding into the next MAGE cycle

Fig. 2 Oligos mediated allelic replacement efficiency.

a Mismatch mutations introduced up to 30 bp.

b Insertion of exogenous sequences of up to 30 bp.

c Deleting up to 45 kbp of chromosomal sequence via single oligo and **d** two-state hybridization energy delta G between the oligo and the targeted sequence.

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lesser frequency of incorporation than highly homologous sequences. MAGE also provides a highly efficient, inexpensive and automated solution to concurrently modify many genomic locations across different length scales from the nucleotide to genome level (Wang et al. 2009).

Wang et al. (2009) have further explored the potential of MAGE for optimisation of the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway in *E. coli* for the over-production of lycopene. A total of 20 endogenous genes (*dxs*, *dxr*, *ispD*, *ispE*, *ispG*, *ispH*, *idi*, *ispA*, *appY*, *rpoS*, *crl*,

elbA, *elbB*, *yjiD*, *purH*, *rnlA*, *yggT*, *ycgZ*, *ymgA*, *ariR*) are documented for increasing the lycopene yield that were targeted for fine tuning of translation through the degenerate RBS which is flanked with gene homology (Fig. 3a). The replaced RBS (red) could enhance the translational efficiency of genes. Whereas, 4 targeted genes for knockout (blue) were used by introducing the two nonsense mutations that interrupted gene function. As shown in Fig. 3b, the black bars show the growth rate of isolated variants (EcHW2a–f) relative to the ancestral EcHW1 strain whereas the white bars show the lycopene production. Colour-coded labels in each white bar represent the genetic changes observed by sequencing. As shown in

Fig. 3c, the changes of lycopene biosynthesis pathway of isolated strains (EcHW2a–f) with relevant genes, blue labels indicate knockout genes and red labels indicate RBS tuning (Wang et al. 2009), generating over 4.3 billion combinatorial genomic variants after which screening revealed variants that were now capable of producing five-fold more lycopene.

However, one of major identified limitations of MAGE (Wang et al. 2009) was found in the *E. coli* ECNR2 strain (knockout of *mutS*). This strain in losing *mutS* can suffer mutations more frequently, impacting in vivo fitness and experimental fidelity. In order to address this issue, Ryu et al. (2014) have recently developed the lambda red

Fig. 3 Optimization of DXP pathway for lycopene production in *E. coli*. **a** Genomic locations of 24 targeted genes with the RBS optimization (red) and knockout (blue). The gene knockout was done by introducing two nonsense mutations in oligo. **b** Black bars show the growth rate of high lycopene producing strains (EcHW2a–f) relative to the ancestral EcHW1 strain. Whereas white bars show lycopene production in ancestral and mutant strains. Colour-coded

labels in each white bar show genetic changes obtained by sequencing and **c** Genetic modifications lycopene pathway of isolated variants EcHW2a–f; and blue labels indicate knockout targets and red labels indicate RBS tuning. Figure reproduced with permission from Nature (Wang et al. 2009) © 2009 Macmillan Publishers Ltd. (Color figure online)

portable system which transiently inactivates the *mutS* gene. This system is strain independent and thus may function within any *E. coli* strain, and after screening of best producer strains, the system can be removed from the hosts. This system has found specific use in the randomising the RBS of *DXS* and *IPI* genes for high lycopene production.

Exploring the potential of MAGE

Recently, MAGE has expanded into a wide range of biotechnological and industrial applications, although the technique is still in its infancy stage and not implemented yet into a wide number of industrial organisms. However, MAGE represents a versatile and powerful toolkit because of its simplicity, rapid experimental practice and highly efficient genome engineering capability (Jeong et al. 2013).

Replacement of stop codons

MAGE has been used for the site-specific replacement of all 314 TAG stop codons with synonymous TAA codons (Fig. 4) in parallel across 32 *E. coli* strains. The genome was divided into 32 regions each containing the 10 TAG stop codons. In parallel, MAGE has been performed to all 10 TAG::TAA codon changes in a single strain for each

genomic location. These partially recoded strains are paired where a targeted position of one strain (called as donor) was transferred into a second strain (called as recipient), allowing the modified genomic regions by conjugative assembly genome engineering (CAGE). This 5-stage process is used to transfer the genomic fragments (~154 kb to ~2.3 Mb) in a controlled manner to a single recoded strain, which lacks the TAG stop codon. Therefore, all TAG codons have been converted to TAA, and the *prfA* gene was deleted to inactivate TAG translational termination (Isaacs et al. 2011). It was further demonstrated that this approach could be useful for measuring the individual recombination frequencies, confirming viability and also identifying related phenotypes. This group has also developed the CAGE technique to combine these sets of codon changes into genomes with 80 precise changes towards the higher-order strain engineering. A number of advantages of this method that are chromosomes can be easily editable and they can also be employed as evolvable templates for permitting the investigation of huge genetic landscapes (Isaacs et al. 2011).

A similar recent study by Lajoie et al. (2013) has constructed a genomically recoded organism (GRO) by replacing all known UAG stop codons (*E. coli* MG1655) with UAA codons. This GRO permits the deletion of release factor 1 (RF1) and allows the reassignment of UAG

Fig. 4 Experimental set up for re-assigning all 314 TAG codons to TAA in *E. coli*. The genome was divided into 32 regions, each containing 10 TAG stop codons and MAGE has been performed all 10 TAG::TAA codon changes in a single strain for each genomic region. These partially recoded strains were paired such that a targeted genomic region of one strain (donor) was transferred into a second strain (recipient), which allows the changes in genomic

regions by CAGE. In a controllable manner the genomic fragments have been transferred from size of ~154 kb to ~2.3 Mb and lacks of TAG stop codon. Once All TAG codons are changed into TAA, the *prfA* gene will be deleted to inactivate TAG translational termination. Figure reproduced with permission from Science (Isaacs et al. 2011) © 2011 AAAS

translation function. There is a three step procedure to engineer GRO with reassigned UAG codon (Fig. 5). As we know that wild-type *E. coli* strain (MG1655) has 321 known UAG codons that are decoded as translation stops by releasing factor (RF1). In the first step, all UAG codons are converted to UAA, alleviating the dependence on RF1 for termination and in second step they were used to eliminate the natural codon function through abolishing the

Fig. 5 Design of a GRO with a re-assigned UAG codon. Wild-type *E. coli* MG1655 has 321 known UAG codons that are decoded as translation stops by RF1 (UAG and UAA). In a first step (1) Removal of codons that are converted all known UAG codons into UAA. (2) Elimination of natural codon function by abolishing the UAG translational termination by deleting RF1, and (3) Expanding the genetic code by introducing orthogonal aminoacyl-tRNA synthetase (aaRS) and tRNA to reassign UAG which allows the incorporating nonstandard amino acids (NSAAs). Figure reproduced with permission from Science (Lajoie et al. 2013) © 2013 AAAS

UAG translational termination by deleting RF1. In the final step, genetic codons could be expanded by introducing the orthogonal aminoacyl-tRNA synthetase (aaRS) and tRNA to re-assign UAG which offers a sense codon capable of integrating the non-standard amino acids (NSAAs) that leads to a new chemical property (Lajoie et al. 2013). This study offers an expansion of the in vivo chemical diversity of proteins. The GRO exhibited increased resistance against T7 phage infection that demonstrates new genetic codes could be beneficial for increased viral resistance. This study can be further expanded within *E. coli* and also towards other diverse bacteria which may have utility in industrial applications.

Insertion of T7 promoters

Whilst Wang et al. (2012a) have used MAGE to scarlessly modify genomes, insertions of more than 10 bases remains inefficient. Insertion limits could be improved significantly by the selection of highly modified chromosomes, through the newly developed technique of 'coselection' MAGE (CoS-MAGE), used recently to optimize the biosynthesis of aromatic amino acid derivatives by the combinatorial insertion of multiple T7 promoters simultaneously into 12 genomic operons. In this procedure, cells undergoing cycles of MAGE were enriched by a coselection phase through phenotypic selection of a revertible genomic locus (*malK*, *galK*, *cat* or *bla*) and used for iterative MAGE cycles. Twelve genomic operons were targeted for insertion of T7 promoter sequence upstream of the first relevant open reading frame. For instance, T7 promoter sequence targeting *trpE* was inserted upstream of the first relevant ORF. TrpR box, tryptophan repressor binding sequence, sigma 70 and endogenous promoter have been targeted (Fig. 6).

Insertion of hexa-histidine sequences

In another study, Wang et al. (2012b) have employed MAGE to modify and co-purify large protein complexes from *E. coli* to reconstitute functional synthetic proteomes in vitro. This is one of the incredible pieces of work that has demonstrated the utility of genomic editing for purification of any desired enzymes or proteins. There, more than 110 MAGE cycles were performed and the insertion of hexa-histidine sequences (towards assisting protein purification) into 38 essential genes was made in vivo encoding the complete translation machinery of the cell. In related work, this was also used for in vitro protein synthesis using the PURE (Protein synthesis Using Recombinant Elements) translation system (Shimizu and Ueda 2010) that can be used for cell free protein synthesis with high yield recovery. Nowadays, PURE systems are available for industrial scale production of desired valuables

Fig. 6 CoS-MAGE set up for enriching highly modified genomes. MAGE is used to enrich by a coselection stage through phenotypic selection of a revertible genomic locus (*malK*, *galK*, *cat* or *bla*) and also used for iterative MAGE cycles (left). Twelve 12 genomic

locations have been targeted by insertion of T7 promoter sequence (bottom right) upstream of *trpE* for example. Figure reproduced with permission from Nature Methods (Wang et al. 2012a) © 2012 Macmillan Publishers Ltd

including chemicals, drugs, enzymes, proteins and more from the cell free systems.

One of the most high throughput applications of MAGE was performed by Bonde et al. (2015) who investigated large-scale mutagenesis that requires 100–1000 unique oligos, which are expensive for synthesis and not feasible for scale-up. A novel method has been developed to amplify the oligos from microarray chips that could be directly used in MAGE to manipulate thousands of genomic loci. The feasibility of large-scale mutagenesis by inserting T7 promoters upstream of 2585 operons in *E. coli* has been demonstrated.

With increasing concerns of climate change and environmental issues, an urgent need arises to develop bio-based processes for the production of chemicals, fuels and materials from renewable sources. Metabolic engineering has the potential to produce high yields of desired products in a cost effective manner by the extension of pathways or editing of genomes towards industrial scale at competitive prices. MAGE shows a rapid, specific and efficient tool for generating billions of genome diversity that can be useful for high level production and optimization of biosynthetic pathways (Wang and Church 2011; Jeong et al. 2013; Isaacs et al. 2011; Lajoie et al. 2013) and this technique has dramatically increased our ability to engineer cells in a directed and combinatorial manner towards the development of flexibly programmable cells (Carr et al. 2012; Esvelt and Wang 2013).

Conclusions and future perspectives

MAGE is still its in infancy, and has been developed primarily for *E. coli*. There is a further arising need to expand the MAGE technique also into other industrially significant

organisms. The price of oligos remains high, however there is a hope in near future that costs may be reduced by the establishing of advances in gene synthesis technology. One of other major limitations of MAGE is that it is hard to incorporate foreign gene of more than 1 kb into the genome. Therefore, needs improvement beyond its current state whereby only small regions of genome can be modified. It can be reasonably anticipated that in the present and near future we would wish to integrate or modify complete sets of pathways for production of desired valuable products.

One of potential applications is in the genetic engineering of natural *E. coli* strains towards the production of high levels of proteins or enzymes through the exchange of promoters and/or RBS regions. Typically, making the genetic constructs for the high level production of recombinant proteins or enzymes requires huge laboratory practice, time and expensive enzymes. MAGE can play a remarkable role towards exchanging promoters or RBSs in natural strains for the optimization of biosynthetic pathways, followed by screening assays for the best industrial strains for recovered proteins, enzymes, drugs, metabolites and biofuels. Recent advances in this field and the exploration of the full potential of MAGE will be useful for better understanding of molecular mechanisms and robust strain engineering, an essential tenet of our abilities in synthetic biology.

Acknowledgments The authors thank D.K. Chaudhary and S. Prakash for their fruitful discussion and suggestion for the improvement of the manuscript. The authors also appreciate anonymous reviewers of the journal for their valuable comments and suggestions to improve the quality of the manuscript.

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