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Direct transfer of a *Mycoplasma mycoides* genome to yeast is enhanced by removal of the mycoides glycerol uptake factor gene *glpF*

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Abstract

We previously discovered that intact bacterial chromosomes can be directly transferred to yeast host cell where they can propagate as centromeric plasmids by fusing bacterial cells with *Saccharomyces cerevisiae* spheroplasts. Inside the host any desired number of genetic changes can be introduced into the yeast centromeric plasmid to produce designer genomes that can be brought to life using a genome transplantation protocol. Earlier research demonstrated that the removal of restriction-systems from donor bacteria, such as *Mycoplasma mycoides*, *Mycoplasma capricolum*, or *Haemophilus Influenzae* increased successful genome transfers. These findings suggested that other genetic factors might also impact the bacteria to yeast genome transfer process. In this study, we demonstrated that the removal of a particular genetic factor, the glycerol uptake facilitator protein gene *glpF* from *M. mycoides*, significantly increased direct genome transfer by up to 21-fold. Additionally, we showed that intact bacterial cells were endocytosed by yeast spheroplasts producing organelle-like structures within these yeast cells. These might lead to the possibility of creating novel synthetic organelles.

Keywords: *Saccharomyces cerevisiae*, spheroplasts, whole genome transfer, synthetic cell, synthetic organelle.

Research into genetically altered and synthetic cells is of great interest, both in furthering the understanding of protocell evolution as well as opening up numerous possibilities for novel designs^{1,2}. In recent years, cell design has been explored from a variety of perspectives³. A top-down approach is frequently used in which genes are consecutively removed from or added to the starting organism⁴. A more powerful technology for creating designer microbes is bottom-up *de novo* synthesis of genome fragments followed by assembly of whole genomes in yeast⁵ and finally “booting up” the assembled genome by genome transplantation from yeast into a recipient cell cytoplasm⁶. This breakthrough technology was used to create a bacterial cell (JCVI-syn1.0) with a chemically synthesized genome⁷ and more recently the reduced derivatives JCVI-syn2.0 and JCVI-syn3.0. The latter has a greatly reduced synthetic genome driven by a near minimal set of essential genes⁸. Homologous recombination-based assembly in yeast has been used to construct large synthetic DNA molecules from multiple species^{9–11}; however at present, genome transplantation has only been achieved in the atypical bacteria called mollicutes^{12,13}. We aspire to expand this to other bacterial species in order to enable the possibility of creating designer microbes that can act as engineering platforms or chassis for various functional tests.

The first step in this technology is to demonstrate that the bacterial genome of interest can be cloned in yeast host cells. To reach this milestone, our group previously showed that many bacterial species governed by a standard genetic code could be successfully cloned in yeast^{14–17}. During the process of cloning intact genomes in yeast host cells, we found that genomes can be directly transferred from intact bacteria to yeast in the presence of polyethylene glycol^{16,18} without the necessity of first purifying the genomic DNA. We also showed that the removal of restriction endonuclease systems from the donor bacteria increased the number of successful bacteria to yeast genome transfers in all species tested (*M. mycoides*, *M. capricolum* and *H. influenzae*); however, the success of genome transfer between some species differed significantly (~ 15,000 yeast colonies formed when genomes transferred from *M. mycoides*, ~29,000 yeast colonies formed when genomes transferred from *M. capricolum* and only ~900 yeast colonies formed when genomes transferred from *H. influenzae*)¹⁶. This encouraged us to search for other genetic factors that could improve the frequency of genome transfers.

In our search efforts, we employed intermediate strains generated during the creation of JCVI-syn3.0⁸. All these strains have been assembled in yeast and all contain centromere (CEN6) and auxotrophic selection marker (HIS3) which allows for replication in yeast. We began screening eight RGD1 (Reduced Genome Design) strains whose genomes were comprised of 7/8th JCVI-syn1.0⁷ DNA and 1/8th of a reduced genomic fragment. A combination

of all 1/8th reduced fragments was used to construct the strain designated as JCVI-syn2.0⁸. These eight RGD1 variations, designated sequentially as RGD1-1 (segment 1 reduced, segments 2,3,4,5,6,7,8 wild type) to RGD1-8, each with a different 1/8th reduced fragment, were tested and the number of yeast colonies observed was our measure of genome transfer efficiency¹⁶.

The number of genome transfer events observed with each of the RGD1 strains was compared to the number of transfer events obtained with the original minimal JCVI-syn1.0 genome (**Figure 1**). This showed that removal of genes in RGD1-2, RGD1-3 and RGD1-6 improved genome transfer, with removal of genes in RGD1-3 demonstrating the greatest positive effect. RGD1-2 showed an increase in number of transformants between four and six times that of the original, whereas RGD1-6 showed slightly less effect with either similar numbers or up to four times the number of transformants. In contrast, a consistently higher increase was seen with RGD1-3, which displayed an increase of between seven and nineteen times that of the original bacterial genome. The other five RGD1 strains did not make a substantial difference to the genome transfer process and were not examined further.

Next, two of the three RGD1 strains that produced the greater number of genome transfers were investigated more closely to determine the genes within these strains that were responsible for the variances in the genome transfer efficiencies. Further experiments with RGD1-2 could not attribute any individual genes to substantially increase genome transfers and it was concluded that any improvement was most likely a synergistic effect rather than the effect of a single gene (Data not shown). We then focused on the RGD1-3 strain. It was retested together with a similar strain (RGD1-3 *glpOKF*) to which three genes as a single cluster were added (*glpO*, *glpK* and *glpF*). One of the initial reasons for choosing this cluster was that glycerol metabolism in some mycoplasma species can result in formation of virulence factors (hydrogen peroxide)¹⁹. As shown in **Figure 2a**, we observed a dramatic decrease in genome transfer efficiency as compared to RGD1-3 when using this strain. We suspected that one or all three genes within this cluster was affecting genome transfer. We then created four additional strains in our best-performing strain, JCVI-syn1.0 Δ RM, which lack six restriction systems¹⁶: JCVI-Syn1.0 Δ RM Δ *glpO*, JCVI-Syn1.0 Δ RM Δ *glpK*, JCVI-Syn1.0 Δ RM Δ *glpF* and JCVI-Syn1.0 Δ RM Δ *glpOKF*. These four strains prevent the production of an oxidase, kinase and a facilitator, respectively, either separately or all at once. In **Figure 2b** we reproduced our previous data showing that the removal of restriction modification systems resulted in an increase in efficiency of genome transfer and also showed that the additional deletion of either the oxidase or kinase gene yielded comparable numbers of genome transfer events to the control. Interestingly, the removal of the facilitator protein gene yielded a very

significant increase in efficiency, which was comparable to the removal of all three proteins simultaneously. Thus, we conclude that the facilitator protein was the sole contributor to the observed improvements in obtaining yeast transformants.

Finally, we compared genome transfer efficiency from our key strains: JCVI-syn1.0, JCVI-syn1.0 Δ RM, JCVI-syn1.0 Δ glpF, JCVI-syn1.0 Δ RM Δ glpF and JCVI-syn2.0 (restriction systems are removed in this strain), JCVI-syn2.0 Δ glpF (**Figure 3**). Once again, we confirmed that the removal of either restriction systems from JCVI-syn1.0 or the *glpF* gene greatly increased the number of gene transfer events while combining all deletions resulted in a multiplicative effect (JCVI-syn1.0 Δ RM Δ glpF). The genome transfer efficiency was subsequently compared to JCVI-syn2.0, with an engineered organism that had 512 of the genes present in JCVI-syn1.0 removed (including the 6 restriction-modification systems), and JCVI-syn2.0 with an additional excision at the *glpF* gene locus. We showed that removal of 499 genes from JCVI-Syn1.0 Δ RM resulted in a subdued effect with regards to genome transfer efficiency as compared to removal of *glpF* in the same background (**Figure 3**). Furthermore, we showed that when *glpF* is deleted in JCVI-syn2.0 the genome transfer improved again. To check whether transferred genomes were fully intact, we genotyped 15 yeast colonies that came from Δ RM Δ glpF using a multiplex PCR approach¹⁶. All 15 colonies carried intact genomes (**Figure S1**).

In our previous studies we observed that JCVI-syn2.0 produced cells with significantly increased cell size⁸. This prompted us to use this strain to investigate the mechanism of genome transfer as the larger cell size should make visualization easier. We previously hypothesised that the mechanism for direct bacterial genome transfer to yeast occur by one of three mechanisms: (i) genome is directly released into the yeast cytoplasm, (ii) an extracellular release of the genome occurs followed by its active uptake into the yeast host cell or (iii) active bacterial engulfment into the yeast host cell. In all scenarios, the genome would eventually enter the yeast nucleus. Based on our previous study, an extra-cellular release of the donor genome (theory “ii”) was the least likely mechanism¹⁶. To determine whether hypothesis “i” or “iii” remained as viable options, we used electron microscopy in an attempt to directly visualize the process.

Yeast spheroplasts were mixed with JCVI-Syn2.0 cells and incubated for 20 minutes in the presence of PEG, followed by removal of the PEG, and resuspension in recovery media (see methods). After 15 minutes incubation in the recovery media, the sample was fixed and prepared for transmission electron microscopy. We observed various events where the “donor” mycoplasma cells appeared attached to the yeast spheroplasts (**Figure 4c**), either

partially (**Figure 4d and e**) or completely (**Figure 4f**) engulfed by the yeast spheroplasts. The mechanisms resembled endocytosis where in similar situations the bacteria are actively engulfed by the yeast spheroplasts; an expected scenario as hypothesised in our previous publication ¹⁶. In recent publication Mehta et al. ²⁰ have also demonstrated that whole bacteria cells can be introduced into yeast cell.

Concluding remarks. This investigation identified several RGD1 strains that improved the process of genome transfer, with strain RGD1-3 being the superior strain that notably improved this genomic transfer process. After thorough investigation, we identified a single gene called the *glycerol uptake facilitator protein glpF* within RGD1-3, whose deletion was shown to vastly improve direct genome transfer as compared to previously published strains ¹⁶. Removing conserved glpF gene in other bacterial species might result in similar outcome. The reasoning behind this intriguing finding is not evident; however, it is believed that *glpF* may alter the regulatory processes responsible for osmotic regulation. Further studies will need to be performed to decipher the links between the removal of the *glpF* gene product and the observed increase in efficiency. Nevertheless, we have again concluded that direct genome transfer process can be improved by removing key genetic factors. Finally, we revealed that in at least some cases, intact bacterial cells were observed within the yeast recipient leading to the intriguing notion of transient existence of organelle-like structures, which could be a first step in creation of synthetic organelles.

ONLINE METHODS

Strains used and cultured conditions.

Mycoplasma mycoides strains ^{5,7,8}, Yeast strain VL6-48. *M. mycoides* cells were cultured in SP-4²¹ and yeast cells were grown in YPD supplemented with adenine hemisulfate salt or yeast synthetic media lacking histidine (Teknova, Inc.) supplemented with adenine hemisulfate salt with or without 1M sorbitol.

Preparation of yeast spheroplasts.

As described in ¹⁶

Preparation of mycoplasma cells.

As described in ¹⁶

Yeast-mycoplasma PEG induced direct genome transfer.

As described in ¹⁶

TEM protocol

Cells were fixed with 2% formaldehyde (made from paraformaldehyde), 2.5% glutaraldehyde, 1% sorbitol, 2 mM CaCl₂ in 0.1 M sodium cacodylate, pH 7.0) for 5 minutes at room temp then placed on ice. Cells were spun for 5 minutes @ 1000 RPM to produce a loose pellet and supernatant is removed and replaced with fixative and gently mixed and the cells are placed on ice. Cells were fixed overnight in the fridge and the next morning were lightly spun (as above) and rinsed for 5 minutes six times in 0.1 M sodium cacodylate buffer. Cells were post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 hour on ice. Cells were rinsed in ice cold DDW for 5 minutes six times and placed in 1% uranyl acetate in DDW for 1 hours. Cells are rinsed again 3 minutes six times in ice cold DDW and dehydrate in cold ethanol and embedded in Durcupan ACM resin (Electron Microscopy Sciences). Ultrathin section were cut with an ultramicrotome and imaged on a JEOL 1200 transmission electron microscope operated at 80 keV.

Creation of deletion strains glpO, glpK, glpF

For deletions of glpO, glpK, glpF or glpOKF cluster, the URA3 gene was amplified from pYAR-RC vector as described in ¹⁴ using primers listed in Table S1. The amplified URA3 cassettes were transformed into yeast carrying JCVI-Syn1.0, JCVI-Syn1.0 Δ RM or JCVI-Syn2.0 using lithium acetate transformation ²². Yeast colonies were selected on media lacking histidine and uracil, and correct clones were identified by PCR at either side of the URA3 insertion junction (see Table S1). Clones identified as having the correct insertion by PCR were transplanted⁶ and used in our experiments.

Supporting Information

One additional figure (Figures S1), and one table (Table S1) are available via the Internet at <http://pubs.acs.org>.

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Conflict of interest statement. J.C.V. and H.O.S. are Scientific Advisors of Synthetic Genomics, Inc. D.G.G. is a Vice President of Synthetic Genomics, Inc. All of these authors and the J. Craig Venter Institute hold Synthetic Genomics, Inc. stock.

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BJK, NGM, TJD performed the experiments., BJK, NGM, TJD, DGG, JCV, HOS, JIG designed experiments and interpreted results, and BJK, NGM, HOS, JIG wrote the paper.

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Graphical Abstract

Figure 1: Identification of reduced genome design (RGD1) variants of JCVI-syn1.0 that increase genome transfer events between mycoplasma and yeast. (a) Mycoplasma genomes containing RGD1-2, RGD1-3 and RGD1-6 show increased genome transfer compared to the complete JCVI-syn1.0 positive control. (b) & (c) Repeat experiments demonstrate that genomes containing RGD1-3 consistently produce the highest numbers of genome transfer. Error bars showing standard deviation were produced from two repeats (a) & (c) or three repeats (b). Numbers above bars illustrate the factor of increase in the strain compared to JCVI-syn1.0.

Figure 2: Identification of the genetic factor *glpF* whose absence from RGD1-3 is responsible for improved genome transfer. (a) In this experiment RGD1-3 shows significant improvement of genome transfers as compared to control strain JCVI-Syn1.0. When cluster *glpOKF* was added back to RGD1-3 the effect disappeared. (b) Comparison of removal of all or individual genes from cluster *glpOKF* tested in a restriction system minus strain, Δ RM, which is JCVI-Syn1.0 lacking six restriction systems¹⁶. Removal of *glpO* and *glpK*, oxidase and kinase genes respectively, show no increase compared to the Δ RM. Removal of *glpF* shows significant improvement as compared to Δ RM. Removal of the whole cluster Δ RM Δ *glpOKF* produced similar results as removal of *glpF* alone.

Figure 3: Generation of a strain with the best genome transfer properties.

The number of genome transfers, as represented by number of yeast colonies, are displayed on a logarithmic scale. Removal of *glpF* from JCVI-syn1.0 results in similar improvements in genome transfer capability as we observed with the previously published strain of JCVI-syn1.0 without restriction systems (Δ RM). When removal of *glpF* is combined with removal of the restriction modification systems, a massive increase in genome transfer events of over six hundred times compared to JCVI-syn1.0 can be seen. In JCVI Syn2.0, which has 512 genes removed (including restriction systems) in comparison to JCVI-syn1.0, removal of *glpF* still causes an increase in genome transfer capability of approximately forty-five times. Numbers above bars illustrate the factor of increase in the strain compared to JCVI-syn1.0.

Figure 4: Transmission electron microscopy (TEM) images of yeast spheroplasts mixed with JCVI Syn2.0 and incubated in the presence of polyethylene glycol. a) Yeast spheroplasts only, b) JCVI-Syn2.0, (c-f) Yeast spheroplasts mixed with JCVI-Syn2.0 bacterial cells. JCVI-Syn2.0 donor bacteria are identified by arrows (c-f). Scale bar (black line) represent 500 nm.