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Direct transfer of whole genomes from bacteria to yeast

Bogumil J Karas¹, Jelena Jablanovic¹, Lijie Sun¹, Li Ma², Gregory M Goldgof^{1,3}, Jason Stam¹, Adi Ramon¹, Micah J Manary³, Elizabeth A Winzeler³, J Craig Venter^{1,2}, Philip D Weyman¹, Daniel G Gibson¹, John I Glass², Clyde A Hutchison III¹, Hamilton O Smith¹, and Yo Suzuki¹

¹Department of Synthetic Biology and Bioenergy, J. Craig Venter Institute, San Diego, California, USA

²Department of Synthetic Biology and Bioenergy, J. Craig Venter Institute, Rockville, Maryland, USA

³University of California, San Diego, School of Medicine, La Jolla, California, USA

Abstract

Transfer of genomes into yeast facilitates genome engineering for genetically intractable organisms, but this process has been hampered by the need for cumbersome isolation of intact genomes before transfer. Here we demonstrate direct cell-to-cell transfer of bacterial genomes as large as 1.8 megabases (mb) into yeast under conditions that promote cell fusion. Moreover, we discovered that removal of restriction endonucleases from donor bacteria resulted in the enhancement of genome transfer.

Techniques for genome engineering are severely limited outside of *Escherichia coli* and yeast. Cloning whole bacterial genomes as centromeric plasmids in yeast¹ is a breakthrough that makes available an arsenal of DNA-manipulation tools^{2–6} for editing genomes of genetically intractable organisms. The creation of the first synthetic cell demonstrated the utility of cloning whole bacterial chromosomes in yeast⁷, opening the door to building genomes consisting of any desired sequences. Other whole genomes cloned in yeast include *Mycoplasma genitalium* (0.6 Mb)^{1,8,9}, *M. pneumonia* (0.8 Mb)⁹, wild-type *M. mycoides* (1.1 Mb)^{7,10}, *Acholeplasma laidlawii* (1.5 Mb)¹¹ and *Prochlorococcus marinus* MED4 (1.6 Mb)¹².

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Correspondence should be addressed to B.J.K. (bkaras@jcv.org) or Y.S. (ysuzuki@jcv.org).

AUTHOR CONTRIBUTIONS

B.J.K., J.J., J.C.V., P.D.W., D.G.G., C.A.H., H.O.S. and Y.S. designed the research. B.J.K., J.J., L.S., L.M., J.S., A.R., P.D.W., D.G.G., C.A.H. and Y.S. performed experiments. G.M.G., M.J.M. and E.A.W. performed genome sequencing and analysis. B.J.K., J.J., J.S., A.R., P.D.W., D.G.G., C.A.H., H.O.S. and Y.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

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Primers are listed in **Supplementary Note 7**.

Cloning genomes in yeast requires the insertion of selectable marker(s), centromere and autonomously replicating sequence(s) that function in yeast (or a 'yeast vector' providing these elements) into a bacterial genome before genome transfer^{9,13}. One can then isolate bacterial genomes for yeast transformation, but large DNA molecules are susceptible to breakage because of shear forces and require a protecting matrix during extraction. To eliminate this requirement, we sought to directly transfer whole genomes from bacteria into yeast under conditions that promote cell fusion.

Cell fusion can result from electrical stimulation¹⁴ or treatment with chemicals such as polyethylene glycol (PEG)^{15–17} between cells lacking a cell wall, such as those of *Mycoplasma*. PEG-mediated fusion has been used to transfer small DNA molecules between bacterial protoplast and yeast spheroplast¹⁸, but transfer of complete bacterial genomes has never been demonstrated.

When we combined the *M. mycoides* strain YCpMmyc1.1 containing a yeast vector integrated in the genome¹⁰ with spheroplasts of the yeast strain VL6-48 (ref. 19) in the presence of PEG, we obtained over 100 yeast colonies. To see how much of the *Mycoplasma* genome along with the integrated vector entered the yeast cells, we examined the colonies for the presence of *Mycoplasma* sequences using multiplexed PCR. Analysis of seven of ten colonies resulted in the band pattern expected for an intact genome (**Supplementary Fig. 1a,b**). In these seven strains, it was possible that the yeast cell contained multiple damaged genomes that collectively covered all the amplicons, rather than maintaining one complete genome as a single molecule. To distinguish between these possibilities, we analyzed the size of the *Mycoplasma* genome in three strains using clamped homogenous electric field (CHEF) gel electrophoresis. The result was consistent with the possibility that clones 1 and 3 contained the whole genome (**Supplementary Fig. 1c**). Analysis of clone 9, which only yielded a subset of amplicons with multiplex PCR, resulted in a smaller band on the CHEF gel.

Complete transferred genomes must contain a complete set of genes in the donor *Mycoplasma*, including hundreds of genes essential for viability. To demonstrate the accuracy of genomes transferred with our method, we transplanted the genomes cloned in yeast back into bacterial recipient cells¹⁰. We recovered *Mycoplasma* colonies using genomic DNA samples from yeast clones 1 or 3, whereas the negative control (DNA from clone 9) did not produce any colony (**Supplementary Table 1**). The obtained colonies were *M. mycoides* based on multiplex PCR analysis. To exclude the concern that colonies are derived from contaminating cells, we also introduced a unique change in a genome directly transferred into yeast (**Supplementary Note 1**) and found that bacterial cells contained the introduced change after genome transplantation. Finally, whole-genome sequencing revealed only one new mutation in the genomes of these transplants relative to the genome sequence determined with a population of cells before cloning and genome transfer into yeast. This frequency is consistent with the mutation rate of *Mycoplasma*²⁰. Therefore, it is likely that no mutation in these genomes is attributed to our transfer procedure (**Supplementary Note 1**).

These structural and functional tests confirmed to our knowledge the first direct transfer of the complete genome from *M. mycoides* to yeast without the intermediate purification step using an agarose matrix. In additional experiments we determined factors that influence the efficiency of genome transfer to yeast (**Supplementary Fig. 2**).

When we applied this cell-to-cell genome transfer method to an *M. mycoides* strain with the genome missing all six restriction-modification systems, we obtained up to ~15,000 yeast colonies per experiment (**Fig. 1a** and **Supplementary Note 2**). A corresponding strain with intact restriction systems (JCVI-syn1.0) produced only ~2,000 colonies in comparable experiments ($P = 0.001$). Genome transfer using an intermediate strain missing one, two, four or five restriction-modification systems did not result in drastic increase in transfer efficiency compared to that in the intact strain (**Fig. 1a**). To determine whether the restriction nuclease or the set of two methyltransferases of the sixth system attenuates genome transfer, we generated a *Mycoplasma* strain with only these two methylases in active form but lacking all the rest of the genes in the restriction-modification systems (**Supplementary Note 2** and **Supplementary Fig. 3**). When combined with yeast, this strain and the strain missing all restriction-modification systems produced comparable increases in colony count (**Fig. 1a**), suggesting that nucleases, but not methyltransferases, limit genome transfer.

To demonstrate the broad applicability of our method, we selected another member from the mollicute family, *M. capricolum*, and a Gram-negative bacterium with a complex cell wall structure, *Haemophilus influenzae* Rd strain KW20. When we mixed *M. capricolum* strains containing a yeast vector in the genome with yeast spheroplasts, the wild-type strain generated ~4,000 colonies, whereas a strain lacking the sole restriction enzyme gene in this organism produced ~29,000 colonies (**Supplementary Fig. 4** and **Supplementary Note 3**). Of the 20 yeast colonies generated with the wild-type strain and the strain lacking the restriction gene, 19 and 20, respectively, had the complete genome as assayed by multiplex PCR.

When we introduced a yeast vector into genomes of *H. influenzae* strains and combined these strains with yeast spheroplasts (**Supplementary Note 4**), we also obtained yeast colonies containing cloned genomes. To our surprise, this procedure was successful without any specific treatment intended to remove the cell wall from *H. influenzae* cells (**Fig. 1b** and **Supplementary Fig. 5**), suggesting that PEG-treated cell wall does not constitute a barrier to genome transfer and potentially simplifying the protocol for Gram-negative bacteria.

As with other species, removal of restriction enzymes increased the frequency of genome transfer with *H. influenzae* cells (**Fig. 1b** and **Supplementary Fig. 5**). Strains containing two restriction enzymes (HindII and HindIII), only HindII, only HindIII and no restriction enzyme produced 0 colonies, 0 colonies, 157 colonies ($P = 0.001$) and 949 colonies ($P = 0.00006$), respectively (**Fig. 1b**). Of 30 yeast colonies containing the double-mutant genome, 26 had the complete genome as assayed by multiplex PCR. The recurring observation that removal of donor restriction systems markedly improves genome-transfer efficiency suggests the involvement of a common mechanism in all bacteria. Genetic engineering in

yeast enables the restoration of restriction enzyme genes if downstream applications require these genes to be present in the final genomes.

We postulate three possible mechanisms for PEG-mediated direct transfer of bacterial genomes to yeast (**Fig. 2a**): (i) bacterial cells fuse with yeast to release the genome into the cytoplasm, (ii) PEG treatment results in the release of the bacterial genome in the extracellular space followed by uptake of the genome by yeast and (iii) engulfment of the bacterial cells by yeast. These are followed by the migration of the genome into the nucleus, or in the case of the third possibility, the engulfed bacteria may survive as symbionts.

To examine the second possibility, we treated *Mycoplasma* cells before PEG treatment with DNase I (**Fig. 2b**). Treatment with active DNase I completely abolished yeast transformation with isolated genomic DNA but only resulted in about 15% fewer yeast colonies with *Mycoplasma* cells compared to numbers obtained with heat-inactivated DNase I (**Fig. 2b** and **Supplementary Table 2**). This result indicates that DNA is protected from externally added DNase I in most cases during genome transfer, consistent with either the formation of continuous cytoplasm between the donor cell and the recipient cell where the genome travels or a mechanism such as engulfment followed by decomposition of *Mycoplasma* that can limit the exposure of DNA to DNase I.

To determine whether *Mycoplasma* cells reside in yeast as symbionts, we plated spheroplasts treated with a hypotonic buffer that would break open the spheroplasts without affecting *Mycoplasma* cells after the gene-transfer protocol, but this procedure did not result in any *Mycoplasma* colonies (**Supplementary Note 5**). We also examined the methylation state of the *M. mycoides* genome in yeast. The sequence GATC is methylated in *Mycoplasma* containing restriction-modification systems, but not in the yeast nucleus. Methylated and unmethylated GATC sequences are substrates of the restriction enzymes DpnI and MboI, respectively. We found that *M. mycoides* DNA recovered from yeast was insensitive to DpnI and sensitive to MboI, suggesting that the *Mycoplasma* genome resides in the yeast nucleus (**Supplementary Fig. 6** and **Supplementary Note 6**).

With the bacterial cells protecting DNA from shearing forces until they contact yeast cells, we expect our method to be suitable for testing the upper size limit for genome cloning and finding requirements for clonable genomes such as the distance between sequences that can act as an autonomously replicating sequence in yeast¹³. Our rapid technology facilitates the first step of the scheme we envision for engineering genetically intractable organisms involving genome cloning, editing and rebooting.

ONLINE METHODS

Strains used and culture conditions

M. mycoides strains YCpMmyc1.1 (ref. 10) and JCVI-syn1.0 (ref. 7), *M. capricolum* subsp. *capricolum* (strain California Kid)¹⁰, *H. influenzae* Rd strain KW20 and *Saccharomyces cerevisiae* strains VL6-48 (ref. 19) and BY4741 were used. *Mycoplasma* were cultured in SP-4 (ref. 21), *H. influenzae* in brain heart infusion (BHI) medium²² supplemented with 4 mg l⁻¹ nicotinamide adenine dinucleotide and 20 mg l⁻¹ hemin, and yeast cells in yeast

extract peptone dextrose (YPD)⁶ medium supplemented with adenine or yeast synthetic complete medium lacking histidine supplemented with adenine (Teknova, Inc.).

Preparation of yeast spheroplasts

YPD medium (10 ml, 2× concentrated) supplemented with adenine (120 mg l⁻¹) was inoculated with VL6-48 and incubated overnight at 30 °C with 225 r.p.m. agitation. In the morning, the culture was diluted tenfold and grown to OD₆₀₀ of ~2.5–3.0 (50 ml of culture was used for ten experiments). When the desired OD₆₀₀ was reached, cells were centrifuged at 2,500 relative centrifugal force (RCF) for 5 min (using a 50-ml centrifuge tube from Corning) at 10 °C, and supernatant was decanted. Next, the pelleted cells were resuspended in 50 ml sterile dH₂O (the cells were first resuspended in 20 ml of dH₂O by vortexing, followed by the addition of 30 ml of dH₂O and mixing by inverting). The tube was centrifuged at 2,500 RCF for 5 min, and the supernatant was decanted. Next, the pelleted cells were resuspended in 50 ml of 1 M sorbitol (first the cells were resuspended in 20 ml of 1 M sorbitol by vortexing, followed by the addition of 30 ml of 1 M sorbitol and mixing by inverting). For some experiments, yeast cells were kept in 1 M sorbitol for 1–5 h at 4 °C before proceeding to the next step. The cells were centrifuged at 2,500 RCF for 5 min, and the supernatant was then decanted. The pelleted cells were then resuspended by vortexing in 20 ml of SPEM solution (1 M sorbitol, 10 mM EDTA, pH 8, 2.08 g l⁻¹ Na₂HPO₄·7H₂O and 0.32 g l⁻¹ NaH₂PO₄·1H₂O), 30 µl of β-mercaptoethanol and 40 µl of Zymolyase-20T solution (200 mg Zymolyase (USB), 9 ml of dH₂O, 1 ml of 1M Tris-HCl pH 7.5 and 10 ml of 50% glycerol; stored at –20 °C). After incubation for 30 min at 30 °C with 75 r.p.m. agitation, OD₆₀₀ was determined for: (i) 0.2 ml of SPEM solution containing yeast cells combined with 0.8 ml of 1 M sorbitol and (ii) 0.2 ml of the SPEM solution containing yeast cells combined with 0.8 ml of dH₂O. When the ratio (i/ii) was in the range 1.8–2.0, 30 ml of 1 M sorbitol was added to the remaining SPEM solution containing yeast cells, and the resulting solution was mixed by inverting. Spheroplasts were collected by centrifugation at 1,000 RCF for 5 min at 10 °C with the supernatant removed. When the i/ii value was below 1.8, yeast cells were incubated longer sometimes with additional Zymolyase. Next, the pellet was gently resuspended in 50 ml of 1 M sorbitol (20 ml of 1 M sorbitol was first added, with the pellet resuspended by passing the solution 5–10 times through a 10-ml pipette, followed by the addition of 30 ml of 1 M sorbitol and mixing by inverting), and again centrifuged at 1,000 RCF for 5 min with the supernatant decanted. Finally the pellet was resuspended in 2 ml of STC solution (1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂ and 2.5 mM MgCl₂), and the generated spheroplasts were kept at room temperature for 5–15 min.

Preparation of *M. mycoides* cells

SP-4 medium (20 ml) was inoculated with *M. mycoides* glycerol stock and incubated overnight at 37 °C without agitation. The next morning, the culture was diluted tenfold and grown to pH 6.5–7.0 (50 ml of culture was used for approximately ten experiments). At that time chloramphenicol was added to the final concentration of 100 mg/l, and cells were incubated for an additional 1.0–1.5 h at 37 °C. Then the culture was spun down at 10,000 RCF for 5 min at 10 °C (in a 50-ml centrifuge tube; Corning). The supernatant was removed, and the pellet was washed with 50 ml of resuspension buffer (0.5 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂ and 2.5 mM MgCl₂; pH was adjusted to 7.5). Next, the

cells were centrifuged at 15,000 RCF for 5 min at 10 °C, the supernatant was decanted, and cells were resuspended in 0.5× resuspension buffer to make the final volume ~500 µl. The 50-µl aliquots were prepared for PEG-induced cell fusion.

Preparation of *H. influenzae* cells with lysozyme treatment

Forty milliliters of BHI medium (supplemented with 4 mg l⁻¹ of nicotinamide adenine dinucleotide and 20 mg l⁻¹ hemin) was inoculated with 1 ml of *H. influenzae* glycerol stock (collected at OD₆₀₀ of ~0.3) and incubated for 4 h at 37 °C with 200 r.p.m. agitation. After 80 µl of puromycin (50 g/l) was added, the culture was incubated for an additional 30 min. The culture was spun down at 7,000 RCF for 5 min at 10 °C (in a 50-ml centrifuge tube). The supernatant was decanted, and the cells were resuspended in 9 ml of resuspension buffer (0.5 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂ and 2.5 mM MgCl₂; pH adjusted to 7.5). Then 500 µl of 0.5 M EDTA (pH 8.0) and 500 µl of lysozyme (25 g/l, resuspended in dH₂O) were added, and the cells were incubated for 30 min in a 37 °C water bath. Next, the cells were centrifuged at 7,000 RCF for 4 min at 10 °C and washed in 40 ml of resuspension buffer (first resuspended in 1 ml by gentle pipetting, followed by the addition of 39 ml and mixing by inverting). The final centrifugation at 7,000 RCF for 4 min at 10 °C was followed by resuspension in 1× resuspension buffer to make the final volume 300 µl. For PEG-induced genome transfer, 50-µl aliquots were prepared.

Preparation of *H. influenzae* cells without lysozyme treatment

The procedures were identical to the procedures described above except that *H. influenzae* was cultured until OD₆₀₀ reached ~0.7 starting with the same 1-ml stock, that 40 µl (instead of 80 µl) of puromycin (50 g/l) was added and that a resuspension buffer (0.5 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, and 2.5 mM MgCl₂; pH adjusted to 7.5) without EDTA or lysozyme was used during the final 30-min incubation.

Treatment of yeast and bacteria with PEG for genome transfer

We added 200 µl of the yeast spheroplast solution (see above) to the 50-µl aliquot of bacterial cells. The resulting solution was mixed by gently flicking the tube and incubated at room temperature for 5 min. Next, 1 ml of 20% PEG (PEG 8000; USB) solution (20% PEG, 10 mM Tris-HCl, pH 8.0, 10 mM CaCl₂ and 2.5 mM MgCl₂; pH adjusted to 8.0) equilibrated at 37 °C was added, and the resulting solution was mixed by inverting (usually 6–10 times). Next, the yeast spheroplast–*Mycoplasma* cell solution was kept at room temperature for 20 min, followed by centrifugation at 1,500 RCF for 7 min. The pellet was resuspended in 1 ml of SOS medium (1 M sorbitol, 6 mM CaCl₂, 2.5 g l⁻¹ yeast extract and 5 g l⁻¹ Bacto Peptone) and incubated for 30 min at 30 °C. During the incubation time, top agar (synthetic complete medium lacking histidine) was melted and 8-ml aliquots of this medium were equilibrated at 50 °C. We added 100 µl–1 ml (depending on the expected number of colonies for the ease of counting colonies) of the mixture of cells (yeast spheroplasts and bacterial cells) in SOS medium to the equilibrated top agar, and the resulting solution was mixed by inverting a few times and plated on agar plates containing synthetic complete medium lacking histidine that were prewarmed to 37 °C. Once the agar solidified (in about 5 min), the plates were moved to 30 °C. Transformants usually appeared

after 2–5 d. The number of transformants per 1 ml of the SOS mixture was calculated for all experiments.

PCR analysis of clones

Before isolation of DNA from selected clones, yeast cells were serially transferred 2–5 times to a fresh plate to remove any contaminating bacterial cells or free bacterial DNA. It is important to note that *M. mycoides*, *M. capricolum* or *H. influenzae* does not grow in a standard yeast medium. Multiplex PCR was performed using the Multiplex PCR kit (Qiagen).

Statistical analysis

Welch's *t*-tests (two-tailed) were used to evaluate the differences in yeast colony numbers obtained with various genomic samples. All statistical tests used the preselected threshold (α) of 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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figure 1.

Effects of disrupting restriction-modification systems on genome transfer. **(a)** Number of yeast colonies generated when *M. mycoides* JCVI-syn1.0 strains were combined with yeast spheroplasts. Wild type contains all systems; 1 lacks system 1 (*mmyCIIR/M*); 1–2 lacks systems 1 and 2 (*mmyCIIR/M1/M2*); 1–4 lacks systems 1–3 (*MMSYN1_0590/mmyCImod*) and 4 (*mmyCIVR/M*); and 1–5 lacks systems 1–5 (*mmyC5VR/M*); 1–5 R_6 strain lacks systems 1–5 and *mmyCVIR* but maintains methylase genes *mmyCVIM1/M2*; and 1–6 lacks systems 1–6 (*mmyCVIR/M1/M2*). **(b)** Number of yeast colonies generated when combined with *H. influenzae* strains lacking zero (wild type), one (Δ HindIII or Δ HindII) and two (Δ HindII/III) nuclease genes without any procedure for cell wall removal for *H. influenzae*. Error bars, s.d. ($n = 3$ (a) and $n = 4$ (b)). * $P < 0.05$ (α) compared to wild type.

figure 2.

Modes of cell-to-cell genome transfer from bacterium to yeast. **(a)** Possible scenarios include bacterial cells fusing with yeast spheroplasts (i), lysed bacterial cells releasing the genomic DNA (red circle), which is taken up by yeast (ii), and engulfed bacterial cells undergoing decomposition, followed by migration of the genomes to the nucleus (iii, A) or surviving as symbionts (iii, B). **(b)** Number of yeast colonies after *M. mycoides* JCVI-syn1.0 cells (wild type and $\Delta 1-6$ containing and lacking all restriction-modification systems, respectively; see **fig. 1a**) and purified genomic DNA (gDNA, positive control) were incubated with heat-inactivated DNase I (HI) or active DNase I (A) before combination with yeast. Error bars, s.d. ($n = 5$ for cells and $n = 4$ for gDNA). $P = 0.04$, 0.006 and 0.005 for wild type, $\Delta 1-6$ and gDNA, respectively, for treatment with HI compared to A DNase I.