Creating Bacterial Strains from Genomes That Have Been Cloned and Engineered in Yeast

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We recently reported the chemical synthesis, assembly, and cloning of a bacterial genome in yeast. To produce a synthetic cell, the genome must be transferred from yeast to a receptive cytoplasm. Here we describe methods to accomplish this. We cloned a *Mycoplasma mycoides* genome as a yeast centromeric plasmid and then transplanted it into *Mycoplasma capricolum* to produce a viable *M. mycoides* cell. While in yeast, the genome was altered by using yeast genetic systems and then transplanted to produce a new strain of *M. mycoides*. These methods allow the construction of strains that could not be produced with genetic tools available for this bacterium.

We have described the translation of the genome of *Mycoplasma mycoides* subspecies *capri* (1–3) from its native cellular environment into a related species, *Mycoplasma capricolum* subspecies *capricolum* (4). We have also described the complete chemical synthesis of the 580-kb *Mycoplasma genitalium* genome (5, 6). Initial stages of the synthesis were carried out by in vitro assembly reactions, and pieces up to a quarter of a genome in size were cloned in *Escherichia coli*. We overcome difficulties in cloning larger segments of DNA in *E. coli* by using homologous recombination in the yeast *Saccharomyces cerevisiae* to assemble the subgenomic synthetic DNA segments into a complete *M. genitalium* genome. To complete our construction of a living microbe, we must isolate our synthetic genome from yeast and transfer it into a cellular environment that will accept and execute the genetic instructions sufficient to produce a replicating organism. In this paper, we describe methods for transplanting natural 1.1-Mb *M. mycoides* genomes cloned in yeast into *M. capricolum* recipient cells. These species are more convenient experimental organisms than *M. genitalium* because of their faster growth rate.

*M. mycoides* was transformed (7) with a vector containing a selectable tetracycline-resistance marker and a β-galactosidase gene for screening. The vector also contained a yeast auxotrophic marker, a yeast centromere, and a yeast autonomously replicating sequence, for selection and propagation in yeast as a yeast centromeric plasmid (YCp). Direct genomic sequencing (8) of one clone (YCpMmyc1.1) showed that the entire vector integrated into the genome. This clone grew robustly and transplanted efficiently into *M. capricolum* (9), so it was chosen for cloning into yeast. The genome of this clone will be called YCpMmyc1.1 throughout this paper, regardless of the cellular source. YCpMmyc1.1 can refer to: (i) the original *M. mycoides* strain (the “native” *M. mycoides* YCpMmyc1.1 genome), (ii) the same genome cloned in yeast, (iii) the genome transplanted from *M. mycoides* or from yeast, or (iv) this genome as free DNA from any of these sources.

YCpMmyc1.1 genomes were isolated from *M. mycoides* (9) and transformed into yeast spheroplasts (10) of strains VL6-48N (11) and W303a. Clones were analyzed for completeness and size by multiplex polymerase chain reaction (PCR) and clamped homogenous electric fields (CHEF) gel electrophoresis. To test whether deletions occur during routine propagation in yeast, we screened 40 individual colonies derived from a single intact clone of YCpMmyc1.1 in W303a. All appeared to contain complete genomes (fig. S1), which indicates that this bacterial genome is stable in yeast. Sequences between the amplicons were not interrogated in this experiment; however, sizable deletions would have been detected by this approach, and none were observed. Sequencing of a complete genome transplanted from yeast (see below) provided a definitive demonstration of stability of the *M. mycoides* genome in yeast.

We engineered YCpMmyc1.1 in yeast by creating a seamless deletion in a nonessential Type III restriction endonuclease gene (Fig. 1). This modification cannot be made with the genetic tools available for this bacterium. We first transformed a YCpMmyc1.1 yeast clone with a cassette containing a seamless deletion in a nonessential Type III restriction enzyme. In this case, we deleted a 3.36-kb DNA segment of the genome, corresponding to a 3.1-kb DNA fragment in the YCpMmyc1.1 yeast clone with a cassette containing a seamless deletion in a nonessential Type III restriction enzyme.

![Diagram](https://www.sciencemag.org/content/325/5942/693/F1.large.jpg) **Fig. 1.** Generation of Type III restriction enzyme deletions. (A) To make an *M. mycoides* Type III restriction enzyme gene (tyrells) deletion in yeast (iii), we constructed a linear DNA fragment, knockout cassette, by fusing two PCR products, CORE and tandem repeat sequence (TR) (i). This cassette was then transplanted into a yeast W303a strain harboring the YCpMmyc1.1 *M. mycoides* genome (ii). Growth on −His (−)Ura medium selected for replacement of the Type III restriction enzyme open reading frame (ORF) by the cassette (iii). Growth on −His (−)Ura medium selected for replacement of the Type III restriction enzyme open reading frame (ORF) by the cassette (iii). (B) The arrows above the DNA in (A) represent PCR primers (P299 and P302) used to verify the presence or absence of the knockout cassette. PCRs of representative transplant clones with (iii) and without (iii) the knockout cassette are shown. PCRs of the YCpMmyc1.1 clone in yeast (i) are shown for comparison. The expected sizes are obtained for each amplicon.
We isolated YCpMmyc1.1 from yeast and attempted transformation into wild-type *M. capricolum* cells. However, we did not recover any transplants (Table 1). We reasoned that the principal obstacle was a restriction endonuclease in the recipient *M. capricolum* that degraded the unmethylated YCpMmyc1.1 donor DNA isolated from yeast (Fig. S4).

Two methods were used to overcome the *M. capricolum* restriction barrier. First, we inactivated the single restriction enzyme in *M. capricolum* by integration of a puromycin-resistance marker into the coding region of the gene. No detectable restriction enzyme activity was seen in extracts of this altered strain (Fig. S1). Removal of *M. capricolum* restriction activity should allow donor *M. mycoides* YCp genomes isolated from yeast to survive initial contact with the *M. capricolum* cytoplasm. The second method was to protect the donor DNA isolated from yeast by in vitro methylation, using *M. capricolum* extracts. An extract of *M. mycoides* also protected the incoming donor DNA, because *M. mycoides* contains an ortholog of the system found in *M. capricolum* (Fig. S6). The additional restriction-modification systems present in the *M. mycoides* donor genome did not affect transformation.

We isolated YcpMmyc1.1 from *M. mycoides* and transformed it into wild-type *M. capricolum* and *M. capricolum RE(-) recipient cells (Fig. S7). Results were scored by selecting for growth of blue colonies on SP4 medium containing tetracycline at 37°C. Successful transformations were obtained using YCpMmyc1.1 from yeast with both recipient cells (Table 1). Colonies were obtained using *M. capricolum* RE(-) as recipient cells when the donor genomic DNA was untreated, mock-methylated, treated with *M. capricolum* or *M. mycoides* extracts, or treated with purified *M. mycoides* methyltransferases. However, transformation using wild-type *M. capricolum* recipient cells occurred only when the donor YCp genome from yeast was methylated with *M. capricolum* extract, *M. mycoides* extract, or purified *M. mycoides* methyltransferases. No colonies were obtained when mock-treated or untreated YCpMmyc1.1 was transformed into wild-type *M. capricolum* recipient cells. Thus, avoidance of the *M. capricolum* recipient restriction system is vital for successful transformation of *M. mycoides* YCp genomes from yeast.

YCpMmyc1.1, as well as the engineered YCp genomes (YCpMmyc1.1-ΔtypeIlires::URA3 and YCpMmyc1.1-ΔtypeIlires), were also isolated from yeast strain W303a. Transformation of all three YCp genomes into *M. capricolum* recipient cells resulted in similar numbers of tetracycline-resistant blue colonies (Table 1). The large deletion clone (YCpMmyc1.1-Δ500kb) discussed above served as an appropriate control because it lacks many presumed essential genes yet retains the YCp element and tetM. As expected, no colonies were recovered when this genome was transplanted into *M. capricolum* recipient cells.

Recovery of colonies in all these transformation experiments was dependent on the presence of both *M. capricolum* recipient cells and an *M. mycoides* genome. The experiments described here used donor YCp genome DNA that included yeast genomic DNA. However, purifying the donor YCp genome DNA away from yeast genomic DNA did not substantially alter transformation results, which suggests that the recipient *M. capricolum* cells are able to tolerate the presence of nonspecific or carrier DNA (Table 1).

Positive transformation results were obtained with donor YCp genome DNA isolated from four independent transformant cultures of strain VL6-48N and four of strain W303a. Thus, bacterial genomes can be stably cloned in both yeast strains.

**Table 1.** Transplantation of *M. mycoides* YCp genomes from yeast into wild-type and RE(-) *M. capricolum* recipient cells. The number of tetracycline-resistant, blue colonies obtained after the transplantation of *M. mycoides* YCp genomes from yeast into *M. capricolum* recipient cells was counted. Wild-type *M. capricolum* and *M. capricolum RE(-) transformation was performed using methods described in Fig. S1. For untreated samples, yeast plugs were digested with β-agarase (melting step) and transplanted into both recipient cells. The treated samples were methylated and treated with proteinase K before the melting step. The mock-methylated sample was treated the same as the methylated samples, except that no extract or purified methyltransferases were added. VL6-48N yeast agarose plugs used in this experiment carried YcpMmyc1.1. W303a yeast agarose plugs carried YCpMmyc1.1, YCpMmyc1.1 that was engineered in yeast (YCpMmyc1.1-ΔtypeIlires::URA3 or YCpMmyc1.1-ΔtypeIlires), or YCpMmyc1.1-Δ500kb. The number of transplants is the average of at least three experiments. The error reported is the absolute mean deviation.

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Genome</th>
<th>Methylation treatment</th>
<th>Number of transplants (colonies or plugs)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>M. capricolum</em> RE(-)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Wild-type <em>M. capricolum</em></td>
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<td>VL6-48N</td>
<td>YCpMmyc1.1</td>
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<tr>
<td></td>
<td></td>
<td>M. capricolum extracts</td>
<td>32 ± 13</td>
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<tr>
<td></td>
<td></td>
<td>M. mycoides extracts</td>
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<tr>
<td></td>
<td></td>
<td>Mock-methylated</td>
<td>34 ± 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M. mycoides purified methylases</td>
<td>20 ± 17</td>
</tr>
<tr>
<td>W303a</td>
<td>YCpMmyc1.1</td>
<td>Untreated</td>
<td>22 ± 5</td>
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<tr>
<td></td>
<td>YCpMmyc1.1-ΔtypeIlires::URA3</td>
<td>Untreated</td>
<td>52 ± 10</td>
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<tr>
<td></td>
<td>YCpMmyc1.1-ΔtypeIlires</td>
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<td>Not done</td>
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<tr>
<td></td>
<td>YCpMmyc1.1-Δ500kb</td>
<td>Untreated</td>
<td>Not done</td>
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*Yeast plugs were cleared of yeast genomic DNA by digestion with a cocktail of Asl I, Ksr II, and Fse I followed by pulsed-field gel electrophoresis.† Yeast plugs were cleared of yeast genomic DNA by using pulsed-field gel electrophoresis.*
Full-length clones of mycoplasma genomes have proven stable in yeast during routine propagation and genome transplantation. As described, 40 individual colonies derived from a complete YCp clone of the *M. mycoides* genome all contained full-length genomes. Furthermore, eight independent full-length yeast clones of the *M. mycoides* genome yielded viable bacteria when the genome was transplanted. Finally, the complete genome sequence of *M. mycoides* was unchanged during cloning into yeast and transplantation back into a bacterial cell. We have never seen deletions in our YCp clones except after selection following DNA transformation.

We previously reported transplantation of naked genomic DNA purified from *M. mycoides* cells (4). The transplant events were rare, and there remained the possibility that they resulted from damaged cells that could be somehow repaired in the presence of recipient cells, or from genomes that were in complex with some *M. mycoides* component other than genomic DNA. Transplantation from yeast of the nonmethylated *M. mycoides* genome into the *M. capricolum* recipient cells eliminates the possibility that component(s) of the *M. mycoides* cells are required for transplantation (fig. S7).

Our original transplant experiments used genomes that were resistant to the restriction enzyme of the recipient cells because the donor cells contain the same restriction modification system as the recipient cell (4). *M. mycoides* DNA sequences from yeast lack the specific methylations imparted by *M. mycoides* restriction modification systems. The natural DNA sequences encoding the methylases cannot be expressed because they contain UGA tryptophan codons, which function as stop codons in yeast. Transplantation from yeast was achieved either by methylation of the donor genome in vitro or by inactivation of the restriction enzyme in the recipient cell. It was unnecessary to protect the *M. mycoides* genome from its own restriction systems, because either inactivation of the recipient cell’s endonuclease or methylation with the recipient cell’s methylase was sufficient to allow transplantation. When transplanting other bacterial genomes from yeast, it may be necessary to methyle the donor genome in vitro to protect it from its own restriction enzymes.

Genetic manipulation of *M. mycoides*, and mycoplasmas in general, is limited. Integration of plasmid DNA by single crossover events allows the targeted addition or disruption of genes in *M. mycoides* (13). However, because there are only a few selection markers, the number of genetic alterations that can be performed in a single *M. mycoides* cell is limited. The maintenance of the *M. mycoides* genome in yeast allowed us to access the powerful repertoire of yeast genetic methods and to produce an *M. mycoides* strain that had not previously existed. Thus, we report engineering of a bacterial cell by altering its genome outside of its native cellular environment.

It is now possible to readily generate *M. mycoides* strains with multiple targeted gene deletions, insertions, and rearrangements. It would also be possible to engineer bacterial genomes in yeast by using random mutagenesis methods so that transplantation would yield populations of altered bacteria. After screening for a desired trait, these methods could be reapplied in a cyclical manner to introduce new traits (Fig. 3). This transplantation system potentially allows *M. mycoides* and closely related species to be model systems for exploring the pathogenicity and biology of mycoplasmas. The *mycoides* group of mycoplasmas causes major diseases of ruminants, and there is an urgent need for better therapeutic strategies.
need for vaccines (14, 15). This technology could accelerate the construction of live vaccine strains.

Many medically or industrially important microbes are difficult to manipulate genetically. This has severely limited our understanding of pathogenesis and our ability to exploit the knowledge of microbial biology on a practical level. We hope that the cycle presented here can be applied to other species, to help solve these problems.

References and Notes

3. Manso-Silvan et al. (2) report the very recent renaming of Mycoplasma mycoides subspecies mycoides Large Colony to Mycoplasma mycoides subspecies capri.

On Universality in Human Correspondence Activity

R. Dean Malmgren,1,2* Daniel B. Stouffer,1,3 Andriana S. L. O. Campanharo,1,4 Luís A. Nunes Amaral1,5,6*

The identification and modeling of patterns of human activity have important ramifications for applications ranging from predicting disease spread to optimizing resource allocation. Because of its relevance and availability, written correspondence provides a powerful proxy for studying human activity. One school of thought is that human correspondence is driven by responses to received correspondence, a view that requires a distinct response mechanism to explain e-mail and letter correspondence observations. We demonstrate that, like e-mail correspondence, the letter correspondence patterns of 16 writers, performers, politicians, and scientists are well described by the circadian cycle, task repetition, and changing communication needs. We confirm the correspondence patterns are driven primarily by the need to respond to other individuals. This is formalized by a priority queueing model (19), which, under certain limiting conditions, reproduces the asymptotic scaling of empirically observed heavy-tailed correspondence statistics. In particular, the heavy-tailed statistical properties of e-mail correspondence are reportedly reproduced by a fixed-length queue with a single task type (19, 20), whereas the heavy-tailed statistical properties of letter correspondence are reportedly reproduced by either a variable-length queue with a single task type (21, 20) or by a fixed-length queue with multiple task types (22). The fact that there are different exponents for the two modes of correspondence has been taken as evidence that human correspondence falls into one of two universality classes (20). When interpreted in the statistical mechanics sense of universality, one would conclude that e-mail and letter correspondence are fundamentally different activities.

In contrast, we hypothesize that human correspondence patterns are not driven by responses to others but by more prosaic mechanisms: the circadian cycle, task repetition, and changing communication needs. We formalize these mechanisms with a cascading, nonhomogeneous Poisson process that we have previously shown to be statistically consistent with e-mail communication patterns (14). We hypothesize that the same model is capable of describing letter correspondence and that the heavy-tailed correspondence statistics primarily arise from the variation in an individual’s communication needs over the course of his or her lifetime.

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Supporting Online Material

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