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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 transplantation 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 overcame 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 con-

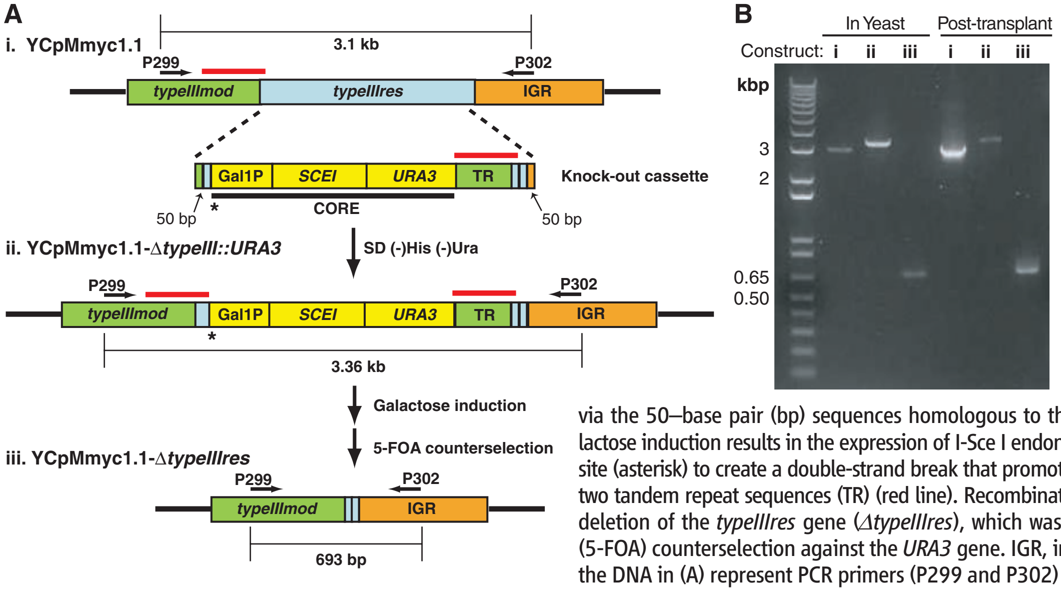

Fig. 1. Generation of Type III restriction enzyme deletions. (**A**) To make an *M. mycoides* Type III restriction enzyme gene (*typeIIIres*) deletion in yeast (iii), we constructed a linear DNA fragment, knockout cassette, by fusing two PCR products, CORE and tandem repeat sequence (TR) (i). This cassette was then transformed 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 via the 50–base pair (bp) sequences homologous to the target sites (Δ*typeIIIres::URA3*). Galactose induction results in the expression of I-Sce I endonuclease, which cleaves the 18-bp I-Sce I site (asterisk) to create a double-strand break that promotes homologous recombination between two tandem repeat sequences (TR) (red line). Recombination between the TRs creates a seamless deletion of the *typeIIIres* gene (Δ*typeIIIres*), which was isolated following 5-flouroorotic acid (5-FOA) counterselection against the *URA3* gene. IGR, intergenic region. (**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 (ii) 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.

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taining a *URA3* marker and the *SCEI* endonuclease gene under the control of the *GAL1* promoter. We selected for insertion of the cassette into the Type III gene. Four of five clones contained intact genomes, and one contained a genome with a large deletion (YCpMmyc1.1-Δ500kb) (figs. S2 and S3). The *URA3* cassette was removed by cleavage at an I-Sce I recognition site near one end of the cassette (Fig. 1). Counter selection with 5-fluoroorotic acid (*12*) produced clones that had lost the *URA3* cassette. Thus, we obtained two *M. mycoides* YCp genomes, one that contained the *URA3* cassette and the other that contained a seamless deletion of the Type III restriction enzyme gene (Fig. 1A). The changes to the genome were verified by PCR (Fig. 1B).

We isolated YCpMmyc1.1 from yeast and attempted transplantation 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 [*M. capricolum* RE(–)] (fig. S5). 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 transplantation.

We isolated YCpMmyc1.1 from *M. mycoides* and transplanted 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 transplantations 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, transplantation 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 transplanted into wild-type *M. capricolum* recipient cells. Thus, avoidance of the *M. capricolum* recipient restriction system is vital for successful transplantation of *M. mycoides* YCp genomes from yeast.

YCpMmyc1.1, as well as the engineered YCp genomes (YCpMmyc1.1-*ΔtypeIIIres::URA3* and YCpMmyc1.1-*ΔtypeIIIres*), were also isolated from yeast strain W303a. Transplantation 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 transplantation 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 transplantation results, which suggests that the recipient *M. capricolum* cells are able to tolerate the presence of nonspecific or carrier DNA (Table 1). Positive transplantation 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.

We verified that the recovered colonies were *M. mycoides* by Southern blot analysis using an *M. mycoides*–specific IS1296 element as probe (Fig. 2A). We showed that the Type III restriction gene was deleted in the engineered bacterium by PCR (Fig. 1B), by Southern blot analysis using the Type III restriction gene sequence as probe (Fig. 2B), and by sequencing the locus (Fig. 2C).

To confirm that our transplants were entirely *M. mycoides* and not chimeras containing yeast sequences or *M. capricolum* recipient cell sequences, we sequenced the genome of one transplant from which the Type III restriction gene had been deleted (GenBank accession no. CP001668) (*9*). All of the assembled genome matched *M. mycoides*, except for those regions that matched the YCp vector. In addition, except for alterations we made in the genome, our transplant YCpMmyc1.1-*ΔtypeIIIres* genome sequence was identical to *M. mycoides* YCpMmyc1.1, which was used to generate the original *M. mycoides* yeast clone. Thus, there was no recombination of either yeast or recipient cell genomes with the *M. mycoides* YCp donor genome, and these bacterial genome sequences are stable as YCps.

Here we describe methods to transfer a genome between branches of life, from a bacterium to a eukaryote, where it can be genetically altered, and then back again into a bacterium (Fig. 3). Our method of yeast vector insertion uses bacterial selection to ensure a viable integration point. This avoids a potentially lethal genome disruption, which would prevent transplantation.

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* recipients was counted. Wild-type *M. capricolum* and *M. capricolum* RE(–) transplantation 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-*ΔtypeIIIres::URA3* or YCpMmyc1.1-*ΔtypeIIIres*), or YCpMmyc1.1-Δ500kb. The number of transplants is the average of at least three experiments. The error reported is the absolute mean deviation.

Yeast strain	Genome	Methylation treatment	Number of transplants (colonies or plugs)	
			M. capricolum RE(–)	Wild-type *M. capricolum*
VL6-48N	YCpMmyc1.1	Untreated	37 ± 3	0
		M. capricolum extracts	32 ± 13	9 ± 4
		M. mycoides extracts	15 ± 8	22 ± 8 [13 ± 4]* [10 ± 4]†
		Mock-methylated	34 ± 17	0
		M. mycoides purified methylases	20 ± 17	13 ± 10
W303a	YCpMmyc1.1	Untreated	22 ± 5	Not done
	YCpMmyc1.1-*ΔtypeIIIres::URA3*	Untreated	52 ± 10	Not done
	YCpMmyc1.1-*ΔtypeIIIres*	Untreated	52 ± 12	Not done
	YCpMmyc1.1-Δ500kb	Untreated	0	Not done

*Yeast plugs were cleared of yeast genomic DNA by digestion with a cocktail of Asi SI, Rsr 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.

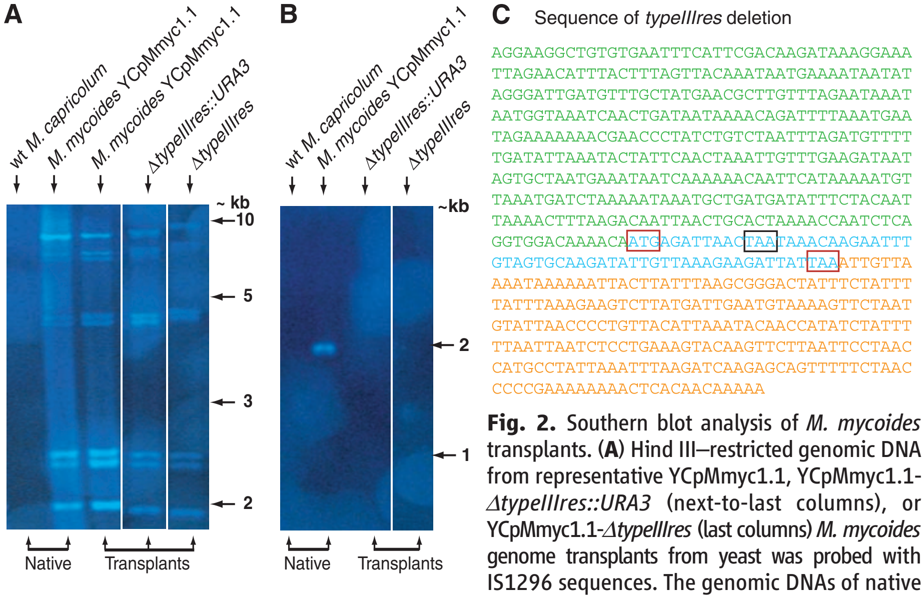
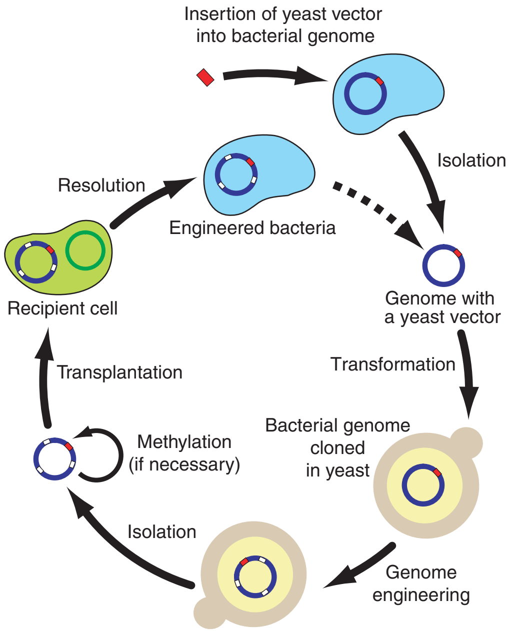

A
wt M. capricolum
M. mycoides YCpMmyc1.1
M. mycoides YCpMmyc1.1
ΔtypeIIIres::URA3
ΔtypeIIIres
~ kb
10
5
3
2
Native
Transplants
B
wt M. capricolum
M. mycoides YCpMmyc1.1
ΔtypeIIIres::URA3
ΔtypeIIIres
~kb
2
1
Native
Transplants
C
Sequence of typeIIIres deletion
AGGAAGGCTGTGTGAATTTCATTCGACAAGATAAAGGAAA
TTAGAACATTTACTTTAGTTACAAATAATGAAAATAATAT
AGGGATTGATGTTTGCTATGAACGCTTGTTTAGAATAAAT
AATGGTAAATCAACTGATAATAAAACAGATTTTAAATGAA
TAGAAAAAAACGAACCCTATCTGTCTAATTTAGATGTTTT
TGATATTAAATACTATTCAACTAAATTGTTTGAAGATAAT
AGTGCTAATGAAATAATCAAAAAACAATTCATAAAAATGT
TAAATGATCTAAAAATAAATGCTGATGATATTTCTACAAT
TAAAACTTTAAGACAATTAACTGCACTAAAACCAATCTCA
GGTGGACAAAACAATGAGATTAACTAATAAACAAGAATTT
GTAGTGCAAGATATTGTTAAAGAAGATTATTAAATTGTTA
AAATAAAAAATTACTTATTTAAGCGGGACTATTTCTATTT
TATTTAAAGAAGTCTTATGATTGAATGTAAAAGTTCTAAT
GTATTAACCCCTGTTACATTAAATACAACCATATCTATTT
TTAATTAATCTCCTGAAAGTACAAGTTCTTAATTCCTAAC
CATGCCTATTAAATTTAAGATCAAGAGCAGTTTTTCTAAC
CCCCGAAAAAAAACTCACAACAAAAA

Fig. 2. Southern blot analysis of *M. mycoides* transplants. (**A**) Hind III–restricted genomic DNA from representative YCpMmyc1.1, YCpMmyc1.1-*ΔtypeIIIres::URA3* (next-to-last columns), or YCpMmyc1.1-*ΔtypeIIIres* (last columns) *M. mycoides* genome transplants from yeast was probed with IS1296 sequences. The genomic DNAs of native *M. mycoides* YCpMmyc1.1 cells and *M. capricolum* recipient cells were also probed for comparison. All of the selected transplant colonies contain the same IS1296 pattern as native *M. mycoides* YCpMmyc1.1. (**B**) The engineered *M. mycoides* transplants were tested for the absence of the Type III restriction enzyme gene by Southern blot analysis (representative clones shown). Eco RV–restricted genomic DNA from YCpMmyc1.1-*ΔtypeIIIres::URA3*, and YCpMmyc1.1-*ΔtypeIIIres* *M. mycoides* cells derived by genome transplantation was probed with the *typeIIIres* gene sequence. The genomic DNAs of native *M. mycoides* YCpMmyc1.1 cells and *M. capricolum* recipient cells were also probed for comparison. The *typeIIIres* gene is absent in the engineered genomes but present in the native *M. mycoides* YCpMmyc1.1 genome. (**C**) Sequencing of the seamless deletion region of the YCpMmyc1.1-*ΔtypeIIIres* *M. mycoides* genome transplant verified that the Type III restriction gene was removed as designed. The sequence text colors are the same as the gene region colors in the genetic maps in Fig. 1. A small portion of the *typeIIIres* gene remained after the deletion because of the overlap between the *typeIIImod* and *typeIIIres* genes. The start and stop codons of the *typeIIIres* gene are boxed in red; the stop codon of the *typeIIImod* gene is boxed in black.

Fig. 3. Moving a bacterial genome into yeast, engineering it, and installing it back into a bacterium by genome transplantation. A yeast vector is inserted into a bacterial genome by transformation. That genome is cloned into yeast. After cloning, the repertoire of yeast genetic methods is used to create insertions, deletions, rearrangements, or any combination of modifications in the bacterial genome. This engineered genome is then isolated and transplanted into a recipient cell to generate an engineered bacterium. Before transplantation it may be necessary to methylate the donor DNA in order to protect it from the recipient cell's restriction system(s). This cycle can be repeated starting from the newly engineered genome (dashed arrow).


Insertion of yeast vector
into bacterial genome
Isolation
Genome with
a yeast vector
Transformation
Bacterial genome
cloned
in yeast
Genome
engineering
Isolation
Methylation
(if necessary)
Transplantation
Recipient cell
Resolution
Engineered bacteria

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* RE(–) recipient cells eliminates the possibility that components 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 methylate 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 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.

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Supporting Online Material

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Table S1
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On Universality in Human Correspondence Activity

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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 universality of these mechanisms by rescaling letter and e-mail correspondence statistics to reveal their underlying similarity.

Power law statistics are a hallmark of critical phenomena. A less obvious characteristic of criticality is the emergence of universality classes that capture the similarity of seemingly disparate systems. For example, despite the fact that water and carbon dioxide have different chemical properties, they were observed to behave in the same manner when close to their respective critical points (*1*). This is because idiosyncrasies, such as the existence of electric dipoles or the ability to form hydrogen bonds, become irrelevant near the liquid/gas critical point. For physical systems, renormalization group theory (*2*, *3*) has enabled researchers to understand the deep connection between the symmetries of a system and the mechanisms that underlie its behavior. The similarity of different fluids near their respective liquid/gas critical points is often demonstrated by rescaling their statistics so that they collapse onto the same universal curves (often power law curves),which have particular scaling exponents. By grouping different substances into the same universality class, as identified by its scaling exponents, one discovers that fluids are described by the same statistical laws near the liquid/gas critical point as uniaxial magnets are near their paramagnetic critical point (*1*). One can also differentiate the behavior of these systems from the behavior of polymers near the sol/gel transition, which belong to a different universality class (*1*).

In addition to describing critical phenomena, power law scaling has also been widely reported in biology, economics, and sociology (*4*–*10*). Renormalization group theory therefore offers a tantalizing hypothesis for the prevalence of particular power law scaling exponents in social systems: Social systems, in analogy with physical systems, may operate near critical points and can therefore be classified into a small number of distinct universality classes. A heated debate has consequently ensued in the literature concerning the "universality of human systems" (in the statistical physics meaning of the phrase). Is there enough statistical evidence for the asymptotic power law description of the heavy-tailed distributions reported in human systems (*11*–*14*)? Is it reasonable to postulate that social systems, like their physical counterparts (*2*, *3*, *15*), can be classified into universality classes according to scaling exponents (*16*)?

Human correspondence is a paradigmatic area where the matters of power law scaling and universality are contentious issues. One view that has recently received considerable attention in the literature (*17*, *18*) posits that correspondence patterns are driven primarily by the need to respond to other individuals. This is formalized by a priority queuing 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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