Cloning whole bacterial genomes in yeast

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ABSTRACT

Most microbes have not been cultured, and many of those that are cultivatable are difficult, dangerous or expensive to propagate or are genetically intractable. Routine cloning of large genome fractions or whole genomes from these organisms would significantly enhance their discovery and genetic and functional characterization. Here we report the cloning of whole bacterial genomes in the yeast *Saccharomyces cerevisiae* as single-DNA molecules. We cloned the genomes of *Mycoplasma genitalium* (0.6 Mb), *M. pneumoniae* (0.8 Mb) and *M. mycoides* subspecies *capri* (1.1 Mb) as yeast circular centromeric plasmids. These genomes appear to be stably maintained in a host that has efficient, well-established methods for DNA manipulation.

INTRODUCTION

DNA cloning is a defining technique of modern molecular biology. Generally, cloned DNA contains no more than a few genes. Recently, there has been increasing interest in cloning much larger pieces of DNA, including whole genomes. For example, several organelle genomes have been cloned. The 16-kb mouse mitochondrial genome has been cloned in *Escherichia coli* (1,2), *Bacillus subtilis* (1,3) and yeast (4). The 139-kb maize chloroplast genome has been cloned in yeast (5), and the 135-kb rice chloroplast genome has been cloned in *B. subtilis* (1). Viral genomes have been cloned in *E. coli*, including those from a number of herpesviruses (6) as well as baculoviruses (7–9), coronaviruses (10) and a poxvirus (11). A 36-kb human adenovirus type 2 genome (12) and a 178-kb fragment of the human cytomegalovirus genome (13) have been cloned in yeast. As for bacteria, ∼10% of the 1.8-Mb *Haemophilus influenzae* genome has been cloned as two separate episomal elements in *E. coli* (14). The 3.5-Mb *Synechocystis* PCC6803 genome was inserted in three discontiguous regions into the *B. subtilis* genome, with the exception of the two ribosomal RNA operons (15). We have reported the cloning of a completely synthetic 0.6-Mb *Mycoplasma genitalium* genome in yeast as a circular yeast centromeric plasmid (YCp) (16,17). We have also reported the cloning of the natural 1.1-Mb genome isolated from *M. mycoides* subspecies *capri* in yeast as a YCp (18).

The cloning of *M. genitalium* and *M. mycoides* genomes in yeast suggests that whole genome cloning could be valuable for engineering genetically intractable organisms, using standard yeast genetic methods. Genome transplantation, which we have recently demonstrated (18,19), would allow the introduction of a genome modified in yeast back into its original cellular environment. Whole genome cloning also facilitates the study of genomes from organisms that are difficult to culture. In addition, it aids in the construction and propagation of synthetic genomes.

Here we report cloning of the natural genomes from *M. pneumoniae* and *M. genitalium*, and describe in detail cloning of the *M. mycoides* genome, in the yeast *Saccharomyces cerevisiae*, a host which provides efficient, well-characterized methods for transformation and DNA manipulation.

MATERIALS AND METHODS

Construction of vector pmycYACTn

The 10-kb vector pmycYACTn was constructed from six fragments using our *in vitro* assembly method (16). The sequence has been deposited in GenBank with the

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accession number GU593054. Primers (IDT) and templates used for the construction are outlined in Supplementary Table S1. Ten nanograms of plasmid was used as template in a 100-μl polymerase chain reaction (PCR) with Phusion DNA polymerase and HF buffer (NEB), according to the manufacturer's protocol, except that extra magnesium chloride ($MgCl_2$) was added for a final concentration of 2 or 3 mM. Cycling conditions were as follows: 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 46–59°C (cycles 1–5) or 51–64°C (cycles 6–30) for 30 s and 72°C for 90 s, followed by 72°C for 5 min. Specific annealing temperatures (°C) for PCR cycles 1–5 were as follows: *bla* (ampicillin resistance) and pUC19 origin, 56 and 59; *lacZ*, 46 and 48; *tetM*, 46 and 50; transposase, 46; *HIS3* and *ARSH4-CEN6*, 48 and 52. Annealing temperatures for cycles 6–30 were 5°C higher. PCR products of each amplicon were pooled and each amplicon was gel purified and recovered using β-agarase (NEB). The transposase was initially hard to amplify from the template pISM31.1, possibly because one of the PCR primers used contained not only 20 bp of homology to the template at the desired location, but also 26 bp of homology to two copies of the IS256 inverted repeat present in other parts of the plasmid. These IS256 copies were separated from the desired template portion of pISM31.1 with a double digest with PciI and SalI, followed by gel-purification and use of the 3.7-kb fragment. The entire CBA reaction was repaired (16), then phenol extracted, isopropanol precipitated, resuspended and electroporated into EPI300 cells (Epicentre). Transformants were selected for carbenicillin resistance. Clones were screened for size by three separate restriction digests, but the plasmid was not sequenced. Instead, its elements were tested phenotypically. Propagation in *E. coli* ensured functionality of the pUC19 origin, while selection in *E. coli* with carbenicillin and tetracycline and screening with X-gal tested the intactness of the *bla*, *tetM* and *lacZ* markers. Successful transformation of yeast showed that the *HIS3*, *ARSH4* and *CEN6* markers were viable. Function of the transposon was tested by transformation into mycoplasma.

Construction of vector miniTn-Puro-JCVI-1.7

Primers (IDT) and templates used for the construction of miniTn-Puro-JCVI-1.7 are outlined in Supplementary Table S2. The sequence has been deposited in GenBank with the accession number GU593055. Amplicons 1–4 (Supplementary Table S2) were assembled using our one-step recombination method (20). This inserted a selectable mycoplasma marker, yeast centromeric vector features and a BAC into the pCC1 BAC vector (Epicentre). A 9616-bp fragment was then separated from the pCC1 BAC segment by digestion with NotI. Again, using our one-step recombination method, this fragment was combined with amplicons 5 and 6 (Supplementary Table S2) to make the *E. coli*–mycoplasma–yeast shuttle vector MiniTn-Puro-JCVI-1.7. All amplicons were produced using Phusion Hot Start High-Fidelity DNA Polymerase (NEB), according to the

manufacturer's protocol. Cycling conditions were 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 1 min per kilobasepair of amplicon, followed by 72°C for 5 min.

Mycoplasma strains and culture conditions

Mycoplasma strains transformed were *M. genitalium* strain MS5 (21), *M. mycoides* subspecies *capri* strain GM12 (22,23) and *M. pneumoniae* strain M129-B170 (ATCC 29343). All were cultured in SP-4 medium (24) at 37°C and 5% carbon dioxide (CO_2).

Yeast strains and culture conditions

Yeast strains used were VL6-48N (25), VL6-48-Δ54G (*MATα his3-Δ200 trp1-Δ1 ura3-52 lys2 ade2-101 met14 rad54-Δ1::kanMX*) and W303a. For transformation, yeast were cultured according to the transformation protocol (26); otherwise, yeast was cultured following standard protocols (27,28).

Analysis of pmycYACTn insertions into *M. genitalium* and *M. mycoides*

Primers were designed to sequence all of pmycYACTn in both directions, except for *tetM*, the spiralin promoter preceding *lacZ* and *lacZ*. Fourteen sequencing primers (IDT) with the following sequences were used: 5′-GCGC TCATAATAGCCATTTCGTTG-3′; 5′-ATTTCTGATG CGCACAAAGGATTAGTCTCT-3′; 5′-TGGCAGAGA CTAATCCTTTGTGCG-3′; 5′-GGTGCACGAGTGGG TTACATCGAAC-3′; 5′-GCCCGGCGTCAATACGG G-3′; 5′-TAAGCATTGGTAACTGTCAGACCAAGT TTA-3′; 5′-TAGCTCTTGATCCGGCAAACAAACC A-3′; 5′-TGGCGACGACTCCTGGAGC-3′; 5′-CAGAA ATCAGTAGAATTGCCCCATCTAA-3′; 5′-CCAACA AATACTACCTTTTATCTTGCTCTTCCT-3′; 5′-TACA CAGACAAGATGAAACAATTCGGC-3′; 5′-TAATCG TTGAGTGCATTGGTGACTTACA-3′; 5′-TGCCTCAT CCAAAGGCGCA-3′; 5′-TTCCCTCCACCAAAGGTG TTCTTATG-3′.

Analysis of MiniTn-Puro-JCVI-1.7 insertion into *M. pneumoniae*

DNA from a portion of the puromcyin-selected pool of *M. pneumoniae* cells transformed with MiniTn-Puro-JCVI-1.7 was isolated in an agarose plug. This plug was treated with beta-agarase (NEB) and 5 μl were electroporated into ElectroMAX Stbl4 competent *E. coli* cells (Invitrogen) according to the manufacturer's protocol, except that 50 μl of cells was used and the recovery time in SOC medium was 2 h. Transformants were selected at 30°C on LB with 25 μg/ml kanamycin. A single transformant was sequenced using Sanger sequencing with primers (IDT) internal to MiniTn-Puro-JCVI-1.7. The primers had the following sequences: 5′-CGTGAAGGTGAGCCAGTGAGTTG-3′ and 5′-CA GGTCGACTCGATGCAAACC-3′.

Genomic DNA isolation

Intact mycoplasma genomes were isolated in agarose plugs using Bio-Rad's CHEF Mammalian Genomic DNA Plug Kit according to the manufacturer's protocol.

DNA treatment of mycoplasma genomes for yeast transformation

Agarose plugs containing mycoplasma genomes with a yeast vector insertion were dialyzed against 10 mM (6%) PEG6000 (United States Biochemical) and 0.6 M NaCl for several hours (29), except where noted in the results. Plugs were then melted at 65°C for 5 min, after which two volumes of 65°C 10 mM Tris, pH 8.0, 1 mM EDTA was added and the mixture was stirred gently and incubated at 65°C for a further 5 min. After melting, plugs of *M. pneumoniae* were digested with beta-agarase (NEB) instead of diluted. For transformation 20 µl was used. This was DNA from ~10^7 *M. genitalium* cells, 10^8 *M. mycoides* cells and 10^7 *M. pneumoniae* cells.

Yeast transformation

Yeast transformations were performed as cited (26), except that cultures were sometimes grown to less than the recommended optical density (OD; see Table 1).

PCR analysis of yeast clones

Mycoplasma genomes cloned in yeast were screened for completeness by multiplex PCR using primers from IDT with the Qiagen Multiplex PCR Kit. Primers and PCR conditions are listed in Supplementary Tables S3–S5.

Sizing of mycoplasma genomes cloned in yeast

Total DNA from individual yeast clones containing mycoplasma genomes was isolated in agarose plugs using the protocol 'Preparation of Agarose Embedded Yeast DNA' from the Bio-Rad CHEF-DR III manual. To remove linear yeast chromosomal DNA, plugs were sometimes pre-electrophoresed at constant voltage (4.5 or 6V/cm) for several hours. (Under these conditions, large circular DNA molecules will not move out of the plugs.) To increase the efficiency of this step, plugs were sometimes first digested with AsiSI, FseI and RsrII. These enzymes cleave yeast chromosomes but do not have recognition sites in *M. genitalium* or *M. mycoides*. Clones were sized by restriction digestion, followed by field-inversion (Bio-Rad FIGE Mapper) or pulsed-field (Bio-Rad CHEF-DR II or III system) electrophoresis. Restriction digestion was sometimes replaced by linearization by heating at 55°C for 1 h. Southern blots were sometimes performed on these gels, as previously described (16).

Preparation of vector pARS-VN

The vector pARS-VN (30) was linearized by a double digest with ClaI and XhoI, treated with antarctic phosphatase and gel purified using beta-agarase. A sample of 5 ng was used as template in a 100-µl PCR with Phusion DNA polymerase and HF buffer, according to the manufacturer's protocol. All of the vector except for the 9 bp between the unique ClaI and XhoI restriction sites was PCR amplified, producing a 6.5-kb product. Primers were supplied by IDT polyacrylamide gel electrophoresis (PAGE) purified. Their sequences are as follows, with the vector sequence in bold and the ClaI (first primer) or XhoI (second primer) site underlined: 5′-TTAATAACAAAAAAATCTCTATTAAAAAAACC AACTTTAAAGTTGGTTTGAAATTCTAAA**ATCGA TGTCGAAAGCTACAT**-3′ and 5′-GGATAGA**GTGTCT GGCTTCGGACCAGAAGGTTATGGGTTCAAGTC CTATTGGGCGCGCCACTCGAGCCACTATTTATAC C**-3′. The 5′ 60 nts of each oligo are homologous to the *M. genitalium* genome. Cycling conditions were 98°C for 30 s, followed by 25 or 30 cycles of 98°C for 10 s, 57°C or 60°C for 30 s and 72°C for 5 min, followed by a final extension of 72°C for 7 min. A 6.5-kb product was gel purified using beta-agarase. All enzymes were from NEB.

Preparation of vector pTARBAC3

The vector pTARBAC3 (31) was linearized with AatII, treated with antarctic phosphatase and gel purified using beta-agarase (all enzymes from NEB). A sample of 10 ng was used as template in a 100-µl reaction with 5-U Takara LA *Taq* DNA polymerase (Takara) at final concentrations of 1× LA PCR buffer II, 3.5 mM $MgCl_2$, 0.5 µM each primer and 0.4 mM each of the four dTNPs. Cycling conditions were as follows: 94°C for 1 min, followed by 30 cycles of 94°C for 20 s, 55°C (cycles 1–5) or 60°C (cycles 6–30) for 30 s, and 72°C for 12 min, followed by 72°C for 8 min. For insertion at the AscI site in quarter 3 of the synthetic *M. genitalium* genome, pTARBAC3 was amplified using primers (IDT) with the following

Table 1. Cloning in yeast of three mycoplasma genomes, each containing an integrated yeast vector

Genome			Host yeast strain (Mating type)	OD$_{600}$ of yeast at harvest	Number of transformants	Number of PCR amplicons tested	Fraction of clones complete by PCR	Fraction of clones correct size by gel or blot
Species, clone	Size (Mb)	Percent GC						
M. genitalium YCpMgen16–2	0.6	32	VL6-48N (α)	4.9	172	10	22/24	2/3
			W303a (a)	2.8	421	10	5/8	4/5
M. mycoides YCpMmyc1.1	1.1	24	VL6-48N (α)	5.0	174	10	20/48	5/6
			VL6-48-Δ54G (α)	3.5	54	10	19/48	Not done
			W303a (a)	1.6	57	10	8/15	8/8
M. pneumoniae, pool of transformants	0.8	40	VL6-48N (α)	0.6	67	20	13/20	5/9

Clones were first screened for completeness by multiplex PCR with one or two sets of 10 amplicons each. Some or all clones complete by PCR were then tested for size by restriction digestion and gel electrophoresis, followed in some cases by Southern blot.

sequences: 5′-TTAATAACAAAAAAATCTCTATTAAA AAAACCAACTTTAAAGTTGGTTTGAAATTCTAA AG<u>CGGCCGC</u>**ATTGCATCAACGCATATAGC**-3′ and 5′-GGATAGAGTGTCTGGCTTCGGACCAGAAGGT TATGGGTTCAAGTCCTATTGGGCGCGCCA<u>GCG GCCGC</u>**AGAGCCTTCAACCCAGTCAG**-3′ (NotI sites are underlined and vector sequence is in bold). For insertion at the BsmBI site in quarter 3 of the synthetic *M. genitalium* genome, pTARBAC3 was amplified using primers (IDT) with the following sequences: 5′-AGGTTT CTTGCTTGTGGAGTTTACCGCGTTTCATACCTG GTTTTCACCAAGCTCGTCTCTGCGGCCGC**ATTG CATCAACGCATATAGC**-3′ and 5′-GTGATAGCCATA ACGACATAACTAGTCTAAGGTTAATACTATGA TGATGTTTTGAAAGTG**GCGGCCGC**AGAGCCTTC AACCCAGTCAG-3′ (NotI sites are underlined and vector sequence is in bold). The 5′ 60 nts of each oligo are homologous to the *M. genitalium* genome. Each PCR product was gel purified using beta-agarase.

Marker replacement in YCpMgen16-2

To replace *HIS3* with *TRP1*, a 1059-bp *TRP1* fragment with homology to YCpMgen16-2 was amplified by PCR from the plasmid pRS304 (32) using primers with the following sequences: 5′-**TACGAGGCGCGTGTAAGTTAC AGGCAAGCGATCCTAGTACACTCTATAT**CAGAGC AGATTGTACTGAGAGTGCACC-3′ and 5′-**CTACAT AAGAACACCTTTGGTGGAGGGAACATCGTTGGTA CCATTGGGCGCGC**ATCTGTGCGGTATTTCACA CCGC-3′ (YCpMgen16-2 sequence is in bold). This fragment was transformed into yeast using lithium acetate (33). Gene replacement was checked by amplification with two primers (sequences 5′-ATTATTCCATCAT TAAAAGA-3′ and 5′-AGTCAAGTCCAGACTCCT GT-3′). These produce a 1207-bp fragment when *HIS3* is replaced with *TRP1* and a 927-bp fragment when replacement has not occurred.

Vector relocation in a synthetic *M. genitalium* genome yeast clone

A yeast clone of a synthetic *M. genitalium* genome with a yeast vector insertion disrupting *RNase*P (16) was cotransformed with two fragments. One fragment inserted yeast vector sequence containing *URA3, the GAL1* promoter and a centromere into MG411. This 1842-bp fragment was generated by PCR using primers with the sequences 5′-**CAGATGGTATTCCTGAAAGGA TATCAATAATAAGTGAAAGTTTTTTTCTTATTTT GGTT**<u>GCGGCCGC</u>**CTTGATTTCGGTTTCTTTGAAA** T-3′ and 5′-**TTAATTATTGCTAGTTATATAGGGGTTA GAACTTCATTTTTCCTTGTTTATCGATGCA**<u>GCGG CCGC</u>GGGTCCTTTTCATCACGTG-3′ (*M. genitalium* sequence is in bold; NotI sites are underlined). The template for this was a construct we had made previously, which was generated as follows. We modified a yeast clone of a synthetic *M. genitalium* genome, sMgTARBAC37 (16), by integrating a 1.5-kb DNA fragment containing *URA3* and the *GAL1* promoter near the centromere in the clone's vector. The 1.5-kb fragment was generated using the PCR-based fusion method (34) to join together

two overlapping PCR products. The *URA3* gene was PCR amplified from the plasmid pRS306 (32) by primer pair ARS-URA (5′-<u>TTCGTGTGTGGTCTTCTACACAGAC AAGATGAAACAATTCGGCATTAATATTGATTTC</u> GGTTTCTTTGAA-3′) and URA-R-GAL (5′-**CTCGGC GGCTTCTAATCCGT**GGGTAATAACTGATATAAT T-3′), and the *GAL1* promoter was amplified from the plasmid pYES2 (Invitrogen) by primer GAL-F-URA (5′-**AATTATATCAGTTATTACCC**CACGGATTAGAAG CCGCCGAG-3′) and GAL-CEN (5′-<u>GGATCGCCAAC AAATACTACCTTTTATCTTGCT</u>**TTCCTGCTCTC** AGGCCGGTTTTTTCTCCTTGACGTTAA-3′). The 40 nts of overlapping sequence is in bold. The 1.5-kb fusion product was integrated into the target site by homologous recombination at the 50-bp underlined sequences. The other fragment replaced the yeast vector insertion in *RNase*P with *M. genitalium* sequence such that the coding region of this gene was restored. This 302-bp fragment was generated by PCR from *M. genitalium* DNA using primers with the sequences 5′-TTAAAGTC AGTTATTTATCTACAGC-3′ and 5′-CTTTAAAGATA TATTTAAAGTAGAGG-3′. Before cotransformation, *TRP1* was inserted into MG411. An 1177-bp *TRP1* gene fragment with homology to MG411 was amplified from the plasmid pRS304 using two primers with sequences 5′-**CAGATGGTATTCCTGAAAGGATATCAATAATAA GTGAAAGTTTTTTTCTTATTTTTGGTT**CAGAGCA GATTGTACTGAGA-3′ and 5′-**TTAATTATTGCTAG TTATATAGGGGTTAGAACTTCATTTTTCCTTGTT TATCGATGCA**CGCATCTGTGCGGTATTTCA-3′ (*M. genitalium* sequence is in bold). *TRP1* gene insertion was checked with a set of primers (sequences 5′-GCCATTGTT TCACTAATTGC-3′ and 5′-TAATCCTATCTTTGGAG CTT-3′) that amplified 1739 bp if insertion occurred and 680 bp if it did not. Cotransformants which were His-Trp-Ura + were selected. Restoration of *RNase*P was confirmed by PCR amplification of a 513-bp product using primers with sequences 5′-CTCCATCATGCGCAGTAA TA-3′ and 5′-CTTTAAAGATATATTTAAAGTAGAG G-3′. Replacement of *TRP1* with yeast vector sequence was confirmed by PCR amplification of an 1841-bp product using primers with sequences 5′-TTGATTTCG GTTTCTTTGAA-3′ and 5′-CAGGCAGGAATTTGAT TCCC-3′.

RESULTS

Strategies for cloning bacterial genomes in yeast

Large DNA molecules have been stably cloned in yeast by the addition of a yeast centromere (*CEN*), which allows the molecules to be segregated along with the yeast chromosomes. Such molecules have been cloned both in the linear form (35,36), by the addition of telomeres to the ends, and also as circles (37). Since bacterial genomes are generally circular, it is easier to clone them in this form. This is also an advantage because circles can be readily separated from linear yeast chromosomes (16,38).

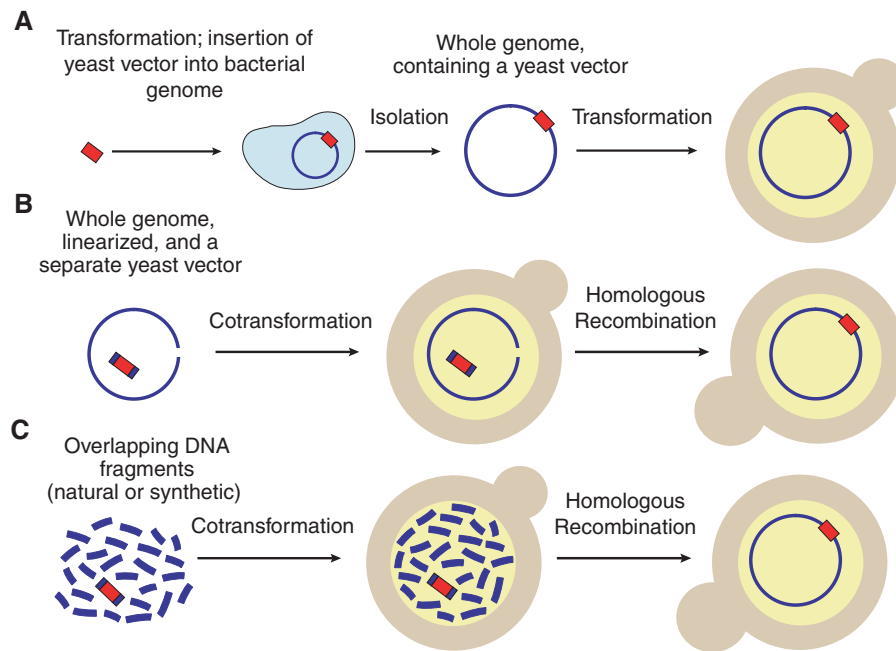We have taken three different approaches to the cloning of bacterial genomes in yeast (Figure 1). The first

Figure 1. Three methods for cloning mycoplasma genomes in yeast. In order to be propagated by yeast (tan, with yellow nucleus) upon transformation, the bacterial genome (blue circle) must contain several yeast sequences (vector, red bar). (**A**) These can be incorporated into the bacterial genome by transformation of the bacterium (light blue). (**B**) Alternatively, they can be inserted by cotransformation of yeast with the vector and the bacterial genome. In this case the two must share overlapping sequences, so that yeast can combine them by homologous recombination. (**C**) The bacterial genome can also be cloned by assembling multiple overlapping fragments.

approach is to insert a yeast vector into the bacterial genome prior to transformation of yeast (18,39,40) (Figure 1A).

In the second approach, the bacterial genome and a yeast vector are cotransformed into yeast (Figure 1B). The linear vector contains terminal regions of homology to a site in the genome and is inserted by homologous recombination. Vector insertion is much more efficient if the genome contains a double-stranded break near the insertion point (41). The second approach can also be done with multiple overlapping fragments (Figure 1C) (16,17).

The approach of Figure 1A selects for vector insertion sites that do not interfere with bacterial viability. This method is favored for a genome that will be transplanted to produce a living cell. Another advantage is that vector insertion may not require knowledge of the genome's sequence. However, this strategy requires that the bacterium be transformable so that the vector can be integrated into the genome. The approach of Figure 1B does not require transformation or even cultivation of the organism, just intact genomic DNA. This method is favored for genetically intractable or uncultured organisms. These may include pathogens and environmental isolates. However, this strategy does not guarantee fitness or viability of the cloned genome. In addition, it may be difficult to find a suitable restriction site for vector insertion, and vector insertion may require some sequence knowledge. The approach of Figure 1C is useful for assembling pieces of DNA, both natural and synthetic. The vector can be included as a separate fragment, or it can be included as a sequence within a larger fragment. In this way genomes can be constructed to study gene function and to create microbial cell factories.

We cloned the *M. genitalium* genome by all of the above approaches. We cloned the *M. mycoides* subspecies *capri* and the *M. pneumoniae* genomes separately in yeast, and the *M. genitalium* and *M. mycoides* genomes as two separate molecules in the same yeast cell, by the strategy depicted in Figure 1A.

Construction of tri-shuttle vectors for cloning of mycoplasma genomes in yeast

To clone mycoplasma genomes by the strategy shown in Figure 1A, we designed and constructed the vectors shown in Figure 2A and B. These plasmids were intended to insert yeast centromeric vector sequences into a mycoplasma genome by transposition. The transposase was constructed outside of the set of IS256 inverted repeats, so that it would be lost after the initial transposition, preventing the inserted sequence from moving within the mycoplasma genome.

The essential features of the pmycYACTn vector (Supplementary Table S1) are: (i) a high copy origin from pUC19 and an ampicillin-resistance marker for propagation in *E. coli*, (ii) the IS256 transposase and inverted repeats for transposition into a mycoplasma genome, (iii) *tetM* and *lacZ* markers, both expressed from spiralin promoters (42,43), for selection and screening in *E. coli* and mycoplasmas and (iv) an *ARS* (autonomously replicating sequence) and a *CEN* for replication and segregation in yeast, and *HIS3* as a selectable
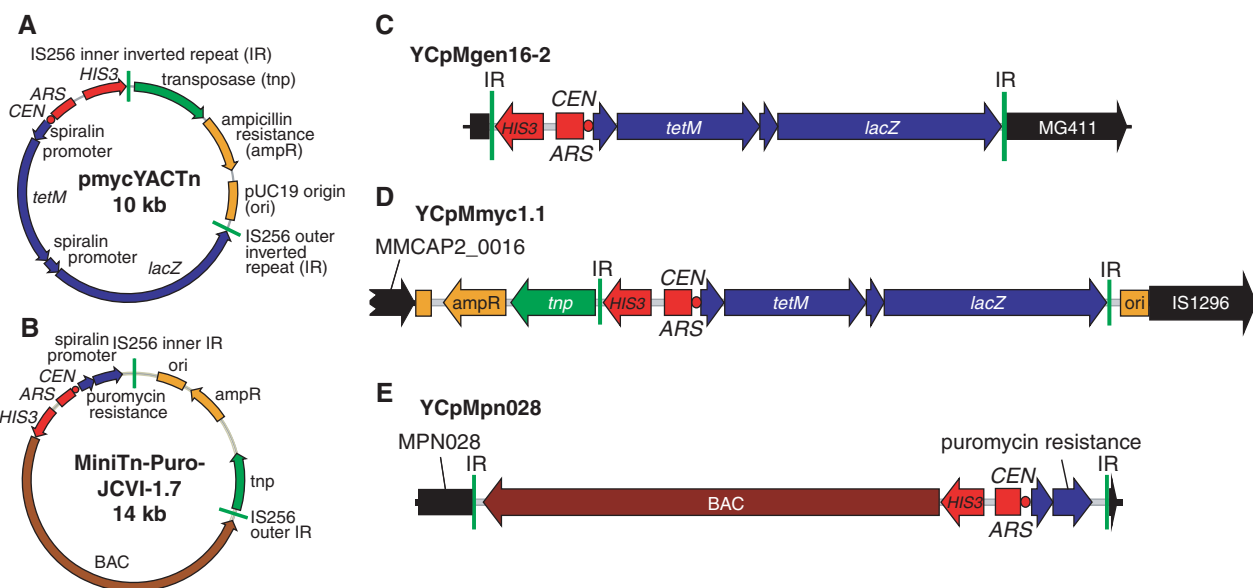
Figure 2. Yeast vector insertions in each of the *M. genitalium*, *M. mycoides* and *M. pneumoniae* genomes, using the approach shown in Figure 1A. (**A** and **B**) The two shuttle vectors used. (**C–E**) The location of the vector insertion in each genome: mycoplasma markers are blue, yeast vector features are red, the *E. coli* plasmid backbone is orange, BAC sequences are brown and transposon elements are green. Mycoplasma sequence is black.

yeast marker. The miniTn-Puro-JCVI-1.7 vector (Supplementary Table S2) differs from pmycYACTn as follows: (i) it does not contain *lacZ* and substitutes a puromycin resistance marker for *tetM* and (ii) it contains a bacterial artificial chromosome (BAC) vector, for possible cloning in *E. coli*.

Cloning mycoplasma genomes that contain an integrated yeast vector

Mycoplasma genitalium strain MS5 (21) was transformed with the vector pmycYACTn by electroporation (44), and transformants were selected with tetracycline. A clone (YCpMgen16-2) was chosen for further analysis. Direct genomic sequencing (44) using primers internal to the vector was performed to determine the site of vector insertion. This clone contained the sequence between and including the two IS256 inverted repeats of pmycYACTn, indicating that transposition had occurred, as designed (Figure 2C). The transposase, pUC19 origin and ampicillin-resistance gene were lost during transposition. The vector inserted within the nonessential MG411 gene (44,45), after the base pair corresponding to 514699 in the *M. genitalium* G-37 sequence (GenBank accession L43967). Base pairs 514694–514699 were duplicated at the insertion site, a phenomenon which has been previously noted (44,46,47).

Mycoplasma mycoides subspecies *capri* strain GM12 (22,23) was transformed with pmycYACTn using PEG (48), and transformants were selected with tetracycline. All four clones were analyzed by direct genomic sequencing to locate the insertion site. Rather than the expected transposition, in all four cases we observed integration of the entire plasmid. In three clones, pmycYACTn was inserted by a crossover within or very close to the pUC origin. In the fourth clone the crossover

occurred within the yeast *HIS3* gene, rendering this genome unsuitable for transformation into yeast. All vector insertions were adjacent to an IS1296 element.

The vector in one of the clones may be able to transpose within the *M. mycoides* genome, since it is bounded on either side by an IS1296 inverted repeat. Since pmycYACTn did not transpose initially, it probably cannot move within the genome by IS256 transposition. We do not know the mechanism by which pmycYACTn inserted into the *M. mycoides* genome. The plasmid contains a functional transposon, since we saw evidence of its transposition into the *M. genitalium* genome. In addition, all four *M. mycoides* clones containing the vector have the correct sequence of the IS256 tranposase and its two inverted repeats. We have not found any published evidence for the functionality of IS256 in *M. mycoides*.

Clone YCpMmyc1.1 (Figure 2D) was selected for cloning into yeast because it grew well and transplanted well into *M. capricolum* (19). We later modified this genome in yeast, sequenced it and deposited the sequence in GenBank (accession CP001668). Base pairs 25040–35183 are pmycYACTn sequence. The insertion of pmycYACTn caused a duplication of several base pairs of the plasmid, and a deletion of several base pairs of *M. mycoides* sequence as compared to the sequence of the other *M. mycoides* clone that is available (GenBank accession CP001621).

M. pneumoniae strain M129-B170 (ATCC 29343) was transformed with MiniTn-Puro-JCVI-1.7 by electroporation (44), and a pool of puromycin-resistant transformants was selected in liquid culture. Selection in liquid culture took several weeks less than plating the transformation mixture and then expanding the resulting individual colonies in liquid medium. When analyzed by
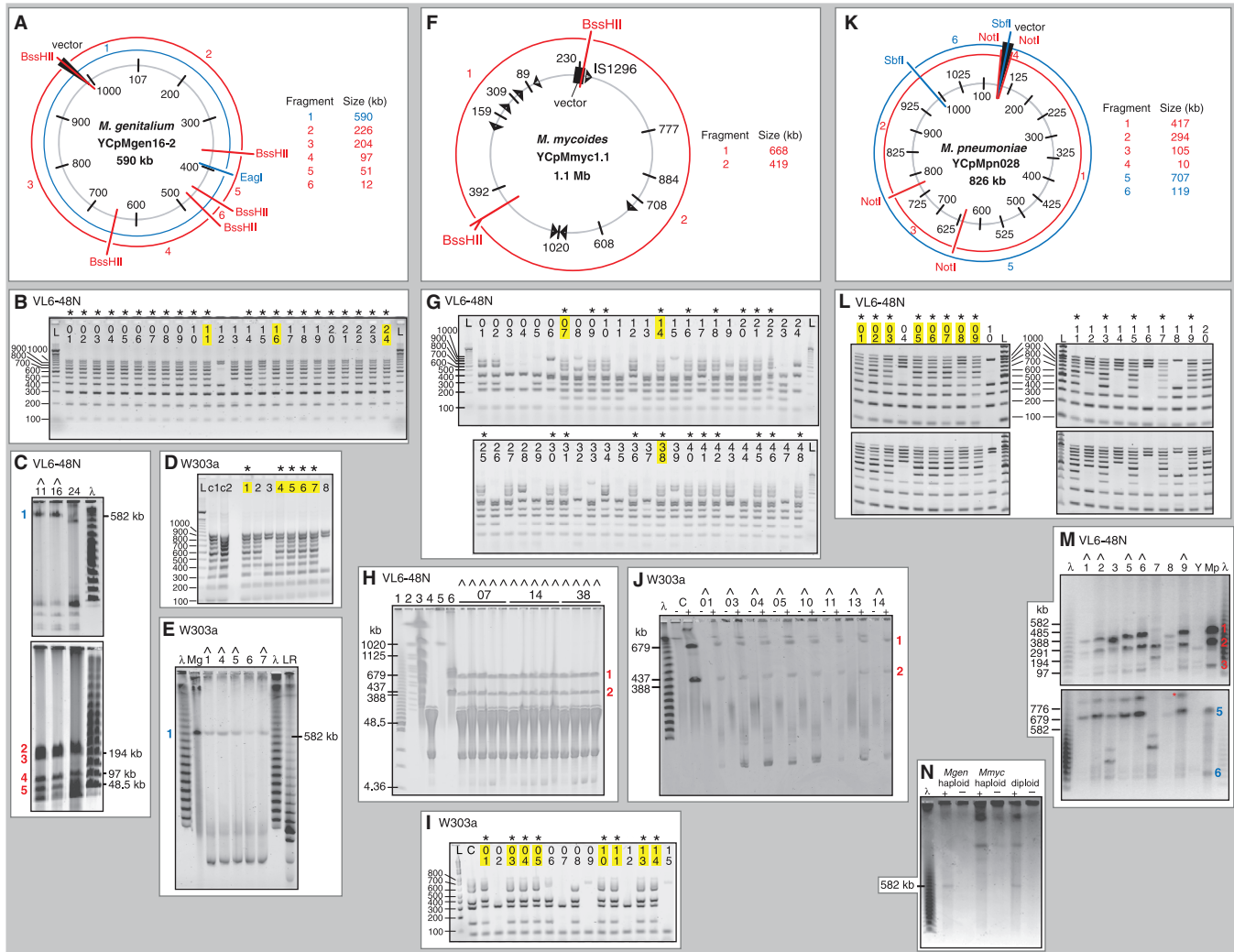
Figure 3. Gel analysis of whole mycoplasma genome clones in yeast. The host yeast strain of each set of clones is marked above the gel. Clones were first assayed for completeness by multiplex PCR (B, D, G, I, L). Complete clones are marked with an asterisk, and those selected for sizing by restriction digestion and gel electrophoresis (C, E, H, J, M) are highlighted in yellow. Clones judged to be the correct size are marked with a caret. L is a 100-bp ladder (Invitrogen), λ is a lambda ladder (NEB) and LR is the Low Range PFG ladder (NEB); selected bands are labeled. (**A**) Map of the *M. genitalium* YCpMgen16-2 genome. The location of the yeast vector insertion is marked. Bars indicate position and numbers indicate size of PCR amplicons. Restriction fragments are numbered and their sizes given in red or blue. (**B**) Multiplex PCR analysis of 24 yeast clones derived from YCpMgen16-2, using primers shown in (A). (**C**) Selected clones assayed as correct in (B) digested with EagI (top gel) or BssHII (bottom gel) and separated by field inversion gel electrophoresis (FIGE). Yeast chromosomal DNA was removed prior to this analysis by digestion with FseI, RsrII, and AsiSI, followed by constant voltage electrophoresis (these enzymes do not have recognition sites in YCpMgen16-2 and large circular DNA molecules remained in the agarose plugs under these conditions). (**D**) Multiplex PCR analysis of eight yeast clones derived from YCpMgen16-2, using primers shown in (A). sMgTARBAC37 (c1) (16) and *M. genitalium* (c2) DNAs were used as positive controls. (**E**) All clones assayed as correct in (D) linearized with EagI and separated by clamped homogenous electric field (CHEF). Before digestion, clones were pre-electrophoresed at constant voltage to remove linear yeast chromosomes. 'Mg' indicates NotI-digested 592-kb sMgTARBAC37 (16). Clone 6 contains a faint extra band of ∼300 kb. (**F**) Map of the *M. mycoides* YCpMmyc1.1 genome. The location of the yeast vector insertion is marked. Arrowheads represent IS1296 elements. Bars indicate position and numbers indicate size of PCR amplicons. Restriction fragments are numbered and their sizes given in red. (**G**) Multiplex PCR analysis of 48 yeast clones derived from YCpMmyc1.1, using primers shown in (F). An additional amplicon of 464 bp, not shown in (F), acted as a positive control by amplifying yeast rDNA. (**H**) Selected yeast clones assayed as correct in (G) digested with BssHII and separated by CHEF. The parent clone (far left in each set) and 3–4 subclones of each were analyzed. Markers and controls are: (1) Low Range PFG Marker (NEB); (2) *S. cerevisiae marker* (Bio-Rad); VL6-48N (3) undigested and (4) BssHII-digested; and YCpMmyc1.1 (5) undigested and (6) BssHII-digested. (**I**) Multiplex PCR analysis of 15 yeast clones derived from YCpMmyc1.1, using primers shown in (F). (**J**) All clones assayed as correct in (I) pre-electrophoresed at constant voltage to remove linear yeast chromosomes, then left undigested (−) or digested (+) with BssHII and separated by CHEF. 'C' indicates YCpMmyc1.1 DNA isolated from *M. mycoides* used as a positive control. (**K**) Map of the *M. pneumoniae* YCpMpn028 genome. The location of the yeast vector insertion is marked. Bars indicate position and numbers indicate size of PCR amplicons. Restriction fragments are numbered and their sizes given in red or blue. (**L**) Multiplex PCR analysis of 20 yeast clones derived from YCpMpn028, using primers shown in (K). (**M**) Selected clones assayed as correct in (L) pre-electrophoresed at constant voltage, then digested with NotI (top gel) or SbfI (bottom gel) and separated by CHEF. Host yeast strain VL6-48N is labeled 'Y' and *M. pneumoniae* DNA transformed into yeast is labeled 'Mp'. A red asterisk marks incomplete digestion (linearization) of the yeast clone. (**N**) A haploid yeast strain containing an *M. genitalium* genome and a haploid yeast strain containing an *M. mycoides* genome are compared to a diploid yeast clone containing the two mycoplasma genomes. Clones were pre-electrophoresed at constant voltage, then left unheated (−) or incubated at 55°C for 1 h for linearization (+). The genomes are too large to move into the gel as circles; they must be linearized to migrate.

restriction digestion, the pool of transformants gave discrete bands whose sizes summed to one *M. pneumoniae* genome (Figure 3M, label 'Mp'). This indicated a predominant vector insertion site. Direct genomic sequencing with primers internal to the vector was unsuccessful. Thus, DNA from the pool was electroporated into *E. coli*. Incubation of transformants with kanamycin selected circularized *M. pneumoniae* fragments that contained the vector, which included BAC sequences. Plasmid DNA from one such transformant was isolated and sequenced. It contained insertion of the vector by transposition within MPN028, after the base pair corresponding to 33901 in the *M. pneumoniae* M129 sequence (GenBank accession U00089), with a duplication of bp 33893–33901. This gene has been shown to be nonessential in *M. genitalium*, but not in *M. pneumoniae*. However, the vector insertion occurred near the 3′-end of the gene, which may not be disruptive to gene function (44,45). The restriction map produced by vector insertion at this location matched our results. This genome was named YCpMpn028 (Figure 2E).

In order to minimize breakage, genomes with yeast vector insertions from all three mycoplasmas were isolated in plugs using low melting-point agarose. Yeast spheroplasts were transformed with DNA from the plugs by a published method (26). Cells were suspended in 1 M sorbitol and treated with zymolyase to remove the cell wall. DNA was recovered from the agarose plugs, sometimes in the presence of 6% PEG and 0.6 M NaCl (29), then incubated with spheroplasts. After recovery in growth medium, cells were plated in selective medium.

All three genomes were transformed into the yeast strain VL6-48N, which has been developed for high transformation efficiency (25). The YCpMgen16-2 and YCpMmyc1.1 genomes were also transformed into the commonly used strain W303a. In addition, we transformed the YCpMmyc1.1 genome into a recombination-deficient yeast strain. We speculated that this genome might be unstable in yeast, possibly due to recombination among the nearly identical 1.5-kb IS1296 copies. Yeast strains defective in the *RAD54* gene have been shown to decrease the occurrence of a variety of recombination events in yeast artificial chromosomes (YACs) (49). The *rad54* mutant strain used (VL6-48-Δ54G) is nearly isogenic with VL6-48N.

Results shown in Figure 3 are summarized in Table 1. Clones were screened first for completeness by multiplex PCR. Amplicons were evenly spaced around the *M. genitalium* and *M. pneumoniae* genomes (Figure 3A and K). Since we speculated that the most likely instability of the *M. mycoides* genome sequence in yeast might be homologous recombination among its copies of IS1296, one or more amplicons was located between each pair of these elements (Figure 3F). We screened some *M. mycoides* genome clones with two additional primer sets. One amplicon lay within the *HIS3* marker of the yeast vector, and an additional primer set amplified yeast rDNA as a positive control for the assay. Some or all clones that appeared complete by PCR were screened for size by restriction digestion and gel electrophoresis.

For *M. genitalium*, we examined 24 individual clones in strain VL6-48N. Of these, 22 appeared to be complete by PCR analysis (Figure 3B). CHEF gel analysis showed two out of three of these to be the expected size (Figure 3C). With strain W303a, five out of eight clones were complete by PCR analysis (Figure 3D). Of these, four were of the expected size when examined by CHEF gel (Figure 3E). For *M. mycoides*, we examined 48 clones in strain VL6-48N and found 20 to be complete by PCR (Figure 3G). We obtained essentially the same PCR result when *M. mycoides* was transformed into the *rad54* recombination-deficient strain (data not shown). Of the 20 complete clones in VL6-48N, six were examined by Southern blot of a CHEF gel and five appeared to be the correct size (blot not shown; restriction analysis of selected clones with subclones shown in Figure 3H). A total of 8 out of 15 clones in strain W303a were complete by PCR (Figure 3I), and all of these were the correct size by CHEF analysis (Figure 3J). Transformations into W303a were carried out in the absence of the PEG and NaCl treatment of the plug. The same was true for transformation of strain VL6-48N with a pool of *M. pneumoniae* genomes containing a yeast vector. A total of 13 out of 20 transformants examined by PCR appeared to be complete (Figure 3L). CHEF gel analysis showed five out of nine of these to be the expected size (Figure 3M).

Cloning *M. genitalium* in yeast by homologous recombination

Vector insertion by homologous recombination (Figure 1B and 1C) is much more efficient if the genome contains a double-stranded break near the insertion point. Such a break can be created by restriction digestion. The *M. genitalium* genome contains three single-cut restriction sites, two of which lie within its rRNA operon, which is essential for viability. The third lies at the 3′-end of a tRNA coding sequence. A vector was inserted by homologous recombination adjacent to this AscI site, since the cloning could be designed to preserve the integrity of the tRNA, which is probably essential.

The yeast cloning vector pARS-VN (30) was used as template for PCR with a pair of primers, each containing 60 bp homologous to the region flanking the AscI insertion site in the *M. genitalium* genome, and 20 bp of homology to the vector. Yeast strain VL6-48N spheroplasts were cotransformed with a mixture of linear vector DNA (140 ng) and *M. genitalium* strain MS5 DNA (from about 10^7 cells). *M. genitalium* DNA was isolated in agarose plugs to minimize breakage. Before transformation these plugs were melted and digested with agarase.

We tested the effect of a double-stranded break at the cloning site produced by AscI digestion of the *M. genitalium* genome (Figure 4A versus C). To increase the fraction of intact genomes, broken DNA was removed from some of the plugs by CHEF gel electrophoresis.

AscI-digested DNA yielded 45 transformants. Of these, 21 were positive for all of 20 PCR amplicons tested. These 21 were examined by Southern blot of a CHEF gel and 15 appeared to be the correct size. Undigested *M. genitalium*
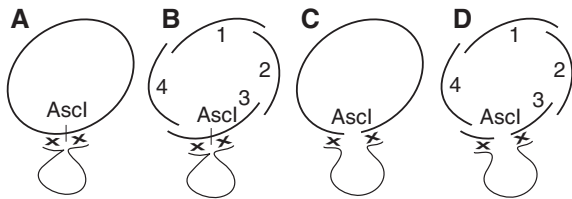
Figure 4. Targeted insertion of a yeast vector using homologous recombination. A yeast vector insertion was attempted with and without a double-stranded break at the insertion point. (**A**) Intact genome and linear vector. (**B**) Overlapping genome fragments and vector with homology internal to one of the fragments. (**C**) Genome cleaved at the integration target and linear vector. (**D**) Overlapping genome and vector fragments.

genomes yielded 50 transformants. Of these, seven were positive for all PCR amplicons. One of these was the correct size. This is consistent with findings that a double-stranded break near a site of homologous recombination increases the efficiency of this event by ~20-fold (41).

Cloning *M. genitalium* by assembly of overlapping fragments in yeast

In Figure 1C we show assembly of genomes from multiple pieces by homologous recombination in yeast. We have previously reported this strategy using pieces derived from *E. coli* BAC clones. We assembled a synthetic *M. genitalium* genome from six pieces, of which one was a vector (16). We have also reported assembly of the genome from 25 pieces (17). The same strategy could be used for assembling any complete set of overlapping genomic clones, natural or synthetic.

When we assembled a synthetic *M. genitalium* genome from six pieces, we started with four quarter genomes (about 144 kb each) cloned as BACs. These quarter genomes were released from their BAC backbones by restriction digestion, yielding four overlapping linear fragments, as described previously (16). Yeast was transformed with a mixture of 120 ng of each quarter genome and 10 ng of linear vector. The vector was produced by amplification of the plasmid pTARBAC3 (31) in a manner analogous to our preparation of vector pARS-VN.

Quarter 3 (Figure 4B and D) was digested at the unique AscI site to produce a double-stranded break at the vector-insertion target. We obtained 65 transformants, which we first assayed for completeness by PCR with a set of six amplicons. One amplicon lay internal to each of the four quarter genome fragments, one lay within the *HIS3* marker of the vector, and one amplified yeast rDNA as a positive control for the assay. Twenty-four clones were correct by this assay. Six out of 17 of these clones were correct when assayed by Southern blot. When we performed the same experiment with the uncleaved quarter we obtained two transformants, neither of which showed a complete *M. genitalium* genome.

We performed another experiment, using the BsmBI site in quarter 3. We obtained 73 transformants, 44 of which were correct as assayed by PCR. Five out of 28 of these

clones examined by Southern blot were correct. With the quarter undigested we obtained two transformants, neither of which showed a complete *M. genitalium* genome. These results agree with the known higher efficiency of homologous recombination at DNA ends (50).

Construction of a diploid yeast strain carrying both *M. genitalium* and *M. mycoides* genomes

Some bacterial genomes consist of more than one chromosome. Thus, for the successful transplantation of these organisms' genomes, it would be necessary to transplant more than one DNA molecule. If cloned in yeast, it would be convenient for all of these chromosomes to be cloned into the same yeast cell. This would also be useful if a bacterial chromosome were cloned as more than one molecule in yeast. This might be convenient for engineering or circumvention of a possible size barrier on individual chromosomes in yeast. To explore this we tested whether yeast could maintain two cloned bacterial genomes.

To produce a diploid strain carrying two mycoplasma genomes, we crossed two haploid strains (51), each of which carried one of the genomes. We mated the W303a strain (mating type a) containing YCpMgen16-2 with the VL6-48N strain (mating type α) containing YCpMmyc1.1 (Table 1). The *HIS3* marker in the *M. genitalium* genome was replaced with a *TRP* marker to allow selection of diploids carrying both genomes on medium lacking histidine and tryptophan. Plugs prepared from individual colonies of these diploid strains were analyzed by CHEF electrophoresis. Five out of five diploids contained both *M. genitalium* and *M. mycoides* genomes (one diploid shown in Figure 3N).

The vector does not require an ARS for maintenance of the *M. genitalium* genome in yeast

For a clone to be propagated in yeast, it must contain sequences that can act as yeast replication origins. One such sequence can be included in the yeast cloning vector. However, one replication origin may not be enough to support propagation of an entire bacterial or archaeal genome. It has been extrapolated that one yeast origin may be sufficient to support the replication of 120–300 kb (52). Yeast contains a replication origin on average every 30–40 kb (53–55). Large eukaryotic sequences can be cloned in yeast without the addition of yeast replication origins; thus, they are assumed to contain sequences that can function as them. The frequency of such sequences in bacterial and archaeal genomes is unknown; the only bacterial ARS reported is from a *Staphylococcus aureus* plasmid (56).

We relocated the yeast vector in the synthetic *M. genitalium* genome from its original site within the *RNaseP* gene to a new site in MG411 so as not to interrupt an essential gene (16). The vector inserted in the new site did not contain an ARS. Because the *M. genitalium* genome is AT-rich, it is likely to contain sequences that can function as *ARS* in yeast. It is known that ARS-like sequences are frequent in eukaryotic AT-rich DNA (57,58).

Stability of genomes cloned in yeast

A significant fraction of yeast transformants showed the presence of complete mycoplasma genomes. Data from five experiments showed that approximately half (range: 35–61%) of the clones were correct by the two assays used (Table 1; percentage of clones complete by PCR multiplied by percentage of those clones correct by size). PCR analysis of the remaining clones showed that most contained some mycoplasma DNA. We do not know how these incomplete clones arose. One or more genome fragments may transform yeast and be circularized in the process by yeast repair mechanisms. Alternatively, these incomplete clones may have arisen from recombination in yeast during the process of transformation with a complete mycoplasma genome.

Once we have identified a full-length clone, we have not observed genetic alterations unless under selection for a recombination event. To examine stability, we started with a glycerol stock of a small culture from a single colony of a re-streaked yeast transformant. This stock was streaked for single colonies on selective medium. Three to eight colonies from this plate (subclones) were individually selected and cultured to saturation in several milliliters of selective medium. DNA from each was isolated and clones were examined for size by restriction digestion followed by pulsed-field gel electrophoresis, and, in some cases, Southern blot. Three out of three YCpMmyc1.1 yeast clones were stable by this analysis (Figure 3H). All 21 synthetic *M. genitalium* yeast clones (16) examined were also stable.

DISCUSSION

We previously reported the assembly and stable cloning of a synthetic 0.6-Mb *M. genitalium* genome in yeast in one step from six and from 25 overlapping pieces of DNA (16,17). Here we cloned natural genomes by transferring them from bacteria to yeast in one piece (18). This contrasts with the approach of assembling multiple smaller fragments into a genome, which is a strategy that others have used in attempting to clone the entire *Synechocystis* (15) and *E. coli* (14) genomes. Cloning a genome by building it from smaller pieces can take a significant number of steps, depending upon how many pieces can be assembled at once.

We have stably cloned in yeast three mycoplasma genomes, ranging in size from 0.6–1.1 Mb, with GC contents of 24–40%. A number of factors may have led to our success. There is no restriction barrier in yeast (59). Gene toxicity is minimized because regulation of gene expression in a eukaryote such as yeast is different from that in prokaryotes. The transcription (60) and translation (61) signals in yeast are different from those in bacteria. In addition, mycoplasmas use the codon UGA for tryptophan rather than as a translation stop signal. Thus, most mycoplasma genes, if expressed, would produce truncated proteins in yeast. This largely avoids the possibility of toxic gene products.

YAC libraries have been constructed for several different bacteria (62–64). Thus, large prokaryotic DNA segments using the 'universal' genetic code can be cloned in yeast. Toxic gene expression does not seem to be a barrier, and it is probable that most prokaryotic genes are not expressed in yeast. If there is a barrier, it may be a replication barrier, rather than a gene expression barrier (58).

During our cloning attempts we have seen molecules as large as ~2 Mb with Southern blot analysis. These are probably clones of concatamers, and suggest that we can clone circular molecules larger than the genomes we have investigated so far. Many bacterial and archaeal genomes are ≤2 Mb in size, and thus in cloning reach.

The mycoplasma genomes that we have cloned in yeast appear to be stably maintained. We previously reported that the sequence of a synthetic *M. genitalium* genome cloned in yeast was correct. A half-genome synthetic *M. genitalium* molecule that was also cloned in yeast was moved to *E. coli*, isolated and sequenced. This too had the correct sequence (16). We have been able to transplant several variations of an *M. mycoides* genome from yeast. This shows that yeast has propagated several different *M. mycoides* genomes with enough fidelity that they are functional. We sequenced a genome from *M. mycoides* cells that was transplanted out of yeast, and aside from changes that we had engineered in yeast, it contained the same sequence as the molecule that was cloned into yeast (18). These results confirm that two different mycoplasma genomes are accurately propagated in yeast.

Cloning whole bacterial genomes in yeast may have considerable utility for the study of organisms that are difficult to cultivate or do not have well-developed genetic tools. These may include pathogens and organisms from environmental sampling. Cloned genomes can be readily manipulated in yeast using standard genetic tools. Genome transplantation, which we have recently shown for mycoplasmas (18,19), allows an engineered genome to be installed as the operating system of a bacterial cell. The cycle of cloning, modification and transplantation could be repeated several times to develop an organism with desired traits. This could enhance our understanding of minimal gene sets and the construction of microbial cell factories.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

REFERENCES

1. Itaya,M., Fujita,K., Kuroki,A. and Tsuge,K. (2008) Bottom-up genome assembly using the *Bacillus subtilis* genome vector. *Nat. Methods*, **5**, 41–43.
2. Yoon,Y.G. and Koob,M.D. (2003) Efficient cloning and engineering of entire mitochondrial genomes in *Escherichia coli* and transfer into transcriptionally active mitochondria. *Nucleic Acids Res.*, **31**, 1407–1415.
3. Yonemura,I., Nakada,K., Sato,A., Hayashi,J., Fujita,K., Kaneko,S. and Itaya,M. (2007) Direct cloning of full-length mouse mitochondrial DNA using a *Bacillus subtilis* genome vector. *Gene*, **391**, 171–177.
4. Wheeler,V.C., Aitken,M. and Coutelle,C. (1997) Modification of the mouse mitochondrial genome by insertion of an exogenous gene. *Gene*, **198**, 203–209.
5. Gupta,M. and Hoo,B. (1991) Entire maize chloroplast genome is stably maintained in a yeast artificial chromosome. *Plant Mol. Biol.*, **17**, 361–369.
6. Adler,H., Messerle,M. and Koszinowski,U.H. (2003) Cloning of herpesviral genomes as bacterial artificial chromosomes. *Rev. Med. Virol.*, **13**, 111–121.
7. Hilton,S., Kemp,E., Keane,G. and Winstanley,D. (2008) A bacmid approach to the genetic manipulation of granuloviruses. *J. Virol. Methods.*, **152**, 56–62.
8. Luckow,V.A., Lee,S.C., Barry,G.F. and Olins,P.O. (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.*, **67**, 4566–4579.
9. Wang,H., Deng,F., Pijlman,G.P., Chen,X., Sun,X., Vlak,J.M. and Hu,Z. (2003) Cloning of biologically active genomes from a *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus isolate by using a bacterial artificial chromosome. *Virus. Res.*, **97**, 57–63.
10. Almazan,F., Galan,C. and Enjuanes,L. (2008) Engineering infectious cDNAs of coronavirus as bacterial artificial chromosomes. *Methods Mol. Biol.*, **454**, 275–291.
11. Domi,A. and Moss,B. (2002) Cloning the vaccinia virus genome as a bacterial artificial chromosome in *Escherichia coli* and recovery of infectious virus in mammalian cells. *Proc. Natl Acad. Sci. USA*, **99**, 12415–12420.
12. Ketner,G., Spencer,F., Tugendreich,S., Connelly,C. and Hieter,P. (1994) Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. *Proc. Natl Acad. Sci. USA*, **91**, 6186–6190.
13. Garcia-Ramirez,J.J., Ruchti,F., Huang,H., Simmen,K., Angulo,A. and Ghazal,P. (2001) Dominance of virus over host factors in cross-species activation of human cytomegalovirus early gene expression. *J. Virol.*, **75**, 26–35.
14. Smailus,D.E., Warren,R.L. and Holt,R.A. (2007) Constructing large DNA segments by iterative clone recombination. *Syst. Synth. Biol.*, **1**, 139–144.
15. Itaya,M., Tsuge,K., Koizumi,M. and Fujita,K. (2005) Combining two genomes in one cell: stable cloning of the *Synechocystis* PCC6803 genome in the *Bacillus subtilis* 168 genome. *Proc. Natl Acad. Sci. USA*, **102**, 15971–15976.
16. Gibson,D.G., Benders,G.A., Andrews-Pfannkoch,C., Denisova,E.A., Baden-Tillson,H., Zaveri,J., Stockwell,T.B., Brownley,A., Thomas,D.W., Algire,M.A. *et al.* (2008) Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science*, **319**, 1215–1220.
17. Gibson,D.G., Benders,G.A., Axelrod,K.C., Zaveri,J., Algire,M.A., Moodie,M., Montague,M.G., Venter,J.C., Smith,H.O. and Hutchison,C.A., 3rd (2008) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome. *Proc. Natl Acad. Sci. USA*, **105**, 20404–20409.
18. Lartigue,C., Vashee,S., Algire,M.A., Chuang,R.Y., Benders,G.A., Ma,L., Noskov,V.N., Denisova,E.A., Gibson,D.G., Assad-Garcia,N. *et al.* (2009) Creating bacterial strains from genomes that have been cloned and engineered in yeast. *Science*, **325**, 1693–1696.
19. Lartigue,C., Glass,J.I., Alperovich,N., Pieper,R., Parmar,P.P., Hutchison,C.A., 3rd, Smith,H.O. and Venter,J.C. (2007) Genome transplantation in bacteria: changing one species to another. *Science*, **317**, 632–638.
20. Gibson,D.G., Young,L., Chuang,R.Y., Venter,J.C., Hutchison,C.A., 3rd and Smith,H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods*, **6**, 343–345.
21. Dhandayuthapani,S., Blaylock,M.W., Bebear,C.M., Rasmussen,W.G. and Baseman,J.B. (2001) Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium*. *J. Bacteriol.*, **183**, 5645–5650.
22. DaMassa,A.J., Brooks,D.L. and Adler,H.E. (1983) Caprine mycoplasmosis: widespread infection in goats with *Mycoplasma mycoides* subsp *mycoides* (large-colony type). *Am. J. Vet. Res.*, **44**, 322–325.
23. Manso-Silvan,L., Vilei,E.M., Sachse,K., Djordjevic,S.P., Thiaucourt,F. and Frey,J. (2009) *Mycoplasma leachii sp. nov.* as a new species designation for *Mycoplasma* sp. bovine group 7 of Leach, and reclassification of Mycoplasma mycoides subsp. *mycoides* LC as a serovar of *Mycoplasma mycoides* subsp. *capri*. *Int. J. Syst. Evol. Microbiol.*, **59**, , 1353–1358.
24. Tully,J.G., Rose,D.L., Whitcomb,R.F. and Wenzel,R.P. (1979) Enhanced isolation of *Mycoplasma pneumoniae* from throat washings with a newly-modified culture medium. *J. Infect. Dis.*, **139**, 478–482.
25. Larionov,V., Kouprina,N., Solomon,G., Barrett,J.C. and Resnick,M.A. (1997) Direct isolation of human *BRCA2* gene by transformation-associated recombination in yeast. *Proc. Natl Acad. Sci. USA*, **94**, 7384–7387.
26. Kouprina,N. and Larionov,V. (2008) Selective isolation of genomic loci from complex genomes by transformation-associated recombination cloning in the yeast *Saccharomyces cerevisiae*. *Nat. Protoc.*, **3**, 371–377.
27. Treco,D.A. and Lundblad,V. (1993) *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc., pp. 13.01.01–13.01.07.
28. Treco,D.A. and Winston,F. (2008) *Current Protocols in Molecular Biology.*, John Wiley and Sons, Inc., pp. 13.02.01–13.02.12.
29. Katsura,S., Yamaguchi,A., Hirano,K., Matsuzawa,Y. and Mizuno,A. (2000) Manipulation of globular DNA molecules for sizing and separation. *Electrophoresis*, **21**, 171–175.
30. Noskov,V.N., Kouprina,N., Leem,S.H., Ouspenski,I., Barrett,J.C. and Larionov,V. (2003) A general cloning system to selectively isolate any eukaryotic or prokaryotic genomic region in yeast. *BMC Genomics*, **4**, 16.
31. Zeng,C., Kouprina,N., Zhu,B., Cairo,A., Hoek,M., Cross,G., Osoegawa,K., Larionov,V. and de Jong,P. (2001) Large-insert BAC/YAC libraries for selective re-isolation of genomic regions by homologous recombination in yeast. *Genomics*, **77**, 27–34.
32. Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
33. Gietz,D., St Jean,A., Woods,R.A. and Schiestl,R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425.
34. Shevchuk,N.A., Bryksin,A.V., Nusinovich,Y.A., Cabello,F.C., Sutherland,M. and Ladisch,S. (2004) Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Res.*, **32**, e19.

35. Burke,D.T., Carle,G.F. and Olson,M.V. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science*, **236**, 806–812.

36. Murray,A.W. and Szostak,J.W. (1983) Construction of artificial chromosomes in yeast. *Nature*, **305**, 189–193.

37. Larionov,V., Kouprina,N., Graves,J. and Resnick,M.A. (1996) Highly selective isolation of human DNAs from rodent-human hybrid cells as circular yeast artificial chromosomes by transformation-associated recombination cloning. *Proc. Natl Acad. Sci. USA*, **93**, 13925–13930.

38. Devenish,R.J. and Newlon,C.S. (1982) Isolation and characterization of yeast ring chromosome III by a method applicable to other circular DNAs. *Gene*, **18**, 277–288.

39. Kouprina,N., Kawamoto,K., Barrett,J.C., Larionov,V. and Koi,M. (1998) Rescue of targeted regions of mammalian chromosomes by *in vivo* recombination in yeast. *Genome Res.*, **8**, 666–672.

40. Zhou,F., Li,Q. and Gao,S.J. (2009) A sequence-independent *in vitro* transposon-based strategy for efficient cloning of genomes of large DNA viruses as bacterial artificial chromosomes. *Nucleic Acids Res.*, **37**, e2.

41. Leem,S.H., Noskov,V.N., Park,J.E., Kim,S.I., Larionov,V. and Kouprina,N. (2003) Optimum conditions for selective isolation of genes from complex genomes by transformation-associated recombination cloning. *Nucleic Acids Res.*, **31**, e29.

42. Lartigue,C., Duret,S., Garnier,M. and Renaudin,J. (2002) New plasmid vectors for specific gene targeting in *Spiroplasma citri*. *Plasmid*, **48**, 149–159.

43. Mouches,C., Candresse,T., Barroso,G., Saillard,C., Wroblewski,H. and Bove,J.M. (1985) Gene for spiralin, the major membrane protein of the helical mollicute *Spiroplasma citri*: cloning and expression in *Escherichia coli*. *J. Bacteriol.*, **164**, 1094–1099.

44. Glass,J.I., Assad-Garcia,N., Alperovich,N., Yooseph,S., Lewis,M.R., Maruf,M., Hutchison,C.A., 3rd, Smith,H.O. and Venter,J.C. (2006) Essential genes of a minimal bacterium. *Proc. Natl Acad. Sci. USA*, **103**, 425–430.

45. Hutchison,C.A., Peterson,S.N., Gill,S.R., Cline,R.T., White,O., Fraser,C.M., Smith,H.O. and Venter,J.C. (1999) Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science*, **286**, 2165–2169.

46. Dyke,K.G., Aubert,S. and el Solh,N. (1992) Multiple copies of IS*256* in staphylococci. *Plasmid*, **28**, 235–246.

47. Rice,L.B., Carias,L.L. and Marshall,S.H. (1995) Tn*5384*, a composite enterococcal mobile element conferring resistance to erythromycin and gentamicin whose ends are directly repeated copies of IS*256*. *Antimicrob. Agents Chemother.*, **39**, 1147–1153.

48. King,K.W. and Dybvig,K. (1991) Plasmid transformation of *Mycoplasma mycoides* subspecies *mycoides* is promoted by high concentrations of polyethylene glycol. *Plasmid*, **26**, 108–115.

49. Le,Y. and Dobson,M.J. (1997) Stabilization of yeast artificial chromosome clones in a *rad54-3* recombination-deficient host strain. *Nucleic Acids Res.*, **25**, 1248–1253.

50. Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) Yeast transformation: a model system for the study of recombination. *Proc. Natl Acad. Sci. USA*, **78**, 6354–6358.

51. Amberg,D.C., Burke,D.J. and Strathern,J.N. (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, 2005 edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 187.

52. Dershowitz,A. and Newlon,C.S. (1993) The effect on chromosome stability of deleting replication origins. *Mol. Cell Biol.*, **13**, 391–398.

53. Beach,D., Piper,M. and Shall,S. (1980) Isolation of chromosomal origins of replication in yeast. *Nature*, **284**, 185–187.

54. Chan,C.S. and Tye,B.K. (1980) Autonomously replicating sequences in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **77**, 6329–6333.

55. Newlon,C.S. and Burke,W. (1980) Replication of small chromosomal DNAs in yeast. *ICN-UCLA Symp. Mol. Cell. Biol.*, **19**, 187.

56. Goursot,R., Goze,A., Niaudet,B. and Ehrlich,S.D. (1982) Plasmids from *Staphylococcus aureus* replicate in yeast *Saccharomyces cerevisiae*. *Nature*, **298**, 488–490.

57. Montiel,J.F., Norbury,C.J., Tuite,M.F., Dobson,M.J., Mills,J.S., Kingsman,A.J. and Kingsman,S.M. (1984) Characterization of human chromosomal DNA sequences which replicate autonomously in *Saccharomyces cerevisiae*. *Nucleic Acids Res*, **12**, 1049–1068.

58. Stinchcomb,D.T., Thomas,M., Kelly,J., Selker,E. and Davis,R.W. (1980) Eukaryotic DNA segments capable of autonomous replication in yeast. *Proc. Natl Acad. Sci. USA*, **77**, 4559–4563.

59. Belfort,M. and Roberts,R.J. (1997) Homing endonucleases: keeping the house in order. *Nucleic Acids Res.*, **25**, 3379–3388.

60. Kornberg,R.D. (1999) Eukaryotic transcriptional control. *Trends Cell Biol.*, **9**, M46–49.

61. Kozak,M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene*, **234**, 187–208.

62. Azevedo,V., Alvarez,E., Zumstein,E., Damiani,G., Sgaramella,V., Ehrlich,S.D. and Serror,P. (1993) An ordered collection of *Bacillus subtilis* DNA segments cloned in yeast artificial chromosomes. *Proc. Natl Acad. Sci. USA*, **90**, 6047–6051.

63. Heuer,T., Burger,C., Maass,G. and Tummler,B. (1998) Cloning of prokaryotic genomes in yeast artificial chromosomes: application to the population genetics of *Pseudomonas aeruginosa*. *Electrophoresis*, **19**, 486–494.

64. Kuspa,A., Vollrath,D., Cheng,Y. and Kaiser,D. (1989) Physical mapping of the *Myxococcus xanthus* genome by random cloning in yeast artificial chromosomes. *Proc. Natl Acad. Sci. USA*, **86**, 8917–8921.