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 contiguous 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 (MgCl₂) 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 (CO₂).

**Yeast strains and culture conditions**

Yeast strains used were VL6-48N (25), VL6-48Δ54G (MATa his3-Δ200 trpl-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'-GGGC TTCATAATGGCCATTTGTG-3'; 5'-ATTCTGTAGGGCGACAAAAGGAGGTATTGTCCTTCT-3'; 5'-TGGCAGAGAAGCTCTTCGTTGAGCTGACGCCACAGCCGACAATACGTCGTAACACC-3'; 5'-GGTGTCGCAAGTCGTGGGTCCATCGACGACTCCTGGAGC-3'; 5'-GCCCCGGGCTCAATACCGGCAAGTGGAATGTGATTTGAAACGACCG-3'; 5'-TAAGCATTGGAACGCTTTGTCAGAAGATGAAACAATTCGGC-3'; 5'-GCCCGGCGTCAAATACGG-3'; 5'-CTAGCTCTTGTGTCGGGGGACGG-3'; 5'-ATACGTGAATTGTCCCGGTCGCTGCAGCAGCGCGCGCG-3'; 5'-CAGAAAAATCACATTACCTTATTGTCGTGCTGCCCTT-3'; 5'-TGGCAGAGAAGCTCTTCGTTGAGCTGACGCCACAGCCGACAATACGTCGTAACACC-3'; 5'-TTCCCTCCACCAAAGGCG-3'.

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

DNA from a portion of the puromycin-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'-CGTGAAAGGTGAGCCAGTGATGG-3' and 5'-CA GGTCGACTCGATGCAACC-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 \( \sim 10^7 \) \( M. \) genitalium cells, 10\(^6\) \( M. \) mycoides cells and 10\(^5\) \( M. \) 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 6 V/cm) for several hours. (Under these conditions, large circular DNA molecules will not move out of the gel or blot.) To increase the efficiency of this step, plugs were sometimes fist digested with AsiSI, FseI and RsrII. These were sometimes pre-electrophoresed at constant voltage (4.5 V/cm) for several hours (29), except where noted in the results. To remove linear yeast chromosomal DNA, plugs were sometimes linearized 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 sites was PCR amplified, producing a 6.5-kb product.

| Sequence |
|----------|
| TGTGAAGGAATTCCACTATTTATACCACTGAGGAGGCACTGACCCGATTTATACACC-3' |
| GACCTTGCAGAACGATATATTCATTTATACCACTGAGGAGGCACTGACCCGATTTATACACC-3' |

Preparation of vector pTARBAC3

The vector pTARBAC3 (31) was linearized by 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 Tag 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 dNTPs. 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 Asel site in quarter 3 of the synthetic \( M. \) genitalium genome, pTARBAC3 was amplified using primers (IDT) with the following conditions are listed in Supplementary Tables S3–S5.

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

| Species, clone | 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 |
|---------------|--------|-------------------------------|--------------------------------|------------------------|--------------------------------|---------------------------------|---------------------------------|
| \( M. \) genitalium YCpMgen16-2 | 0.6 | VL6-48N (a) | 4.9 | 172 | 10 | 22/24 | 2/3 |
| | | W303a (a) | 2.8 | 421 | 10 | 5/8 | 4/5 |
| \( M. \) mycoides YCpMmyc1.1 | 1.1 | VL6-48N (a) | 5.0 | 174 | 10 | 20/48 | 5/6 |
| | | VL6-48-Δ54G (a) | 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 | VL6-48N (a) | 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'-TTAATAACAAAAAATCTCTATTAA
AAAAACCACTTTAAAGTGGTTGTTAAAATT
AGCGGCGCAGTTCACAGCAGTATAGC-3' and
5'-GGATAGGAGTGCTGACGCGGCAAGAGG
TATGGGTCAAGTCTATTGGGGCGCCGCG
CCGCGAGCCTCTCACCCGACG-3' (NotI sites
are underlined and vector sequence is in bold). For insertion
at the BsmBII site in quarter 3 of the synthetic
M. genitalium genome, pTARBAC3 was amplified using
primers (IDT) with the following sequences: 5'-AGGTTTT
CTTCTTCTAGGTAAGAGGCTGGTTCTACCTG
GTTTTCACCAAGTGCTCTGCGCGCCGATTTG
CATCAACGCAGTATAGC-3' and 5'-GTGATAGGCCATA
ACGACATAACTAGCTAAGGTTAATACTATGA
TGATTTTTGAAGTGGCGCGCCGACAGCTTC
AACCCCAGTCAG-3' (NotI sites are underlined and vector
sequence is in bold). The 5' 60 nts of each oligo are homol-
gous 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 fol-
lowing sequences: 5'-TACAGGCGCGGCGTGAAGTTAGT
AGGCAACGCCCTCACTAAGCACAAGCGAC
AGATTTGACTGAGAGCACC3' and 5'-CTCAT
AAAGAACACTTGGGGAGGGAACATCGTTGGT
CCATTTGGCCGCGCATCTGTGCGGTATTCA-3'
(M. genitalium sequence is in bold). This fragment
was transformed into yeast using lithium acetate (33). Gene replacement was checked by amplifica-
tion with two primers (sequences 5'-TTGAATTATTGCTAGTTATATAGGGGTTAGA
CTTTTAAAGATA
TGATTTTTGAAGTGGCGCGCCGACAGCTTC
AACCCCAGTCAG-3' (NotI sites are underlined and vector
sequence is in bold).  The 5.1-kb fusion product was integrated into the target site by homologous recombin-
ation at the 50-bp underlined sequences. The other fragment replaced the yeast vector insertion in
RNaseP 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'-TTAAAGCTC
GATTTATTTATCTACAGC-3' and 5'-CTTTTTAAAGATA
TGATTTTTGAAGTGGCGCGCCGACAGCTTC
AACCCCAGTCAG-3' (NotI sites are underlined and vector
sequence is in bold). The 40 nts of overlapping sequence is in bold. The 1.5-kb fusion product was integrated into the target site by homologous recombin-
ation at the 50-bp underlined sequences. The other fragment replaced the yeast vector insertion in
RNaseP 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'-TTAAAGCTC
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sequence is in bold). The 40 nts of overlapping sequence is in bold. The 1.5-kb fusion product was integrated into the target site by homologous recombin-

The 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 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 marker.
yeast maker. 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 transposase 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 (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, 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 digested 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 (VL6-48Δ54G) 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 10⁷ 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. Ascl-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*
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 Ascl 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 a) 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 such. 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 media. 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 YCpMyc1.1 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.

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SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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