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Cloning the *Acholeplasma laidlawii* PG-8A Genome in *Saccharomyces cerevisiae* as a Yeast Centromeric Plasmid

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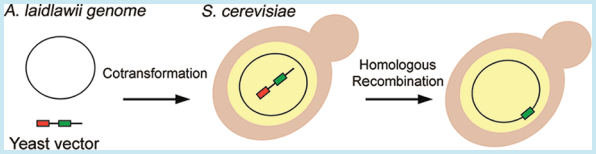
Supporting Information

ABSTRACT: Cloning of whole genomes of the genus Mycoplasma in yeast has been an essential step for the creation of the first synthetic cell. The genome of the synthetic cell is based on *Mycoplasma mycoides*, which deviates from the universal genetic code by encoding tryptophan rather than the UGA stop codon. The feature was thought to be important because bacterial genes might be toxic to the host yeast cell if driven by a cryptic promoter active in yeast. As we move to expand the range of bacterial genomes cloned in yeast, we extended this technology to bacteria that use the universal genetic code. Here we report cloning of the *Acholeplasma laidlawii* PG-8A genome, which uses the universal genetic code. We discovered that only one *A. laidlawii* gene, a surface anchored extracellular endonuclease, was toxic when cloned in yeast. This gene was inactivated in order to clone and stably maintain the *A. laidlawii* genome as a centromeric plasmid in the yeast cell.



KEYWORDS: *Acholeplasma laidlawii, Saccharomyces cerevisiae, yeast centromeric plasmid, transformation associated homologous recombination*

We recently reported the creation of a bacterium controlled by a synthetic genome.[1−3] This experiment demonstrated that it is possible to assemble complete viable genomes from a large number of chemically synthesized DNA oligonucleotides. *Saccharomyces cerevisiae* was chosen as a heterologous host for DNA manipulations because of its well-established molecular biology tools such as seamless gene manipulation technique by homologous recombination[4] or multisite engineering method,[5] cloning of large DNA fragments by PEG transformation,[6−8] and in vivo assembly of multiple overlapping fragments into a single DNA molecule.[9,10]

For the synthetic cell, the genome of *Mycoplasma mycoides*[2] was used as a template. *M. mycoides* was chosen because of its small genome (~1.1 Mb) and because it uses a nonstandard genetic code, where UGA codes for a tryptophan instead of a translation stop signal. The latter feature was thought to be important because bacterial genes might be toxic to the host yeast cell if driven by a cryptic promoter active in yeast. In the case of the *M. mycoides* genome, a toxic gene would presumably not be translated properly in yeast due to truncation of transcripts encoding for protein sequences containing tryptophan. Indeed, cloning wild type and synthetic[1,11] *M. mycoides* genomes and other mycoplasmas that use the mycoplasma code (*Mycoplasma genitalium*[11−13] and *M. pneumonia*[12]) does not appear to cause any significant toxicity in yeast.

As we expanded the range of bacterial genomes cloned in yeast, we chose a bacterium with the universal genetic code. We initiated cloning of *Acholeplasma laidlawii* PG-8A, a member of the Mollicute class to which *M. mycoides*, *M. genitalium*, and *M. pneumoniae* also belong. *A. laidlawii* was selected because it uses the universal genetic code, has a relatively small genome of 1.5 Mb, lacks a cell wall, and has a G+C content of 31%,[14] attributes similar to those of *M. mycoides* and *M. genitalium*, whose genomes have been successfully cloned in yeast.

There are three methods of cloning whole genomes in yeast as reported by Benders et al.[12] The first is to insert a yeast vector (from here on referred to as the "yeast standard vector", YSV) containing a selectable marker (SM), centromere (CEN), and a yeast autonomously replicating sequence (ARS), into the bacterial genome by transformation prior to genome transfer to yeast.[12] Second, the whole bacterial genome can be cotransformed and assembled in yeast using a YSV, flanked by 40−70 bp of sequence homologous to a site within the genome where the vector is to be inserted. Third, this process can be performed with multiple overlapping fragments instead of the full genome, but with the same YSV.

In this paper, we present the successful cloning of the *A. laidlawii* genome and discuss how to identify and address a subset of the potential problems that may occur when cloning genomes heterologously in yeast.

RESULTS AND DISCUSSION

Initial Attempts To Clone a Whole *A. laidlawii* Genome in Yeast. The first method for cloning genomes is preferred, but it requires that the bacterium of interest can be transformed and that insertions of selection markers or replication origins can occur either by homologous recombination, illegitimate recombination, or transposition. Initial attempts to modify the *A. laidlawii* genome by YSV insertion

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Table 1. Summary of Experiments Preformed To Clone Whole or Partial *A. laidlawii* Genomes

vector type	length of homology	type of gDNA digest	size of the fragment	no. of positive/total colonies[b]
YSV	60 bp each	SgrdI, complete	whole genome	0/200 (2)
YSV[a]	60 bp each	SgrdI, complete	whole genome	0/1000 (4)
YSV	60 bp each	no digest	whole genome	0/1000 (4)
YSV	60 bp each	ApaI, complete	F1: 136kb	0/200 (3)
YSV[a]	60 bp each	ApaI, complete	F1: 136kb	1/44 (3)
YSV	60 bp each	ApaI, complete	F2: 897kb	1/150 (1)
YSV	60 bp each	ApaI, complete	F3: 343kb	18/25 (1)
YSV	60 bp each	ApaI, complete	F4: 121kb	11/12 (1)
YSV	60 bp each	ApaI, partial	F2-3: 1240kb	0/1000 (6)
YSV	60 bp each	ApaI, partial	F2-4: 1376kb	0/1000 (5)
YRBV	300 bp, 200 bp	ApaI, complete	F2: 897kb	14/14 (10)
YRBV	300 bp, 200 bp	ApaI, partial	F2-3: 1240kb	12/15 (4)
YRBV	300 bp, 200 bp	ApaI, partial	F2-4: 1376kb	1/2 (4)
YRBV	300 bp, 200 bp	no digest	whole genome	0/30 (20)
YRBV	3000 each	no digest	whole genome	3/18 (20)

[a]Knock out cassette (URA3 gene flanked by 70bp homology regions to *A. laidlawii* endonuclease gene) was also co-transformed. [b]Numbers in brackets represent number of experiments performed.

directly were unsuccessful. Therefore we pursued the second method: cotransformation of the genome of *A. laidlawii* with an appropriate YSV. Vector insertion is much more efficient if the genome contains a double strand break near the insertion point.[15,16] Since the *A. laidlawii* genome has a single recognition site for the SgrDI restriction enzyme, we chose this site for vector insertion. Genomic DNA was isolated in plugs, digested, and cotransformed with the standard vector. This transformation yielded 200 colonies (Table 1), but no clones containing the whole genome were isolated.

Cloning the *A. laidlawii* Genome in Fragments. *A. laidlawii* genomic DNA was digested with the *ApaI* restriction enzyme, which produces four fragments (Figure 1). Four YSVs with appropriate flanking sequences were designed to recombine at ApaI cut sites of these fragments and cotransformed with ApaI digested genomic DNA. On the first attempt three out of the four fragments were successfully cloned (Supplementary Figure 1). Despite several attempts, a 136 kb fragment (fragment F1) could not be cloned (Table 1).

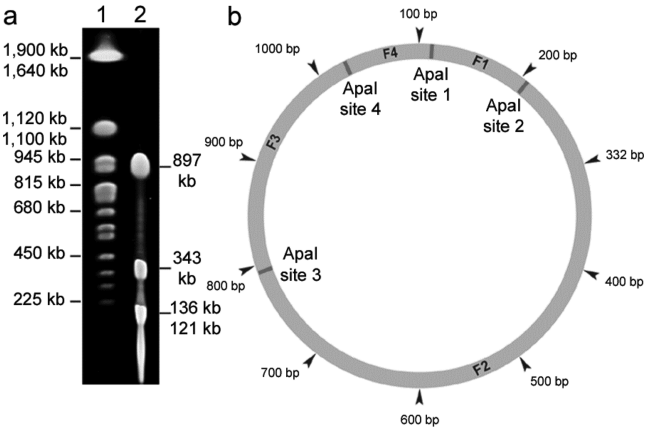

Figure 1. Digest of *A. laidlawii* genomic DNA with *ApaI* restriction enzyme. (a) Lane 1: Yeast chromosome PFG marker (NEB); lane 2: *A. laidlawii* genomic DNA digested with *ApaI*. (b) Map of *A. laidlawii* genome showing the locations of the *ApaI* restriction sites. Fragments are labeled on the basis of relative position in the genome. Locations for multiplex PCR primers are also shown (100–1000).

Fragment F1 was split into smaller regions in hope of being able to assemble them separately (Figure 2a–e). Once again, new YSVs were created for each fragment. Fragments of F1 were created by digesting *A. laidlawii* genomic DNA with the *ApaI* and *PspXI* restriction enzymes (fragments F1-1, F1-2), retrieved from undigested DNA (fragments F1-3 to F1-10), or created by PCR amplification (fragments F1-6 to F1-10). Using these approaches we were able to achieve full coverage of the *A. laidlawii* genome in yeast strains as separately cloned fragments.

Identification of *A. laidlawii* Toxic Gene. It was suspicious that fragments F1-7 and F1-8 were unclonable when genomic DNA was used as a source but cloneable when these regions were derived from PCR amplification (Figure 2d,e). We surmised that during the PCR reaction mutations may have been introduced into toxic genes. To assess this possibility, we sequenced two PCR-generated clones containing fragment F1-7 and one clone containing F1-8. Instead of sequencing all ~20 kb of the fragments, we sequenced only the region that was common to all of the uncloneable fragments. This common region encompassed three open reading frames, annotated[17] as a hypothetical protein (ACL0116), a surface anchored extracellular endonuclease (ACL0117), and a DNA-3 methyladenine glycosylase (ACL0118) (Figure 2f). All three clones contained mutations in residues conserved across long phylogenetic distances in homologues of the endonuclease (Figure 2f,g). We also found a mutation in one clone of the hypothetical protein and mutations in two clones of the DNA glycosylase, one of which was silent (Figure 2f). As the endonuclease was the only gene where mutations were found in all clones, it suggested that endonuclease activity could be responsible for toxicity in yeast.

To confirm toxicity of the endonuclease, we PCR-amplified the mutated version of the endonuclease gene from fragment F1-7 clone 13 (Figure 2e) and cotransformed it into yeast with the appropriate YSV. The wild type version amplified from genomic DNA served as a control. Cotransformation with the mutated version produced 3,150 yeast colonies while the wild type produced only 30. For the mutated version 10 of 10 genotyped clones contained correct assemblies of the vector with the fragment. For the wild type, 3 of 10 were correct. Once again we suspected that these 3 correctly assembled clones that came from the control experiment probably contained

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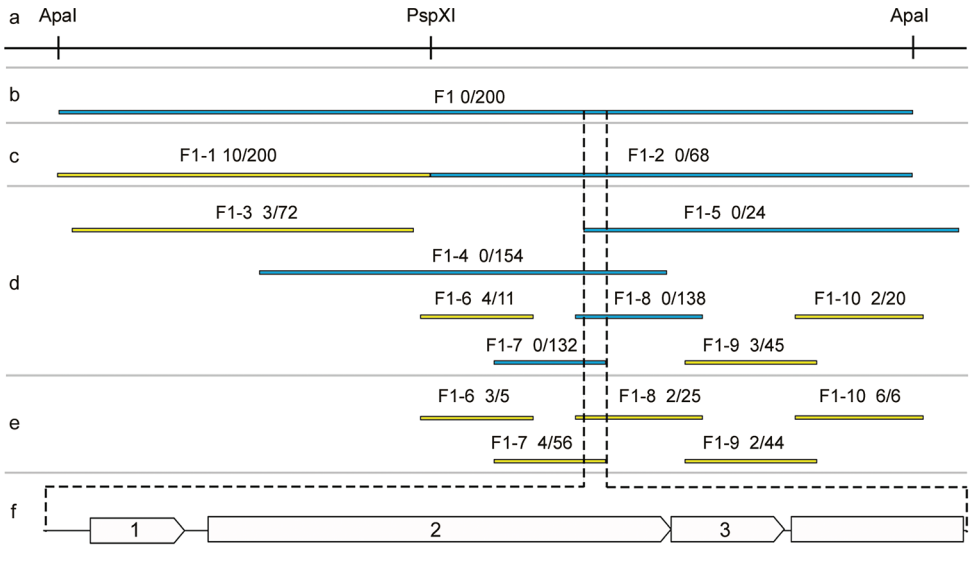

Figure 2. Strategies to clone *ApaI* fragment F1 of the *A laidlawii* genome. (a) *ApaI* and *PspXI* restriction sites were used to linearize the *A. laidlawii* genome. (b, c) Fragments whose cloning were attempted: (b) DNA isolated in plugs, digested with only the *ApaI* restriction enzyme, and cotransformed with the YUV failed to produce clones; (c) DNA isolated in plugs, digested with *ApaI* and *PspXI* restriction enzymes, and cotransformed with YUV to generate a clone bearing F1-1. Fragment F1-2 failed to produce clones. (d) DNA isolated in solution (no digestion) was used to generate fragments F1-3 through F1-10; F1-4, F1-5, F1-7, and F1-8 failed to produce clones. (e) Fragments F1-6 through F1-10 were PCR-amplified and cotransformed with YUVs to clone these fragments. Numbers of successful clones identified from total number of colonies screen are listed for each fragment (b–e) next to the fragment's name. (f) The region common to all of the unclonable fragments contained the open reading frames (ORFs) 1, ACL0116; 2, ACL0117; and 3, ACL0118. All three ORFs were sequenced from F1-7 clone 1 and 13 as well as from F1-8 clone 6. Mutations found in ACL0116: S53P (F1-7 clone1); in ACL0117: Y676H (F1-8 clone 6), W708R (F1-7 clone 1, 13); and in ACL0118: L34L (F1-8 clone 6) and F109L (F1-7 clone 13). (g) Alignment of regions near mutations retrieved from PCR-cloned fragments of the *A. laidlawii* endonuclease gene (ACL0117). Sequences selected were the closest match by BLAST searches in the indicated species. Amino acids highlighted in blue indicate positions of mutations that mitigate gene toxicity (Y676 and W708).

mutations resulting from PCR. Sequencing one of these three clones again revealed a mutation in the same conserved residue of the nuclease gene as found in F1-7 clone 13.

Demonstration That a *A. laidlawii* Endonuclease Gene Is Also Toxic in *Escherichia coli*. It is known that some bacterial genes are toxic when cloned in *E. coli*.[18,19] We were interested if the *A. laidlawii* endonuclease is also toxic in *E. coli*. To that end we amplified the wild type endonuclease gene from genomic DNA and the mutated endonuclease gene from fragment F1-7 clone 13 and ligated them into the pmycYACTnNotI vector,[12] which propagates in *E. coli* and yeast. After transformation of the ligation products into *E. coli* we obtained only 64 colonies with the wild type version and 512 when the mutant version was used (Supplementary Table 1). We genotyped 64 and 25 colonies from the wild type and the mutated versions, respectively, and only identified positive clones for the mutant version (Supplementary Table 1). Next we set up an experiment where we deleted various lengths from the 5′ end of the endonuclease coding sequence to identify the minimal fragment toxic to *E. coli*. For fragments that yielded positive clones for both the wild type and the mutant version we isolated plasmids and used them to transform yeast (Figure 3, Supplementary Table1). For both, yeast and *E. coli* we assumed that a fragment was toxic to the host if substantially

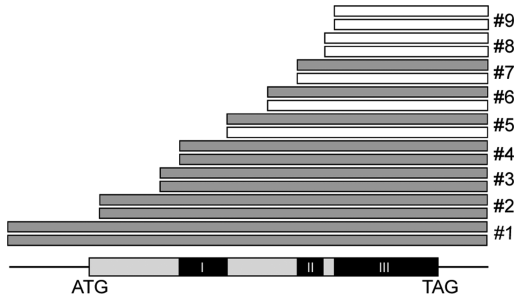

Figure 3. Deletion analysis of the *A. laidlawii* endonuclease coding region. The 2244 bp open reading frame for *A. laidlawii* endonuclease gene flanked by 5′ 503 bp, and 3′ 346 bp sequences are shown at the bottom. Fragments 2–9 had deleted 67, 456, 590, 919, 1,146, 1350, 1,529, and 1,728 bp, respectively, from the 5′end of the open reading frame. Toxicity in yeast (upper bar for each fragment) and in *E. coli* (lower bar for each fragment) is indicated by gray (toxic) or white (none-toxic) colors. Of the 9 fragments shown above, in *E. coli*, wild type versions of fragments 1–4 were toxic, whereas in yeast wild type fragments 1–7 were toxic. On the basis of which fragments were toxic (see Supplementary Table 1) we assigned putative promoter regions for *E. coli* (I) and yeast (II). The third highlighted region (III) represents the domain of the endonuclease gene conserved across many phyla of bacteria.

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fewer colonies were obtained for the wild type version compared to the mutant version (Supplementary Table 1). Based on these experiments we discovered that deleting the 5′ 919 bp region of the 2,244 bp open reading frame inactivates the toxicity in *E. coli* and deleting a longer 1,529 bp region inactivates toxicity in yeast. We assume that these regions (described as regions I (*E. coli*) and II (yeast) in Figure 3) represent putative promoter regions of the cloned fragments in the respective hosts.

Demonstration That the *A. laidlawii* Endonuclease Gene Must Be Expressed To Cause Toxicity in Yeast. We constructed a fusion of the galactose promoter with deletion fragment 8. Fragment 8 was the first fragment that shows no toxicity in yeast (Figure 3). We expressed the wild type and mutated versions of fragment 8 in the high copy 2 μm plasmid. When grown on glucose no significant differences between yeast colonies harboring the wild type versus the mutated version were observed; however, when grown on galactose media significant inhibition of growth was observed with the wild type version (Figure 4).

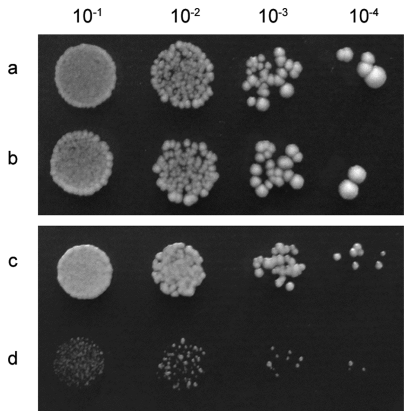

Figure 4. Expression of *A. laidlawii* endonuclease deletion fragment 8 expressed from a galactose promoter on a 2 μm plasmid. Yeast liquid cultures were grown to an OD_{600} of 1, then diluted as indicated above, and grown for 4 days on defined media minus histidine plus glucose (a and b) or minus histidine plus galactose (c and d). The mutant form of the gene (W708R) of fragment 8 (a, c) is compared with the wild type fragment 8 (b, d).

New Strategy To Clone the *A. laidlawii* ApaI Fragment 1. We designed an experiment to clone fragment 1 by cotransforming *ApaI* digested DNA with the same YSV that was used to initially clone this fragment and a cassette that would replace the endonuclease with a URA3 marker. Using this strategy we obtained 44 clones from which one contained fragment 1 (Table 1, Supplementary Figure 2).

Other Strategies To Clone the Whole *A. laidlawii* Genome in Yeast. On the basis of the information that we learned trying to clone ApaI fragment 1, we again attempted to clone the whole genome by cotransformation of *SgrDI* digested *A. laidlawii* genomic DNA and YSV that should insert at the SgrDI site, but this time we also included the URA3 replacement cassette; 1000 colonies were screened of which no successful clones were identified. We also cotransformed an intact bacterial genome with a new YSV designed to recombine directly into the toxic endonuclease open reading frame and completely remove it; 1000 colonies were screened, and again no successful clones were identified.

We speculated that in addition to the toxic effect of the identified endonuclease gene there could be an additional cumulative gene dose effect when attempting to clone the entire genome. Alternatively, we expected that cloning of the whole genome using the described strategies would simply be a very rare event. Either way, identifying yeast clones of the entire genome would be markedly improved by minimizing incorrect assemblies. In our experiments using the YSV we obtained a very high number of background colonies that were probably the result of vector recircularization.[20,21] To reduce the number of background colonies, vectors containing negative and positive selection markers can be used.[22,23] The design of our new vector included all of the elements of the YSV but also featured a URA3[24] (Supplementary Figure 3) and homology regions lengthened to 200−300 bp instead of 40−70 bp (from here on referred to as the "yeast reduced background vector", YRBV). While the URA3 gene is typically used as a positive selection marker, it can also be used as a negative selection marker when 5-fluoroorotic acid[25] (5FOA) is added to the media. Our vector was designed to allow resection of the exposed URA3 sequence only upon recombination with the appropriate fragment. However, if the vector recircularizes, the URA3 gene will still be present and result in cell death when yeast is exposed to 5-FOA. To test this new vector we repeated our experiment to clone ApaI fragment 2 (Figure 1, 897 kb). We obtained 14 colonies all of which were positive, a great improvement over the YSV method where we obtained only 1 positive clone out of 150 colonies (Table 1).

Next we designed YRBVs to test the method for cloning fragments of increasing size: 1.24 Mb (corresponding to ApaI fragments 2−3) and 1.38 Mb (corresponding to ApaI fragments 2−4) fragments, and the 1.5 Mb full genome (Table 1). For the intermediate fragments we used genomic DNA partially digested with ApaI for cotransformation; for the full genome we used intact, circular genomic DNA. For the 1.24 Mb fragment we obtained 12 positive clones out of total of 15 colonies screened. For the 1.38 Mb we obtained 1 positive out of 2 colonies. However, for the full genome, we obtained 10 colonies, and none were positive.

We speculated that longer homology sites could increase the chances of cloning the *A. laidlawii* genome, especially in the case where the genome is not first linearized with any enzyme. Therefore we designed a new YRBV with increased homology regions of 3000 bp on each end. Using this modified YRBV, we obtained 26 colonies of which 3 were positive. Presence of the *A. laidlawii* genome was confirmed by multiplex PCR as well as by CHEF gel electrophoresis (Figure 5). Colonies containing the genomes grew comparably to yeast cells carrying vector only (Supplementary Figure 4). We also compared the rate of *A. laidlawii* to *M. mycoides* genomes lost when yeast cells were grown without selection. *A. laidlawii* genome loss was comparable to genome loss of *M. mycoides* (Supplementary Table 2). On the basis of the experiments described above, we showed that it was possible to heterologously clone the *A. laidlawii* genome in yeast with the exception of one toxic gene.

Summary and Conclusions. There has been increasing interest in cloning of large DNA fragments (>250 kb) as well as complete bacterial genomes (0.6−3.5 Mb). Itaya et al.[26] showed that the 3.5 Mb genome of *Synechocysits* PCC6803 can be inserted as three discontinuous regions into *Bacillus subtilis* with the exception of two rRNA operons. The only other known host organism that has been proven to be suitable for cloning large DNA fragments or whole bacterial genomes is

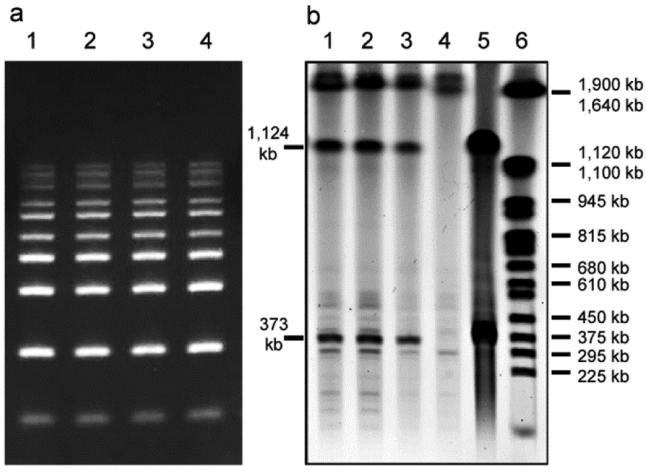

Figure 5. Confirmation of yeast clones carrying the *A. laidlawii* whole genome. (a) Multiplex PCR screen of yeast clones. Lanes 1−3 contain PCR products derived from clones 8, 13, and 14, respectively. Lane 4 contains PCR products from the *A. laidlawii* wild type genomic DNA. (b) CHEF gel analysis of *A. laidlawii* genomes cloned in yeast. DNA isolated from yeast was digested with the *AscI*, *FseI*, and *I-CeuI* enzymes. The *A. laidlawii* genome does not contain any recognition sites for *AscI* or *FseI*; however, it has two *I-CeuI* sites. After digest two bands for *A. laidlawii* genomes were predicted at 1,124 and 374 kb. Lanes 1−3 contain digests from yeast bearing clones 8, 13, and 14, respectively. Lane 4 contains yeast transformed with an empty vector, lane 5 contains *A. laidlawii* wild type genomic DNA, and lane 6 contains the yeast chromosome PFG marker (NEB).

S. cerevisae. We have reported the cloning of *M. genitalium*,[11,13] *M.mycoides*,[2] and *M. pneumonia*[12] in *S. cerevisae*. All the above mycoplasma strains use a nonstandard genetic code. These initial strains were selected to avoid the possibility of introducing toxic gene products to the yeast host. In this paper we described cloning a genome using the universal code. It was possible to clone the whole bacterial genome of *A. laidlawii* with the exception of one gene, a surface anchored extracellular endonuclease (ACL0117) with predicted deoxyribonuclease I function. Inactivation of this gene was necessary to prevent what we believe to be a toxic function in yeast. Interestingly, only a partial domain of the full coding sequence is responsible for toxicity in yeast. Expression of just the endonuclease domain from the yeast 2 μ-plasmid is sufficient to inhibit yeast growth, but the presence of a putative yeast promoter embedded upstream in the *A. laidlawii* coding sequence appears to be necessary for toxic function in the genomic context. Follow up studies will be required to investigate the overall level of transcription of heterologous genome sequences and the resulting expression of bacterial genes or gene fragments.

In cloning *A. laidlawii* genome we identify a toxic gene and improved the vector to reduce background when cloning in yeast. The successful design of this vector included positive selection, negative selection, and increased lengths for the target site homology regions. We have since also successfully cloned the 1.66 Mb genome of cyanobacterium *Prochlorococcus marinus* MED4. Yeast clones carrying complete *P. marinus* genomes showed no obvious toxic gene activity (Tagwerker C., personal communication). This further demonstrates that very few prokaryotic genes will have toxic effects when cloned in yeast.

In addition to compensating for the presence of toxic genes, it also important to take into account the size and the G/C content of candidate genomes. Yeast replication origins, called autonomously replicating sequences (ARS),[27−31] are A/T rich sequences and organisms featuring low genomic G/C content will naturally contain enough ARS sequences (the *A. laidlawii* genome is 31% G/C) to support replication in yeast. However, when cloning bacteria featuring high G/C content such as *E. coli* (G/C content ∼50%) or *Streptomyces lividans* (G/C content ∼60%), it may be necessary to insert additional ARS sequences into the bacterial genome before they can be cloned and maintained in yeast.

METHODS

Strains Used and Culture Conditions. *Acholeplasma laidlawii* PG-8A (ATCC 23206), *Saccharomyces cerevisiae* strain *VL6-48*.[32] *A. laidlawii* cells were cultured in SP-4[33] and yeast cells were grown in YPDE[9] or yeast synthetic media lacking histidine (Teknova, Inc.).

Genomic DNA Isolation. Intact genomic DNA from *A. laidlawii* or *S. cerevisiae* was isolated in agarose plugs according to the Bio-Rads CHEF-Dr III manual. For experiments described in Figure 2d genomic DNA was prepared in solution using Promega Wizard Genomic DNA purification Kit. In addition DNAs that were used for all multiplex PCRs as well as for experiments described in Supplementary Figure 2c were isolated as follows: (1) 4−10 mL of yeast culture was grown to high density. (2) Yeast cells were palleted at 3.2 Krpm for 5 min, supernatant was discarded, and cells were resuspend in 200 μL buffer resuspension buffer P1 (Qiagen). (3) 5 μL of 1.4 M β-mercaptoethanol and 5 μL Zymolyase solution (Zymolyase solution: 200 mg Zymolyase 20T (USB), 9 mL H_2O, 1 mL 1 M Tris pH7.5, 10 mL 50% glycerol, stored at −20 °C) were added, and samples were incubated at 37 °C for 60 min. (4) 200 μL of Lysis buffer P2 (Qiagen) was added, and samples were inverted 5−10 times to mix. (5) 200 μL of Neutralization buffer P3 was added, and samples were inverted 5−10 times to mix. (6) Then samples were spun down at 13.2 Krpm for 10 min. (7) Supernatant was transferred to a clean tube, 600 μL isopropanol was added, and samples were mixed by inversion and spun down at 13.2 Krpm for 10 min. (8) Supernatant was discarded, and pellets were resuspended in 50 μL of TE buffer. (9) Samples were kept at 37 °C for 30−60 min to dissolve. (10) 0.5 μL RiboShredder RNase blend (Epicenter) was added, and samples were incubated at 37 °C for 30−60 min. Steps 11−13 can be omitted; however, they are recommended if it is desired to verify the size of large plasmids by gel electrophoresis (see Supplementary Figure 2c). (11) 50 μL of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed by stirring with a pipet tip 5−10 times or flicking the tube gently 5−10 times. (12) Samples were centrifuged at 13.2 Krpm for 1−2 min. (13) DNA from the aqueous top layer was transferred to new tube.

Yeast Transformation/Colony Selection. Yeast transformations were performed as described in ref 9. Various vector to genomic DNA ratios were added for cotransformation experiments ranging from 50 to 300 ng of the vector with 1−2 μg of genomic DNA isolated in agarose plugs. For YSV transformations increasing the amount of vector resulted in high background; however, increasing the amount of YRBV did not significantly increase the background.

For the YRBV, colonies were selected as follows: transformed spheroplasts were resuspended in 8 mL of liquid

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(equilibrated at 50 °C) -HIS, 1 M Sorbitol, 2% agar media and plated on Petri dish containing the 20 mL of the same media. After 20−24 h 10 mL of media containing 5-FOA (-HIS, Sorbitol, 5-FOA 2 g/L, 2% agar) was added. Colonies were picked after 4 days onto new plates (-HIS, 5-FOA 1 g/L, 2% agar).

PCR Analysis of Clones. *A. laidlawii* genomic fragments or genomes cloned in yeast were screened by completeness by multiplex PCR with the Qiagen Multiplex PCR Kit.

Sizing of *A. laidlawii* Genomic Fragments or Full Genomes Cloned in Yeast. Total DNA from individual yeast clones was isolated in agarose plugs and digested with a cocktail of restriction enzymes selected to cleave yeast DNA into small fragments and one restriction enzyme to linearize the cloned *A. laidlawii* genomic fragment or whole genome. To remove linear yeast chromosomal DNA, plugs were pre-electrophoresed at constant voltage (70 V for several hours). Enzymes used for digests described in Suppleementary Figure 1; fragment 2: ApaI, AscI, I-CeuI, NotI, RsrII, fragment 3: ApaI, AscI, NotI, fragment 4: ApaI, AscI, I-ceuI, NotI. Enzymes used for digests described in Figure 5b: AscI, FseI, I-CeuI.

Preparation of YSVs. All YSV vectors were amplified from the pmycYACTn[12] template vector. Most primers had 20 bp homology to the pmycYACTn vector and 40−70 bp homologies to the insertion site.

Preparation of YRBVs. First the template YRBV sequence was assembled in yeast. This vector contained the URA3 marker from pYAC-RC vector (ATCC 37610), a core element: CEN6, HIS3, and ARS209 from pmycYACTn, and a pUC19 backbone. Also XhoI site was introduced at the junction between URA3 and the core element and another XhoI site was inserted at the junction of the core element and pUC19 (Note: sequences were overlapping each other by 40 bp). The template YRBV was isolated from yeast cells (see genomic DNA isolation above) and transformed into *E. coli*. Final YRBVs were assembled in yeast by cotransforming XhoI digested template RBV DNA isolated from *E. coli*, and two PCR amplified homology regions (these sequences are homologous to a site within the genome where the vector is to be inserted). To get the linear version of the final RBV suitable for cotransformation, vectors were PCR amplified or the plasmids from yeast were moved to *E. coli* (for increased yield), isolated and digested with XhoI enzyme (the design of the homologous sequences was that after assembly only one of the XhoI site would be restored).

Deletion Analysis of the Endonuclease Gene. All *A. laidlawii* fragments were PCR amplified and ligated into pmycYACTn[12] vector at the NotI site. Ligation products were transformed into *E. coli* strain DH10B (Invitrogen). Plasmids were isolated from *E. coli* and transformed into yeast.

Expression of the Toxic Region of the *A. laidlawii* Endonuclease Gene under Galactose Induction. A plasmid containing the fragment of the *A. laidlawii* endonuclease gene (this fragment was identical to deletion fragment 8 with the exception that two nucleotides "AT" were added at the 5′ end to restore the correct ORF) under the control of galactose promoter was constructed as follows: the vector containing a galactose promoter (GAL1) was amplified from pAG423GAL-ccdB[34] (Addgene plasmid 14149); a wild type fragment of endonuclease gene ACL0117 was amplified from *A. laidlawii* genomic DNA and a mutated version was amplified from F1-7 clone 13.

Both constructs were assembled in yeast by cotransformation of the appropriate fragments. Note: Many fewer colonies were obtained for the wild type version; however, some of the contained correctly assembled wild type version of fragment 8.

■ ASSOCIATED CONTENT

Supporting Information

Four additional figures and two tables, as well as information about YRBV and all primer sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors contributed to design of the research; B.J.K. and C.T. performed experiments; all authors contributed to writing of the paper.

Notes

H.O.S. is co-chief scientific officer and on the Board of Directors of SGI. C.A.H. is chairman of the SGI Scientific Advisory Board. Both of these authors and JCVI hold SGI stock.

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