Cloning and functional characterization of *Komagataella phaffii* centromeres by a color-based plasmid stability assay

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Abstract

The yeast *Komagataella phaffii* is widely used as a microbial host for heterologous protein production. However, molecular tools for this yeast are basically restricted to a few integrative and replicative plasmids. Four sequences that have recently been proposed as the *K. phaffii* centromeres could be used to develop a new class of mitotically stable vectors. In this work we designed a color-based genetic assay to investigate genetic stability in *K. phaffii*. Plasmids bearing *K. phaffii* centromeres and the *ADE3* marker were evaluated in terms of mitotic stability in an *ade2/ade3* auxotrophic strain which allows plasmid screening through colony color. Plasmid copy number was verified through qPCR. Our results confirmed that the centromeric plasmids were maintained at low copy number as a result of typical chromosome-like segregation during cell division. These features, combined with high transformation efficiency and *in vivo* assembly possibilities, prompt these plasmids as a new addition to the *K. phaffii* genetic toolbox.

Introduction

Komagataella phaffii is a methylotrophic yeast of great industrial importance which has been used for more than 30 years as a heterologous protein production platform [1]. Its genome was first published in 2009 and has since then been refined and thoroughly studied [2,3]. As a result, in addition to a protein factory, *K. phaffii* has also

been widely considered as a platform for the production of chemicals, biopharmaceuticals, vitamins and other molecules. However, the construction and regulation of new pathways demand complex molecular biology tools which are not readily available for this yeast [4].

K. phaffii genetic manipulation traditionally involves the use of shuttle vectors assembled in *Escherichia coli* and subsequently integrated into the yeast´s genome [5]. Recent studies have described the development of a wide range of genetic parts for use in this yeast, as well as new methods of plasmid assembly and transformation [6]. An alternative to integrative strategies is the use of replicative plasmids, which are usually based on the well-known ARS1 sequence [1]. These plasmids may overcome some drawbacks such as genetic instability in multi-copy strains, non-specific genomic integration and different expression levels depending on the integration *locus* [7-9]. In addition, they present higher transformation efficiency when compared to integrative vectors and can be assembled by *in vivo* recombination, which eliminates the need for bacterial transformation [10,11]. However, replicative plasmids show low mitotic stability when compared to integrative vectors and few vector options are available for use [12]. Stability problems can be circumvented by the creation of centromeric plasmids, which may provide proper segregation during mitosis. Greater mitotic stability as well as low copy number allow stable and constant protein expression [13]. Centromeric plasmids can be constructed *in vivo*, allowing the assembly and cloning of large sequences including whole metabolic pathways and regulatory regions [14]. Therefore, the construction of such vectors would be of great value for *K. phaffii* strain development in the context of synthetic biology.

Centromeres are typically surrounded by large heterochromatin sections in most organisms [15]. Their structure ranges from simple "point" centromeres of only ~125 bp in *Saccharomyces cerevisiae* to epigenetic, sequence-independent centromeres, such as those present in plants and animals. The reason for this phenomenon is that, for most eukaryotes, centromeres are maintained epigenetically and not genetically. Sequence homologies are rare in and between species, hindering the definition of a consensus sequence. In addition, some DNA regions can be centromeric or not depending on its function in previous cell cycles, which highlights the epigenetic nature of the centromere [16].

As for non-conventional yeasts, there are wide variations in centromere size and structure. *Candida glabrata* has centromeres that show some homology to the CDEI and CDEIII regions of *S. cerevisiae* while *Kuraishia capsulata* centromeres have 200-bp conserved sequences [17,18]. On the other hand, *Candida tropicalis, Schizosaccharomyces pombe* and *Candida albicans* have regional centromeres named after their sizes which range from 3 to 110 kb [19–21].

K. phaffii centromeres have recently been identified, bearing no sequence similarities to those of any other yeast [3]. Since centromere function relies strongly on its structure rather than on its sequence, a centromere-specific histone H3 variant (CSE4) was used in the search for centromeric regions in *K. phaffii*. A CSE4 homolog was identified in chromosome 2 and tagged with a fluorescence marker. The corresponding nuclear localization of the histone-DNA complex indicated a centromere pattern typical of budding yeasts [3]. Tridimensional conformation analysis followed the centromere clustering pattern observed in yeasts and narrowed down all four *K. phaffii* centromere locations to 20 kb windows [22].

Considering that a low transcription rate is typical of centromeric regions, RNA-seq analysis allowed researchers to pinpoint the putative centromeric locations for all four *K. phaffii* centromeres [3]. Similarly to *C. tropicalis* and *S.* pombe, *K. phaffii* centromeres are formed by inverted repeats. All four sequences have two inverted repeats of ~2,5 kb, separated by a central segment of 800 to 1300 bp. Chromatin immunoprecipitation sequencing analysis showed that the CSE4 histone binds preferably to the central region, but also along the inverted repeats [23].

K. phaffii centromeric sequences contain early replication peaks with autonomously replicating sequences, characteristics that are also observed in centromeres of other yeasts [24,25]. According to recently published studies, there are native ARS sequences contained within centromeres 2, 3 and 4. These comprise regions within the inverted repeats, as well as unique adjacent sequences [23,26].

In order to expand the functional analysis of the *K. phaffii* centromeres we sought in this study to develop a genetic system based on an *ade2/ade3* auxotrophic strain and a replicative vector carrying the wild-type *ADE3*. Vectors carrying *K. phaffii* centromeres were used to assess plasmid copy number and mitotic stability.

Results and Discussion

In yeasts, the adenine synthesis pathway is used as a tool for auxotrophic selection, gene copy number indicator and for plasmid stability analysis [27]. Many genes from this pathway have been deleted in *S. cerevisiae* in order to create auxotrophic strains, while in *K. phaffii* studies have only focused on *ADE1* and *ADE2* [28,29]. *K. phaffii* LA2, a strain mutant for *ADE2* [30], was used as a starting point for

the construction of a strain that would allow plasmid stability verification. Deletion of *ADE2* results in cells that are auxotrophic for adenine and accumulate a red intermediate [27] while deletion of genes located upstream, such as *ADE1* or *ADE3*, should prevent the formation of such pigment [28]. As expected, *ADE3* deletion in the LA3 strain results in white colonies (Fig 1). The deletion of this gene in *S. cerevisiae* has regulatory effects in the histidine synthesis pathway [31]. Consequently, *ade2 ade3* strains are not only auxotrophic for adenine, but also for histidine. In order to verify if this phenotype is applicable to *K. phaffii*, we plated strains X-33, LA2 and LA3 on MD medium without supplementation, comparing growth and colony color to cells plated on MD medium with adenine and histidine (Fig 1). LA3 strain displayed the expected histidine auxotrophy phenotype, showing that the adenine-histidine pathways in *K. phaffii* and *S. cerevisiae* have common characteristics.

Fig 1. Strain phenotypic analysis on defined media. *K. phaffii* X-33 (wild-type), LA2 and LA3 cultures were spotted on MD medium with or without supplementation.

In order to assess plasmid stability, we first constructed plasmid pPICH-ADE3 bearing the *ADE3* gene (Fig 2). When transformed with pPICH-ADE3, LA3 cells should return to being red and any changes on colony color would allow a simple screening of plasmid loss [27]. Although adenine auxotrophy has been explored for other purposes in *K. phaffii* [29], this particular color-based system has not yet been used for measuring plasmid stability in this yeast.

Fig 2. Map of vector pPICH-ADE3. ARS1 was used as the autonomously replicative sequence and *Sh ble* was used as the zeocin resistance marker. The *Not*I site was used for cloning the *K. phaffii ADE3* gene.

pPICH-ADE3 was used for cloning *K. phaffii* centromeres. Since it revealed extremely difficult to amplify entire centromeric regions, we designed a strategy to amplify centromeres in halves in order to reduce fragment size and to avoid primer annealing inside the inverted repeats (Fig 3). Amplified fragments exhibited in their ends overlapping regions that would allow recombination between each other and with vector pPICH-ADE3. Centromeric primer sequences were designed using *K. phaffii* GS115 genome sequence as reference [2]. The amplified regions corresponded to the following chromosomal coordinates: chromosome 1 position 1401429-1406917 (5488 bp); chromosome 2 position 1543739-1550657 (6918 bp); chromosome 3 position 2204800-2211493 (6693 bp) and chromosome 4 position 1703369-1709958 (6589 bp). Despite several attempts, we were unable to clone the centromere present in chromosome 3, which was excluded from our analysis. We speculate that its repetitive motifs, which were unlike those present in the other *K. phaffii* centromeres, rendered it extremely unstable in *E. coli*.

Fig 3. Strategy for *K. phaffii* centromere amplification. Schematic representation of a typical *K. phaffii* centromere. Inverted repeats are represented by green arrows. Primer annealing regions are shown by small arrows.

Centromeric sequences are known as early replication regions and according to recently published studies there are native ARS contained within centromeres 2, 3 and 4 [12, 23]. Fig 4 illustrates the positions of the ARS within and around these centromeres. In chromosome 2, ARS are located on coordinates 1543374-1543971 (597 bp) and 1549967-1551156 (1189 bp). These were partially amplified in this work, containing 232 and 690 bp, respectively. Regarding chromosome 3, there is an ARS located on coordinates 2204369-2205185 (816 bp) which was also partially amplified with 385 bp. Chromosome 4 has an ARS on coordinates 1703466-1704103 (637 bp), which was fully amplified, as well as a partially amplified one (840 bp) on coordinates 1709118-1710114 (996 bp total) [12].

Fig 4. Relative positions of ARS sequences around centromeres 2, 3 and 4 of *K. phaffii*. Regions in gray represent the centromeric sequences amplified in this work. Black boxes indicate identified ARS sequences [11].

LA3 strain was individually transformed with pPICH-ADE3 and centromeric plasmids pPICH-CEN1, 2 and 4, which were verified for autonomous replication by plasmid rescue in *E. coli* following restriction digestion with *Not*I to verify vector integrity (S1 Fig).

Plasmid stability was firstly verified through colony color in non-selective medium (Fig 5). When plated on YPD non-selective medium, colonies transformed with pPICH-ADE3 lost their color rapidly and presented a red center with large white edges, a result consistent with plasmid instability. In contrast, strains transformed with centromeric plasmids presented a uniform red coloration throughout the colony.

Fig 5. Plasmid stability analysis. LA3 strain transformed with pPICH-ADE3 and the centromeric plasmids was grown on YPD medium for 3 days until color development.

Further stability examination of the centromeric plasmids was performed by growing cells in liquid YPD medium for 144 hours. After diluting and plating cultures on non-selective medium, red and white colonies were counted and compared between each construction (Fig 6). LA3 strain transformed with pPICH-ADE3 did not yield red colonies in any growth period, indicating that the plasmid was mitotically unstable. Conversely, centromeric plasmids presented a higher mitotic stability than pPICH-ADE3. After 96 hours of growth, cells with pPICH-CEN1 started to present white colonies, while the other centromeric plasmids remained stable. After 144 hours, pPICH-CEN1 was lost in most colonies while the other centromeric plasmids were lost in <10% cells. The reason for the instability of pPICH-CEN1 could be related to the absence of an autonomously replicating sequence within the centromere, since centromeres 2 and 4 were cloned with at least partially amplified ARSs. The original replicating sequence in the pPICH-ADE3 plasmid, ARS1, has shown to be less efficient than its modern counterparts, therefore new ARSs contained in the centromeres could have enhanced the centromeric plasmids' mitotic stability [12].

Fig 6. Plasmid stability test. (A) Aliquots of the liquid cultures were collected after 96 and 144 hours of growth and plated on YPD medium. Red colonies represent cells that maintained the *ADE3*-containing plasmid, while white colonies have lost it. Red

portions of the bars represent red colonies; light pink bars represent white colonies. (B) A plate representing a typical result after 144 h growth.

Yeast centromeric plasmids knowingly have a higher mitotic stability under non-selective conditions than common replicative vectors since they are equally segregated between daughter cells and therefore provide a uniform culture of cells containing the plasmid [27]. A centromeric vector containing *K. phaffii* CEN2 has been constructed and it presented an enhanced stability when compared to a replicative plasmid [26]. In addition to *K. phaffii* and *S. cerevisiae*, centromeric plasmids have been developed for other yeasts such as *S. pombe, C. glabrata* and *Scheffersomyces stipitis* and in all cases an enhanced plasmid stability under non-selective conditions was verified [13,18].

Yeast replicative plasmids are normally replicated but are unevenly distributed between daughter cells, which creates both multi-copy and plasmidless cells [27]. Under selective conditions, cells lacking the plasmid are unable to survive and the result is a population of multi-copy plasmid-containing cells. The construction of centromeric plasmids should provide better plasmid segregation and stability and cells should maintain a low and stable plasmid copy number during yeast growth [32]. Plasmid copy number was assessed by qPCR after strains were grown in YPD medium containing zeocin in order to ensure that all cells assayed were harboring the centromeric plasmids. The results were compared to the LA3 strain transformed with pPICH-ADE3 also grown in selective medium and to the LA3 control strain, grown in YPD medium. qPCR results (Fig 7) indicate that the strain transformed with centromeric plasmids carried 1-2 copies per cell while the replicative plasmid was present at approximately 25 copies per cell. The difference in plasmid copy number between the replicative vector

and the centromeric vectors was significant according to a t-test ($p<0.05$). This result illustrates the expected segregation pattern described above for growth in selective conditions and, together with the mitotic stability analysis, provides a clear picture of *K. phaffii* genetic manipulation using centromeric plasmids.

Fig 7. Plasmid copy number determination. The number of plasmids in each cell was estimated by qPCR. LA3 was used as negative control. Statistical analysis comparing each of the centromeric plasmids with the replicative vector was performed through a t-test using GraphPad Prism 5. ($p<0.05$). Error bars depict the standard deviation of the mean ($n = 3$).

S. cerevisiae centromeric plasmids, in comparison to plasmids bearing the 2 micron sequence, presented the same difference in copy numbers when auxotrophic markers were used. However, when *kanMX* G418 resistance marker was used, the plasmid copy numbers did not differ between centromeric and replicative plasmids [37]. This indicates that factors other than the type of replication origin can influence plasmid copy numbers. In a previous study, *K. phaffii* transformed with a plasmid containing centromere 2 was analyzed regarding plasmid copy number and compared to both a replicative plasmid and an integrative strategy [26]. Results exhibited a low number of plasmids per cell in all strategies, which does not correspond to our observation. The difference may be related to the different strains, culture conditions or plasmid constructions, since although both plasmids used the ARS1 replicative sequence and the

zeocin resistance marker, pPICH-CEN1, 2, and 4 contained the *ADE3* gene while the previously reported centromeric plasmid carried an *EGFP* reporter gene.

Overall, our results indicate that centromeric plasmids could be employed as a new tool for the genetic manipulation of *K. phaffii*. Plasmids were maintained for long periods in non-selective medium, indicating that growth can be performed without the addition of antibiotics or any form of selective pressure. The centromeric plasmids' low copy numbers per cell characterize a stable and homogeneous culture that can provide reliable expression results. Finally, their structure as a circular molecule allows *in vivo* plasmid assembly with relatively short homologous sequences when compared to genomic integration techniques where sequences have to be much longer for directed homologous recombination. Simpler assembly may also facilitate the construction of larger and more sophisticated vectors such as yeast artificial chromosomes (YAC), whose stability features may be also analysed by the color-based assay described in this work.

Materials and methods

Strains and Media

DNA cloning was performed using chemically competent *Escherichia coli* XL-10 Gold (Agilent Technologies) grown in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone and 10 g L^{-1} NaCl, pH 7.2). When needed, agar was added to a final concentration of 1.5%. When zeocin (25 µg mL^{-1}) was used for bacterial antibiotic selection, NaCl concentration was reduced to 5 g L^{-1}.

K. phaffii strains were derived from X-33 (Invitrogen). LA2 strain (*amd2 ade2*) was described in previous work [30]. Yeast was routinely grown in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose). Solid medium used 2% agar. Zeocin and geneticin, when used, were added at 100 µg mL^{-1} and 500 µg mL^{-1}, respectively. Hygromycin B was used to a final concentration of 50 µg mL^{-1}. Minimal medium (MD) used 0.34% Yeast Nitrogen Base, 1% $(NH_4)_2SO_4$, 2% glucose, 0.00004% biotin, and 0.0002% adenine or 0.004% histidine, when needed.

PCR

DNA was amplified using Invitrogen Platinum Taq DNA Polymerase (High Fidelity), Promega GoTaq Colorless Master Mix or Sigma-Aldrich Accutaq LA DNA Polymerase. All primers used in this work are shown in Table 1.

Table 1. Primers used in this work.

Primer	Sequence	Enzyme
ADE3up-F	GATAAGCTTGATATCGAATTCCTGCAGCCCCCC GGGACGTAATGGAATAACTGCTGAC	*Sma*I
ADE3up-R	GAAGTTATGGATCCTACGAGGTAATTGAAGGCT CAC	
ADE3lox-F	CTTCAATTACCTCGTAGGATCCATAACTTCGTAT AATG	
ADE3lox-R	CAATCTCTCCCTTGTCATCGGATCCATAACTTCG TATAG	
ADE3dw-F	GAAGTTATGGATCCGATGACAAGGGAGAGATT GAAG	
ADE3dw-R	GGTGGCGGCCGCTCTAGAACTAGTGGATCCCCC CCCGGGTGCAATGTACTGTTGAGTAGG	*Sma*I
ADE3conF	GGGGACCGGAGGTAAAAGAC	
ADE3conR	GTTGGAATAATTGCATGGTCTG	

MUT1(Hpa)	CCATTGACATGGTAAACAGTTGGA	
MUT2(Bam)	GAACACTGGGATCTTGGTTGAGG	
Cen1-F	GAGTTTAAACGGATCCACGAAGCAATGGATAGGCACT	*Bam*HI
Cen1-R	CCGCGAATTCGGATCCTGAAGTCTTTCAGAGAGGAGCA	*Bam*HI
Cen1c-F	CAAGTATGCGTGATCCCAGGT	
Cen1c-R	TACGAATTGTGGGGCTCTGT	
Cen2-F	GAGTTTAAACGGATCCATCTCCGTTGATACTCCCAAC	*Bam*HI
Cen2-R	CCGCGAATTCGGATCCATCGACAAGCAGAACACTAAG	*Bam*HI
Cen2c-F	GAATGGAGGTGCTGGTGGTTA	
Cen2c-R	TGTAATGCTCGCTGGTGAGT	
Cen3-F	GAGTTTAAACGGATCCAAGTGGTACACCAGTCAGCG	*Bam*HI
Cen3-R	CCGCGAATTCGGATCCTCAGTATTCAACTGCAACTGC	*Bam*HI
Cen3c-F	TCAGCCGAATACCCACACTT	
Cen3c-R	TCAGCCGTCAGCGAAATGAT	
Cen4-F	GAGTTTAAACGGATCCCAAACGCACCGTCTTGTTCA	*Bam*HI
Cen4-R	CCGCGAATTCGGATCCAATTGATGTAGACGAGCAGC	*Bam*HI
Cen4c-F	TCAAGAATCGTACTGGCACCT	
Cen4c-R	CAAGCTCGTGAGATGGGATGT	
Cen370-F	CGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTGGTCATGAGATCAGATCTAACATCCAAAGACGAAAGGTTGAATGAGTTTAAACGGATCCAAGTGG	
Cen370-R	GCTGGCCCTCTCTTCCCAGCTCACGAATCAGATCCTAAGTCCTACTCAACAGTACATTGCAGCGGCCGCGTTTAAACGAATTCGGATCCTCAGTATTCAA	
qZEO-F	CGACGTGACCCTGTTCATCA	

qZEO-R	TGGACACGACCTCCGACCA	
qHIS-F	GTGTATCCTGGCTTGGCATCT	
qHIS-R	GCCAAGTACGGTGTGACGTT	

Restriction sites are underlined.

DNA manipulation

All basic DNA manipulation and analysis were performed as previously described [33]. Restriction digestion was performed in accordance to the manufacturer instructions (New England Biolabs), as well as vector dephosphorylation with Shrimp Alkaline Phosphatase (Promega) and ligation with T4 DNA Ligase (USB). In-Fusion Cloning Kit (Clontech) was used for in-vitro assembly of plasmids. Site-directed mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech). PCR and gel purification used Promega Wizard SV Gel and PCR Clean-Up System.

Quantitative PCR (qPCR)

Strains harboring the zeocin resistance plasmids were grown to an OD_{600} (optical density measured at 600 nm) of 1 in 10 mL YPD containing zeocin while LA3 was grown in 10 mL YPD. Cells were collected by centrifugation at 2000 x g for 5 minutes. The cell pellet was resuspended with 1 mL 0.25% SDS and incubated at 98ºC for 8 minutes according to previous work [26]. Finally, cell debris was removed by centrifugation and DNA was diluted 10-fold in water before qPCR reactions.

Quantitative PCR reactions used primers qZEO-F and qZEO-R for plasmid quantification and qHIS-F and qHIS-R as an internal single-copy control. Assays were

carried out with iTaq Universal SYBR Green Supermix (Bio-Rad) in a Rotor-Gene Q (Qiagen) thermal cycler. Analysis used the absolute quantification method and standard curves that ranged from $1x10^4$ to $1x10^8$ copies of the gene of interest. pPIC9 (Invitrogen) and pPICH linearized plasmids were used for construction of the standard curves.

Yeast transformation

K. phaffii was electroporated following two different protocols. For integrative cassettes, we followed the *Pichia* Expression Kit protocol (Invitrogen) and when using replicative plasmids, we proceeded as described previously [34].

Construction of an *ade2 ade3* strain for color-based stability assays

Strain LA2 [30] was transformed with an *ADE3* deletion cassette and had the marker recycled before transformation with the centromeric plasmids. Construction of the deletion cassette used PCR reactions assembled by an "In-Fusion" cloning reaction. Briefly, primers ADE3up-F and R, ADE3dw-F and R were used for PCR amplification of 491 bp and 582 bp, respectively, from the *K. phaffii* genome. These reactions amplified sequences used for directing homologous recombination and substitution of the complete *ADE3* coding sequence. Meanwhile, primers ADE3lox-F and ADE3lox-R amplified the *kanR* geneticin resistance cassette from plasmid pGKL [35]. PCR fragments were assembled and cloned into pBluescript II SK^+ linearized with *Sma*I. A final PCR reaction using primers ADE3up-F and ADE3dw-R amplified the whole

deletion cassette which was used for transformation of *K. phaffii* LA2. Cells were selected in YPD containing geneticin.

The resulting *ade2 ade3* strain was later transformed with pYRCre2 [36] and selected in YPD supplied with hygromycin B. This step promoted a Cre-mediated excision of the *kanR* cassette eliminating geneticin resistance. After PCR confirmation of marker recycling using primers ADE3conF and ADE3conR, the resulting strain was plated in non-selective YPD medium, causing loss of the pYRCre2 plasmid. The resulting strain was named LA3.

Construction of centromeric plasmids containing *ADE3*

Plasmid pPICH [30], which is derived from pPICHOLI (MoBiTec), contains the ARS1 replicating sequence [1]. This sequence is originally located on *K. phaffii* GS115 chromosome 2, coordinates 413701-413856 [2]. The plasmid was digested with *Not*I for cloning of the *K. phaffii* native *ADE3*. The complete gene was amplified from X-33 DNA using primers ADE3up-F and ADE3dw-R following digestion with *Not*I. After vector dephosphorylation, fragments were ligated and transformed into *E. coli* XL-10 Gold. One positive clone was then submitted to site-directed mutagenesis using primers Mut1(Hpa) and Mut2(Bam) for removal of the *Bam*HI restriction site present within the *ADE3* coding sequence. The final plasmid containing ARS1, the *Sh ble* resistance marker and *ADE3* was named pPICH-ADE3.

pPICH-ADE3 was digested with *Bam*HI for cloning of all four *K. phaffii* centromeres. These were amplified from *K. phaffii* X-33 genomic DNA using two PCR reactions for each centromeric sequence. Primers Cen1/2/3/4-F and Cen1/2/3/4c-R amplified the first inverted repeat of each centromere while primers Cen1/2/3/4c-F and

Cen1/2/3/4-R amplified the other half of the sequences. In order to promote *in vitro*/*in vivo* assembly, the amplicons had ~80 bp homology between each other in one end and 15 bp with pPICH-ADE3 in the other end. First, we attempted an "In-Fusion" cloning reaction for each of the four centromeres using linearized pPICH-ADE3 and the two PCR fragments. Plasmids were extracted and analyzed by restriction digestion. Centromeres 1, 2 and 4 were successfully assembled and cloned into the plasmid through this strategy. Centromere 3 did not yield any *E. coli* clones following the "In-Fusion" reaction; we then proceeded to an *in vivo* assembly strategy. Primers Cen370-F and Cen3c-R; Cen3c-F and Cen370-R amplified both inverted repeats adding 70 bp of homologous sequences between the fragments and pPICH-ADE3. Finally, we transformed *K. phaffii* LA3 using the linearized vector and both centromeric fragments, using 85 bp of homology for directing recombination. Clones were selected in YPD supplied with zeocin. However, none of the obtained clones presented the assembled plasmid as it was expected and this centromeric sequence was not used in further analyses.

The resulting plasmids were named pPICH-CEN1, pPICH-CEN2, and pPICH-CEN4. All plasmids were transformed into *K. phaffii* LA3 for subsequent stability and quantification assays.

Stability analysis

LA3 strain transformed with each of the three centromeric plasmids was grown in 20 mL YPD for 16 hours at 28ºC and 200 rpm. This culture was inoculated to 20 mL YPD to an initial OD of 0.1. After 24 h of growth under the same conditions the culture was used as inoculum for another flask containing 20 mL YPD to an OD of 0.1. This

procedure was repeated every 24 h until a total of 144 hours. At 96 and at 144 hours of growth, a small amount of cells was diluted 10^6-fold and 100 µL of this dilution were plated on YPD. Plates were incubated at 30 ºC for 72 h.

References

1. Cregg, J. M., Barringer, K. J., Hessler, a Y., Madden, K. R. *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. 1985; 5(12): 3376–85.

2. De Schutter, K., Lin, Y.-C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., et al. Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat. Biotechnol. 2009;27(6): 561–566.

3. Sturmberger, L., Chappell, T., Geier, M., Krainer, F., Day, K. J., Vide, U., et al. Refined *Pichia pastoris* reference genome sequence. J. Biotechnol. 2016; 235: 121–131.

4. Schwarzhans, J. P., Luttermann, T., Geier, M., Kalinowski, J., Friehs, K. Towards systems metabolic engineering in *Pichia pastoris*. Biotechnol. Adv. 2017;35(6): 681–710.

5. Cereghino, J. L., Cregg, J. M. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 2000; 24(1): 45–66.

6. Obst, U., Lu, T. K., Sieber, V. A modular toolkit for generating *Pichia pastoris* secretion libraries. *ACS Synth. Biol.* 2017; 6 (6): 1016-1025.

7. Camattari, A., Goh, A., Yip, L. Y., Tan, A. H. M., Ng, S. W., Tran, A., et al. Characterization of a panARS-based episomal vector in the methylotrophic yeast *Pichia pastoris* for recombinant protein production and synthetic biology applications. Microb. Cell Fact. 2016;15(139). doi: 10.1186/s12934-016-0540-5

8. Schwarzhans, J.-P., Wibberg, D., Winkler, A., Luttermann, T., Kalinowski, J., Friehs, K. Integration event induced changes in recombinant protein productivity in *Pichia pastoris* discovered by whole genome sequencing and derived vector optimization. Microb. Cell Fact. 2016;15(84). doi: 10.1186/s12934-016-0486-7

9. Berg, L., Strand, T. A., Valla, S., Brautaset, T. Combinatorial mutagenesis and selection to understand and improve yeast promoters. Biomed Res. Int. 2013;926985. doi: 10.1155/2013/926985

10. Yu, X., Wang, R., Zhang, M., Xu, Y., Xiao, R. Enhanced thermostability of a Rhizopus chinensis lipase by in vivo recombination in *Pichia pastoris*. Microb. Cell Fact. 2012;11(102). doi : 10.1186/1475-2859-11-102

11. Lee, C. C., Williams, T. G., Wong, D. W. S., Robertson, G. H. An episomal expression vector for screening mutant gene libraries in *Pichia pastoris*. Plasmid 2005;54: 80–85.

12. Liachko, I., Youngblood, R. A., Tsui, K., Bubb, K. L., Queitsch, C., Raghuraman, M. K., et al. GC-Rich DNA elements enable replication origin activity in the methylotrophic yeast *Pichia pastoris*. PLOS Genet. 2014;10:3. doi: 10.1371/journal.pgen.1004169

13. Cao, M., Gao, M., Lopez-garcia, C. L., Wu, Y., Seetharam, A. S., Severin, A. J., et al. Centromeric DNA facilitates nonconventional yeast genetic engineering. ACS Synth. Biol. 2017; 6(8): 1545-1553.

14. Kouprina, N., Larionov, V. Transformation-associated recombination (TAR) cloning for genomics studies and synthetic biology. Chromosoma 2016; 125(4): 621-632.

15. Lyn Chan, F., Wong, L. H. Transcription in the maintenance of centromere

chromatin identity. Nucleic Acids Res. 2012;40(22): 11178–11188.

16. Malik, H. S., Henikoff, S. Major evolutionary transitions in centromere complexity. Cell 2009;138(6): 1067–1082.

17. Morales, L., Noel, B., Porcel, B., Marcet-Houben, M., Hullo, M. F., Sacerdot, C., et al. Complete DNA sequence of *Kuraishia capsulata* illustrates novel genomic features among budding yeasts (Saccharomycotina). Genome Biol. Evol. 2013;5(12): 2524–2539.

18. Kitada, K., Yamaguchi, E., Arisawa, M. Isolation of a *Candida glabrata* centromere and its use in construction of plasmid vectors. Gene 1996;175(1-2): 105–108.

19. Ketel, C., Wang, H. S. W., McClellan, M., Bouchonville, K., Selmecki, A., Lahav, T., et al. Neocentromeres form efficiently at multiple possible loci in *Candida albicans*. PLOS Genet. 2009;5(3). doi: 10.1371/journal.pgen.1000400

20. Chatterjee, G., Sankaranarayanan, S. R., Guin, K., Thattikota, Y., Padmanabhan, S., Siddharthan, R., et al. Repeat-associated fission yeast-like regional centromeres in the ascomycetous budding yeast *Candida tropicalis*. PLOS Genet. 2016;12(2). doi: 10.1371/journal.pgen.1005839

21. Smirnova, J. B., McFarlane, R. J. The unique centromeric chromatin structure of *Schizosaccharomyces pombe* is maintained during meiosis. J. Biol. Chem. 2002; 277(22); 19817–19822.

22. Varoquaux, N., Liachko, I., Ay, F., Burton, J. N., Shendure, J., Dunham, M. J., et al. Accurate identification of centromere locations in yeast genomes using Hi-C. Nucleic Acids Res. 2015;43(11): 5331–5339.

23. Coughlan, A. Y., Hanson, S. J., Byrne, K. P., Wolfe, K. H. Centromeres of the yeast *Komagataella phaffii* (*Pichia pastoris*) have a simple inverted-repeat structure. Genome

448 Biol. Evol. 2016;8(8): 2482–2492.

449 24. McCarroll, R. M., Fangman, W. L. Time of replication of yeast centromeres and

450 telomeres. Cell 1988;54(4): 505–513.

451 25. Koren, A., Tsai, H., Tirosh, I., Burrack, L. S., Barkai, N., Berman, J. Epigenetically-

452 inherited centromere and neocentromere DNA replicates earliest in s-phase. PLOS

453 Genet. 2010;6(8). doi: 10.1371/journal.pgen.1001068

454 26. Nakamura, Y., Nishi, T., Noguchi, R., Ito, Y., Watanabe, T., Nishiyama, T., et al.

455 Construction of a new stable, autonomously replicating plasmid vector containing

456 *Pichia pastoris* centromeric DNA. Appl. Environ. Microbiol. 2018;84(15). doi:

457 10.1128/AEM.02882-17

458 27. Hieter, P., Mann, C., Snyder, M., Davis, R. W. Mitotic stability of yeast

459 chromosomes : a colony color assay that measures nondisjunction and chromosome

460 loss. Cell 1985;40(2): 381–392.

461 28. Ugolini, S., Bruschi, C. V. The red/white colony color assay in the yeast

462 *Saccharomyces cerevisiae*: epistatic growth advantage of white ade8-18, ade2 cells over

463 red ade2 cells. Curr. Genet. 1996;30(6): 485–492.

464 29. Du, M., Battles, M. B., Nett, J. H. A color-based stable multi-copy integrant

465 selection system for *Pichia pastoris* using the attenuated *ADE1* and *ADE2* genes as

466 auxotrophic markers. Bioeng. Bugs 2012;3(1): 32–37.

467 30. Piva, L. C., De Marco, J. L., de Moraes, L. M. P., Reis, V. C. B., Torres, F. A. G.

468 Acetamidase as a dominant recyclable marker for *Komagataella phaffii* strain

469 engineering. Appl. Microbiol. Biotechnol. 2018;102(6): 2753–2761.

470 31. Rébora, K., Laloo, B., Daignan-Fornier, B. Revisiting purine-histidine cross-

471 pathway regulation in *Saccharomyces cerevisiae*: a central role for a small molecule.

472 Genetics 2005;170(1): 61–70.

473 32. Steensels, J., Snoek, T., Meersman, E., Nicolino, M. P., Voordeckers, K.,

474 Verstrepen, K. J. Improving industrial yeast strains: exploiting natural and artificial

475 diversity. FEMS Microbiol. Rev. 2014;38(5): 947–995.

476 33. Sambrook, J., Fritsch, E., Maniatis, T. Molecular cloning: a laboratory manual. 2nd

477 ed. New York: Cold Spring Harbor Laboratory Press; 1989.

478 34. Wu, S., Letchworth, G. J. High efficiency transformation by electroporation of

479 *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. Biotechniques

480 2004;36(1): 152–154.

481 35. Reis, V. C. B., Nicola, A. M., de Souza Oliveira Neto, O., Batista, V. D. F., de

482 Moraes, L. M. P., Torres, F. A. G. Genetic characterization and construction of an

483 auxotrophic strain of *Saccharomyces cerevisiae* JP1, a Brazilian industrial yeast strain

484 for bioethanol production. J. Ind. Microbiol. Biotechnol. 2012;39(11): 1673–1683.

485 36. Betancur, M. O., Reis, V. C. B., Nicola, A. M., De Marco, J. L., de Moraes, L. M.

486 P., Torres, F. A. G. Multicopy plasmid integration in *Komagataella phaffii* mediated by

487 a defective auxotrophic marker. Microb. Cell Fact. 2017;16(99). doi: 10.1186/s12934-

488 017-0715-8

489 37. Karim, A. S., Curran, K. A., Alper, H. S. Characterization of plasmid burden and

490 copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering

491 applications. FEMS Yeast Res. 2013;13(1): 107-116

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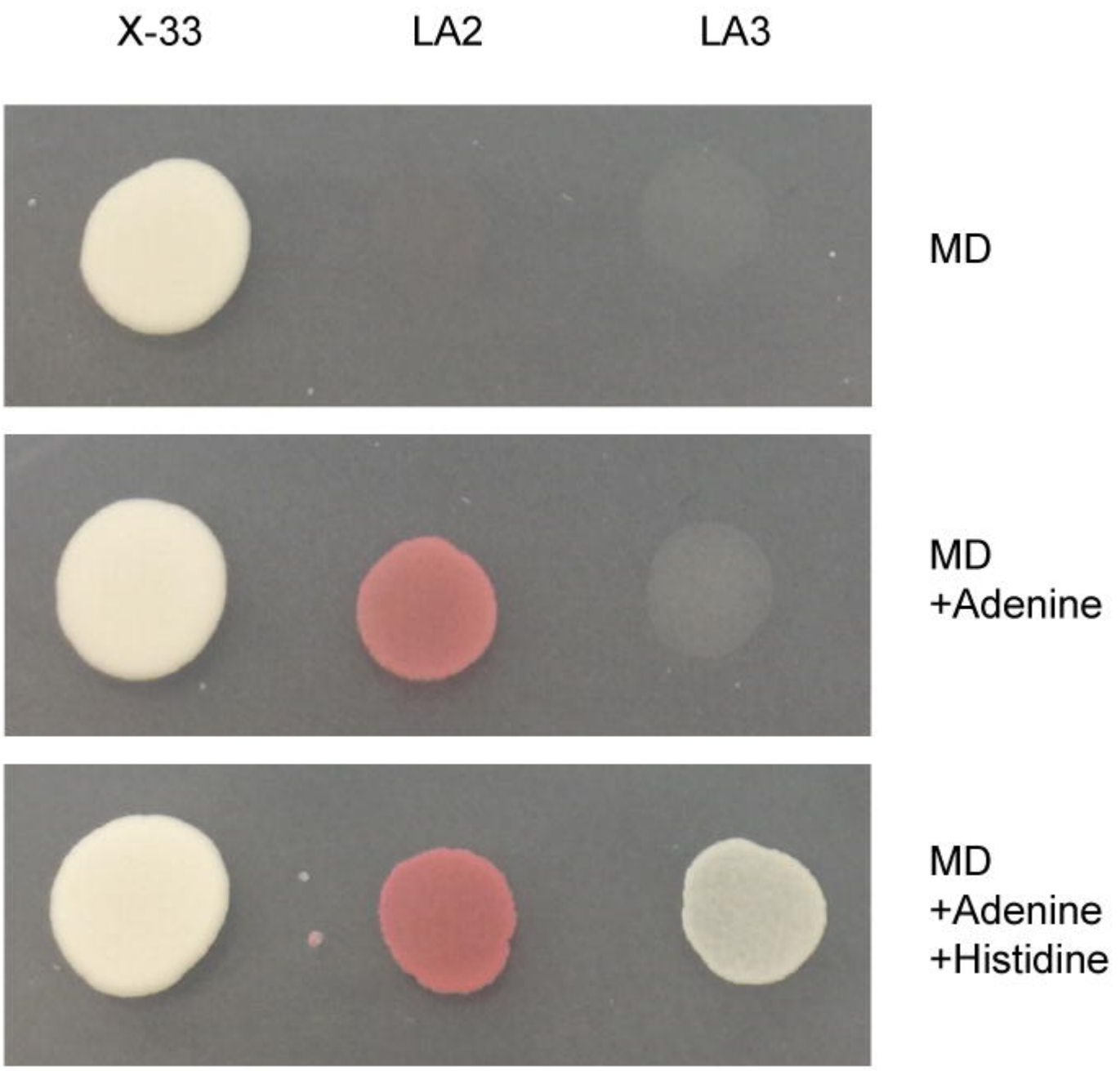
494 Supporting information captions

495 **S1 Fig. Restriction analysis of recovered plasmids pPICH-CEN1, 2, and 4.** Plasmids

were digested with *Not*I and analyzed on 1% agarose gel**.** All digested plasmids yielded a common 4.1 kb band which represents the *ADE3* gene. The size of the upper bands represent the sum of individual centromeres (CEN1 = 7.6 kb; CEN2 = 9.0 kb; CEN4 = 8.7 kb) and other common vector sequences. M: 1 kb Plus DNA Ladder (Thermo Fischer Scientific).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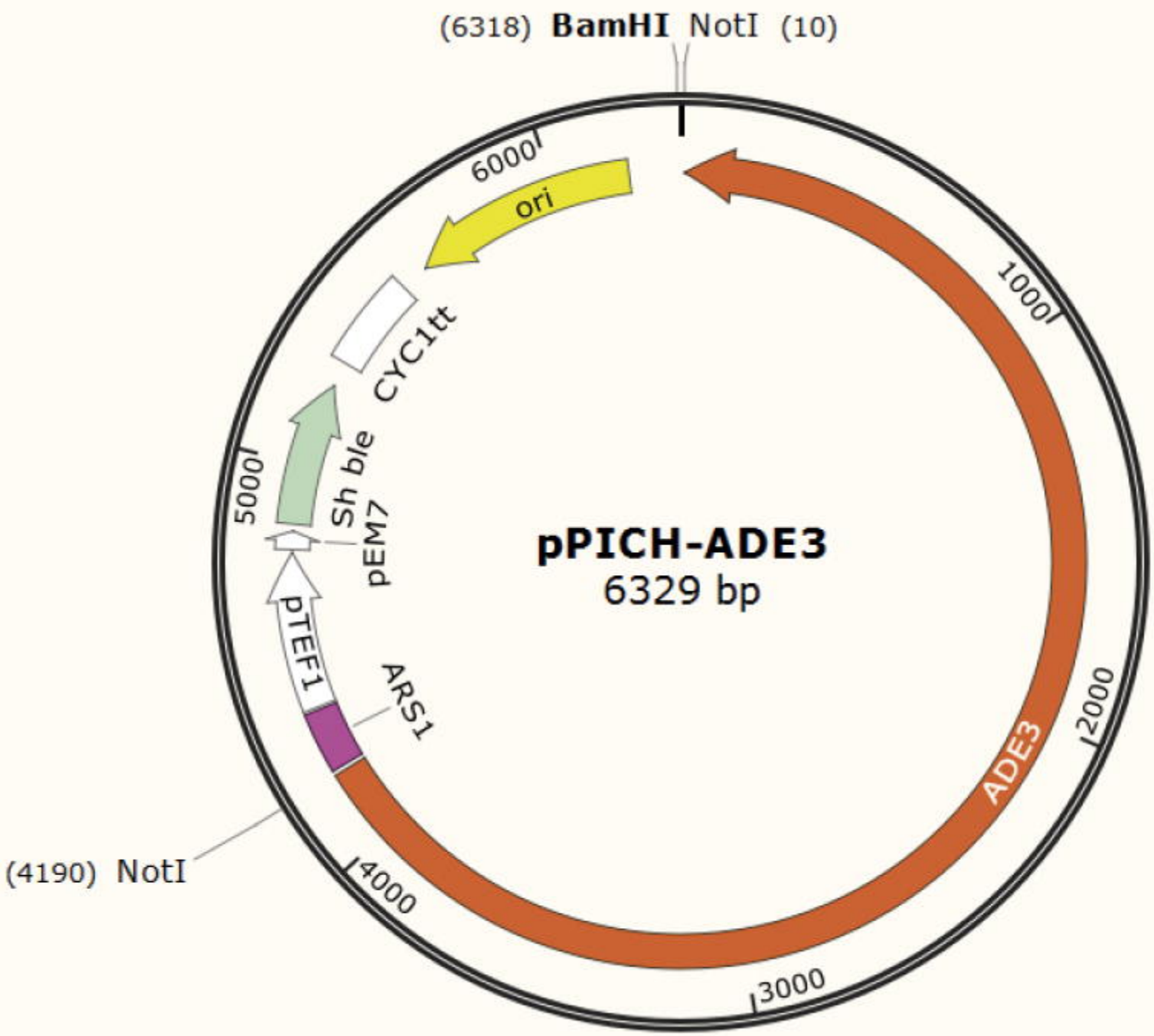
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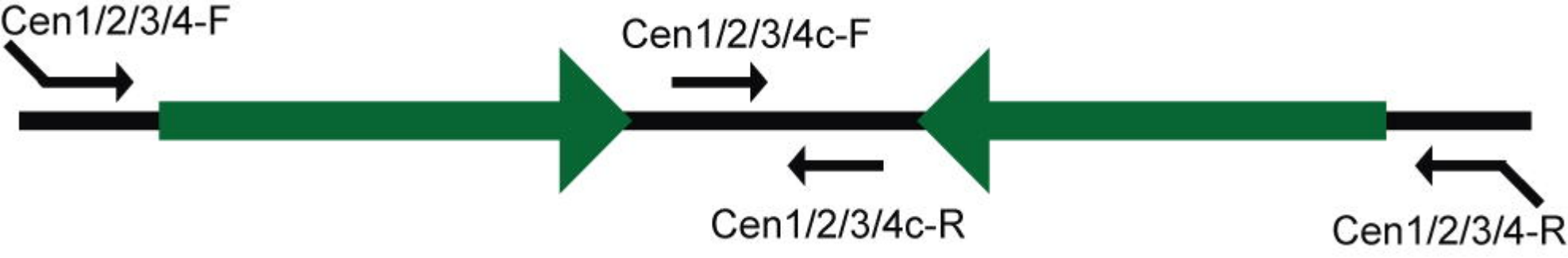
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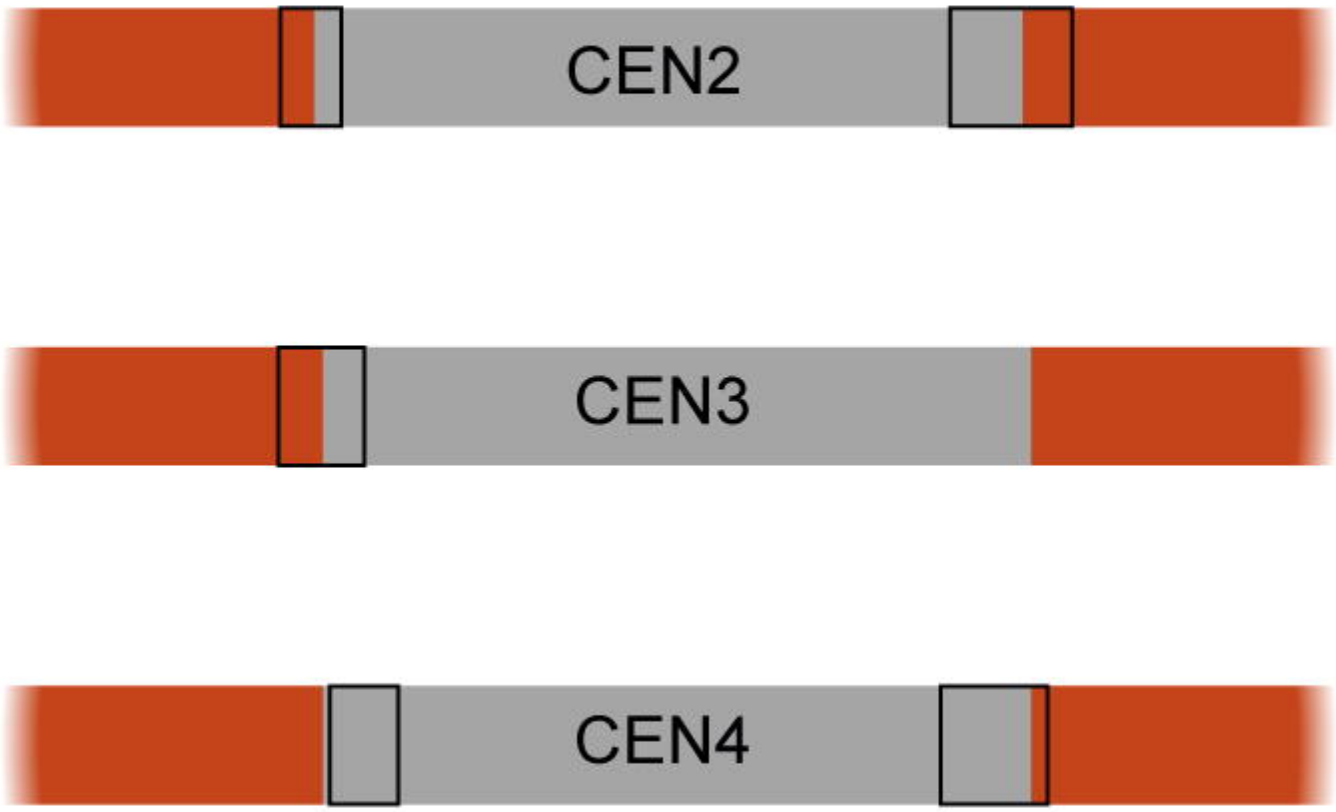
CEN2
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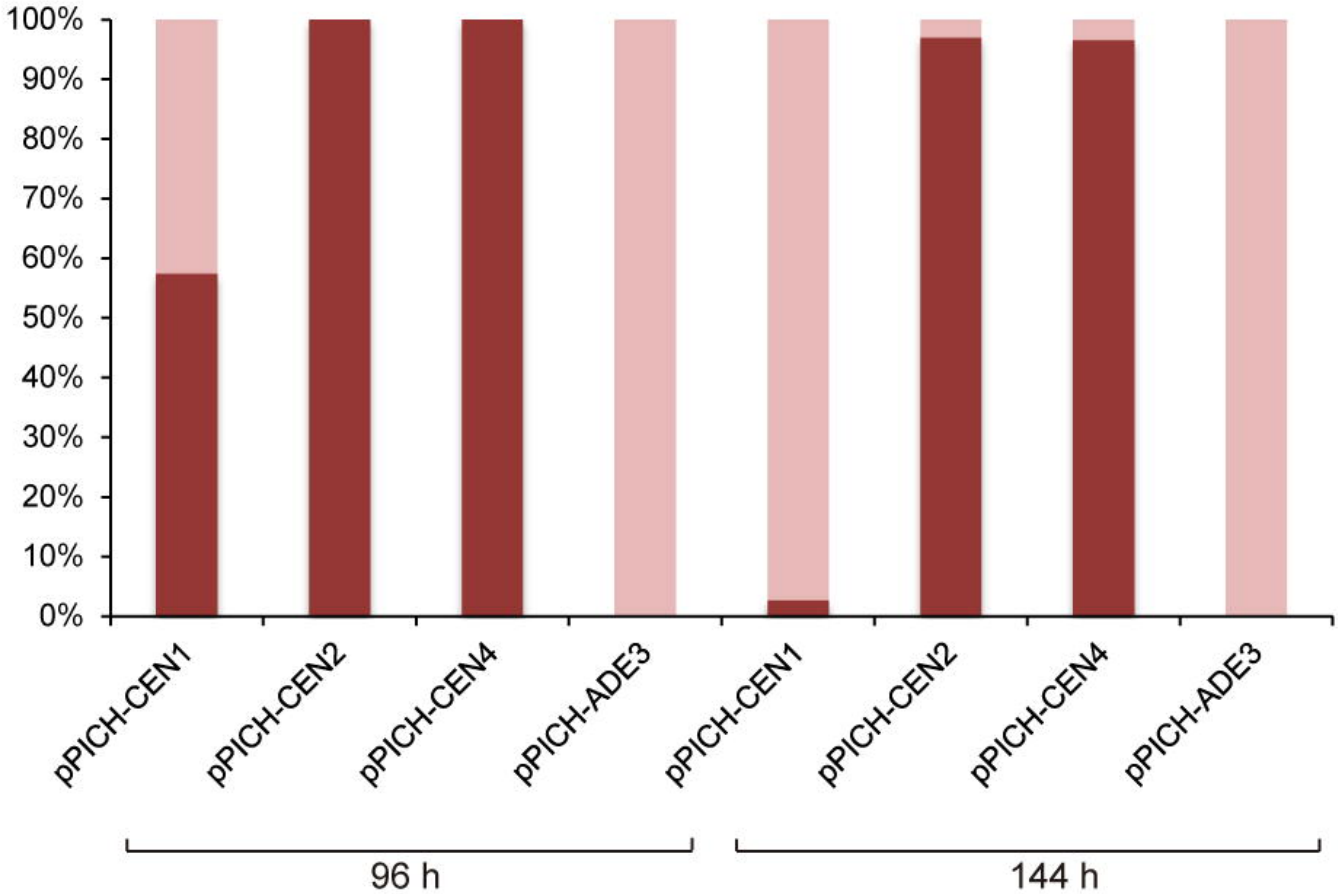
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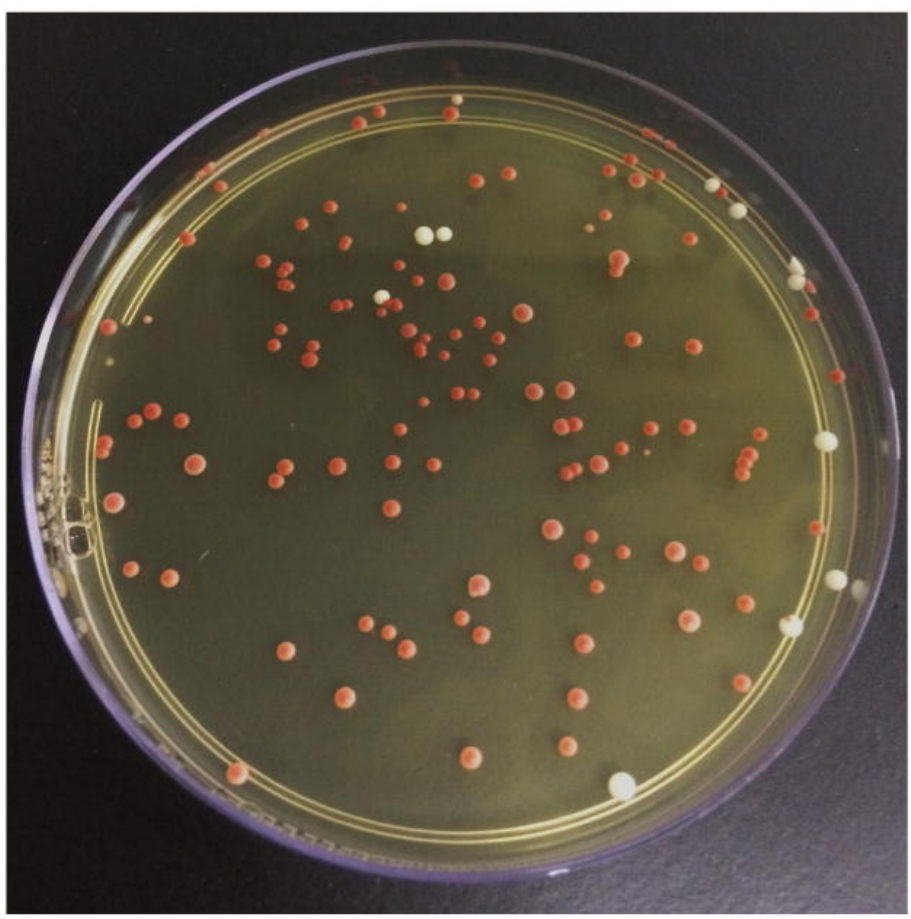

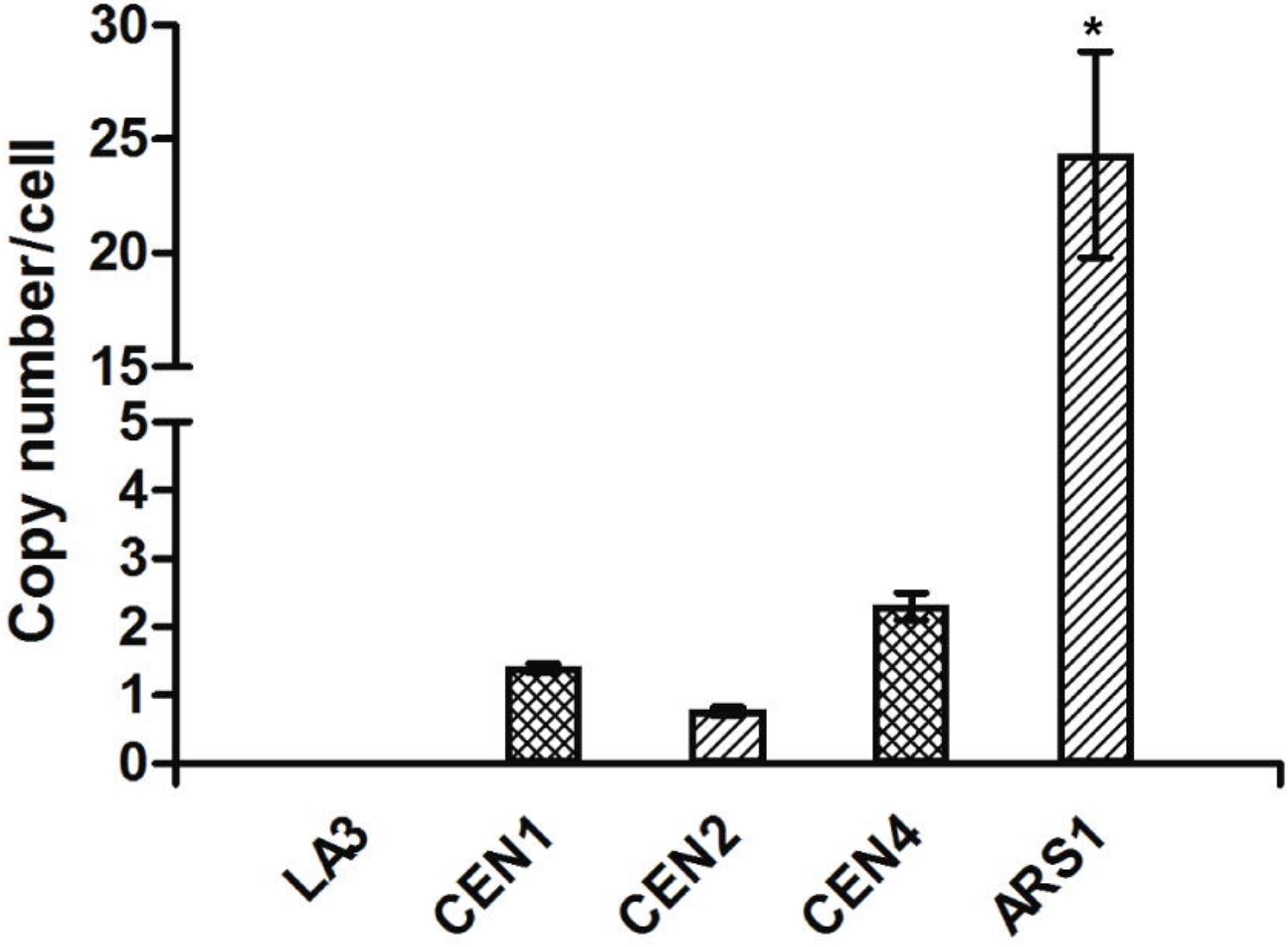
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