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D I P L O M A R B E I T

Genome editing in *Aureobasidium pullulans* via Cas9 ribonucleoproteins and PEG-mediated protoplast transformation

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Kurzfassung

Aureobasidium pullulans ist ein polymorpher hefeähnlicher Ascomycet, der in verschiedensten Lebensräumen und unter verschiedensten Umweltbedingungen zu finden ist. Er wird vorwiegend als Biokontrollmittel in der Landwirtschaft und zur industriellen Herstellung des Biopolymers Pullulan verwendet. Zu den möglichen zukünftigen Anwendungsmöglichkeiten gehören die Produktion extremotoleranter Enzyme, antimikrobieller Verbindungen, Schweröle, Siderophore und anderer Biopolymere. Die Entwicklung einer effizienten Genom-Editierungs-Strategie für *A. pullulans* ist daher notwendig, um weitere Forschung und die Erzeugung leistungsfähiger Stämme für industrielle Anwendungen zu ermöglichen. Diese Arbeit beschreibt die Entwicklung eines Polyethylenglykol (PEG)-vermittelten Protoplastentransformationsprotokolls und einer CRISPR/Cas9-basierten Genom-Editierungs-Strategie unter Verwendung von Cas9-sgRNA Ribonukleoproteinen (RNPs) für *A. pullulans*. Um das Transformationsprotokoll zu testen, wurden die Stämme EXF-150 (Referenzstamm), NBB 7.2.1 und ATCC 42023 mit den Hygromycin-Resistenz vermittelnden Plasmiden pAN7-1 und pRLM$_{EX}$30 transformiert. pAN7-1 und pRLM$_{EX}$30 enthalten das bakterielle Hygromycin B-Phosphotransferase-Gen (*hph*), flankiert von Promotor- und Terminatorelementen von *Aspergillus nidulans* bzw. *Trichoderma reesei*. Für den Referenzstamm wurden Transformationsraten von bis zu 8,6 KBE pro µg Plasmid-DNA erreicht. Southern-Hybridisierung zeigte ektopische Integration des Plasmids pRLM$_{EX}$30 und extrachromosomale Präsenz von pAN7-1. Das entwickelte Transformationsprotokoll wurde anschließend für die Einbringung von RNPs zur Genom-Editierung in *A. pullulans* verwendet. Das *ura3*-Gen (kodierend für Orotidin-5'-Phosphat-Decarboxylase) konnte in allen drei *A. pullulans* Stämmen, nur allein mit RNPs, zerstört werden. Die resultierende Uridin-Auxotrophie konnte mit *ura3* Homologen aus *Trichoderma reesei* (*pyr4*) bzw. *Aspergillus fumigatus* (*pyrG*) kompensiert werden. Darüber hinaus wurden die nicht direkt selektierbaren Gene *praics* (kodierend für ein Gen des Purin-Biosynthesewegs) und *asl* (kodierend für ein Gen des L-Arginin-Biosynthesewegs) im Referenzstamm durch Co-Targeting des selektierbaren Markers *ura3* in einem Multiplexing-Ansatz erfolgreich manipuliert. Die erhaltenen auxotrophen Stämme könnten für weitere Studien verwendet werden. Schließlich konnte die homologe Rekombinationsrate im Referenzstamm durch die Verwendung von RNPs, sogar in Kombination mit kurzen (20 bp) homologen Flanken, auf fast 100% gesteigert werden. Diese Arbeit zeigt, dass Cas9 RNPs mittels PEG in *A. pullulans*-Protoplasten eingebracht werden können und dort eine schnelle und effiziente Genom-Editierung ermöglichen.

Abstract

Aureobasidium pullulans is a polymorphic yeast-like ascomycete that can be found in diverse habitats and environmental conditions. It is used as a biocontrol agent in agriculture and for the industrial production of the biopolymer pullulan. Potential future applications include the production of extremotolerant enzymes, antimicrobial compounds, heavy oils, siderophores and other biopolymers. Thus the development of an efficient genome editing strategy for *A. pullulans* is necessary to allow for further research and generation of powerful strains for application in industry. This work describes the development of a polyethylene glycol (PEG)-mediated protoplast transformation protocol and a CRISPR/Cas9-based genome editing strategy using Cas9-sgRNA ribonucleoproteins (RNPs) for *A. pullulans*. To test the transformation protocol, the strains EXF-150 (reference strain), NBB 7.2.1 and ATCC 42023 were transformed to hygromycin resistance, using plasmids pAN7-1 and $pRLM_{EX}30$ that contain the bacterial hygromycin B phosphotransferase gene (*hph*) flanked by promoter and terminator elements of *Aspergillus nidulans* and *Trichoderma reesei*, respectively. For the reference strain transformation rates of up to 8.6 CFUs per µg plasmid DNA were achieved, and Southern hybridization of transformants revealed integration of plasmid $pRLM_{EX}30$ and presence of pAN7-1 extrachromosomally. The developed PEG-mediated transformation protocol was used for the delivery of RNPs for genome editing in *A. pullulans*. Disruption of the *ura3* gene (encoding for orotidine-5'-phosphate-decarboxylase) was successfully achieved in all three *A. pullulans* strains using only RNPs. The resulting uridine auxotrophy could be complemented with *ura3* homologous from *Trichoderma reesei* (*pyr4*) and *Aspergillus fumigatus* (*pyrG*), respectively. Further, manipulation of not directly selectable genes *praics* (encoding a gene of the purine biosynthesis pathway) and *asl* (encoding a gene of the L-arginine biosynthesis pathway) in the reference strain was achieved by co-targeting the selectable marker *ura3* in a multiplexing approach. The obtained auxotrophic strains could be used for further studies. Lastly, the homologous recombination rate in the reference strain could be increased to nearly 100% by the usage of RNPs, even in combination with short (20 bp) homologous flanks. This work shows that Cas9 RNPs can be delivered into *A. pullulans* protoplasts using PEG and allow for fast and efficient genome editing.

Introduction

Aureobasidium pullulans (DE BARY) ARNAUD is a previous species complex of ubiquitous, polymorphic fungi that belong to the group of so called "black yeasts" due to the production of melanin. (Cooke, 1959; de Hoog, 1993; Di Francesco *et al.*, 2020). The *A. pullulans* complex is allocated in the *Ascomycota* phylum (class: *Dothideomycetes*, order: *Dothideales*) (Schoch *et al.*, 2006) and until recently comprised four varieties: *A. pullulans* var. *pullulans*, var. *melanogenum*, var. *subglaciale* and var. *namibiae* (Zalar *et al.*, 2008). In 2014 the genomes of these four varieties were sequenced, consequently leading to the redefinition as separate species: *A. pullulans, A. melanogenum, A. subglaciale and A. namibiae* (Fig. 1), with *A. pullulans* being the most widely known and studied one (Gostinčar *et al.*, 2014).

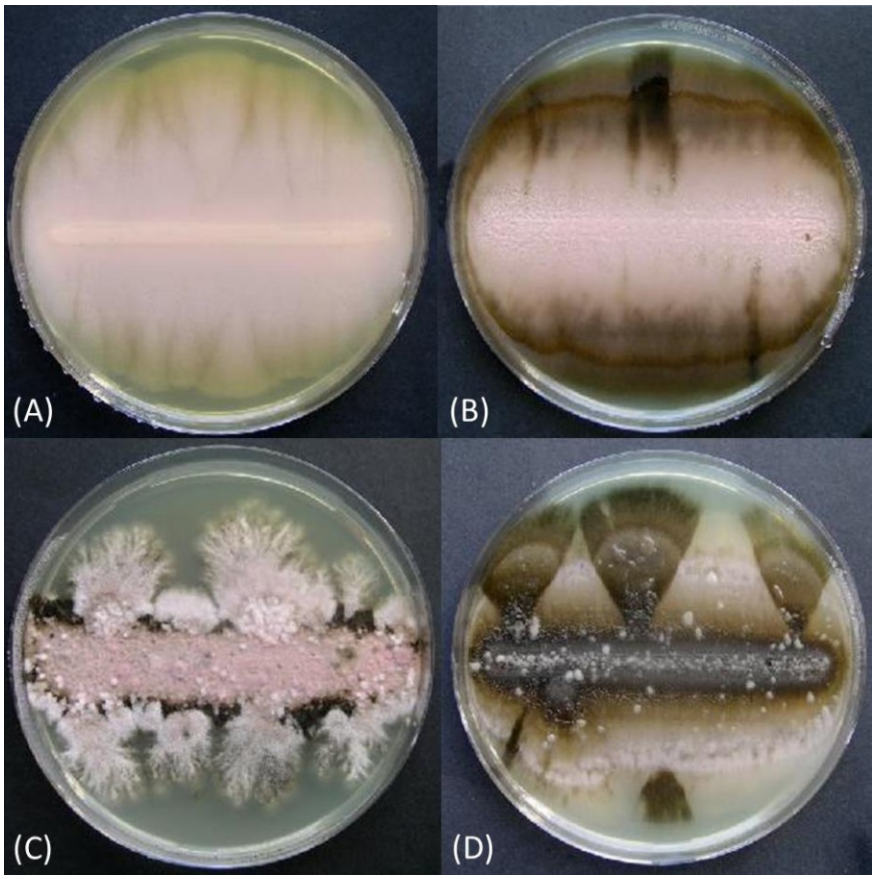

Fig. 1 *One month old cultures of A. pullulans (A), A. melanogenum (B), A. subglaciale (C) and A. namibiae (D) on malt extract agar (Gostinčar et al., 2014).*

These fungi exhibit extreme morphological and physiological variability (Cooke, 1959). They can form yeast-like cells, hyphae, chlamydospores and swollen cells (Fig. 2), depending on the environmental conditions (Cooke, 1959; Lingappa *et al.*, 1963; Ramos and García Acha, 1975; Pechak and Crang, 1977; Sevilla *et al.*, 1977; Bermejo *et al.*, 1981). This phenotypic plasticity together with melanisation, biofilm formation and production of extracellular polysaccharides and other bioproducts are hypothesized to allow *Aureobasidium* spp. to colonize diverse environments all around the world (Onofri *et al.*, 2007; Slepecky and Starmer, 2009; Gostinčar *et al.*, 2011). Habitats range from plant

leaves, feed and fruits (Cooke, 1959; Zalar *et al.*, 2008; Gostinčar *et al.*, 2019; Xiao *et al.*, 2019) to ecological niches like glass (Schabereiter-Gurtner *et al.*, 2001), dishwashers, (Zalar *et al.*, 2011), glacial ice (Zalar *et al.*, 2008; Branda *et al.*, 2010; de Garcia *et al.*, 2012), hypersaline saltern waters (Gunde-Cimerman *et al.*, 2000; Zalar *et al.*, 2008) and radiation-contaminated sites (Zhdanova *et al.*, 2000; Zalar *et al.*, 2008).

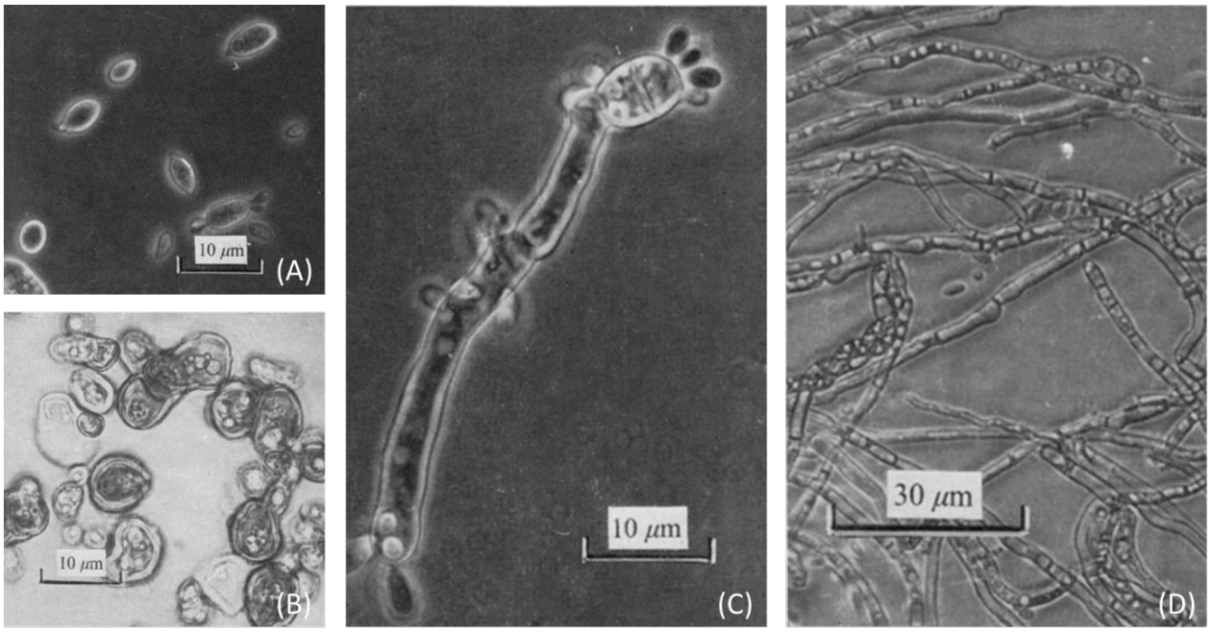

Fig. 2 *Microscopic images of Aureobasidium yeast-like cells (A), chlamydospores (B), a septate swollen cell giving rise to mycelium and yeast-like cells (C) and mycelium (D) (Ramos and García Acha, 1975).*

A. pullulans strains produce of a variety of different enzymes, metabolites, polysaccharides, and proteins of biotechnological significance. Most prominently *A. pullulans* produces the non-toxic and water-soluble exopolysaccharide (EPS) pullulan (Bernier, 1958; Bender *et al.*, 1959; Leathers, 2003). Pullulan is primarily composed of α-(1→6) linked maltotriose units (Fig. 3) and is used to produce adhesives, food additives and films and fibres that resemble petroleum-derived polymers (Leathers, 2003). Some *A. pullulans* isolates also act as biocontrol agents or produce high levels of industrial relevant enzymes, antimicrobial compounds, heavy oils, siderophores, poly(β-L-malic acid) or the EPS aubasidan (Chi *et al.*, 2009; Prasongsuk *et al.*, 2018). Due to its polyextremotolerant character *A. pullulans* isolates are potential sources of extremotolerant enzymes (Niehaus *et al.*, 1999).

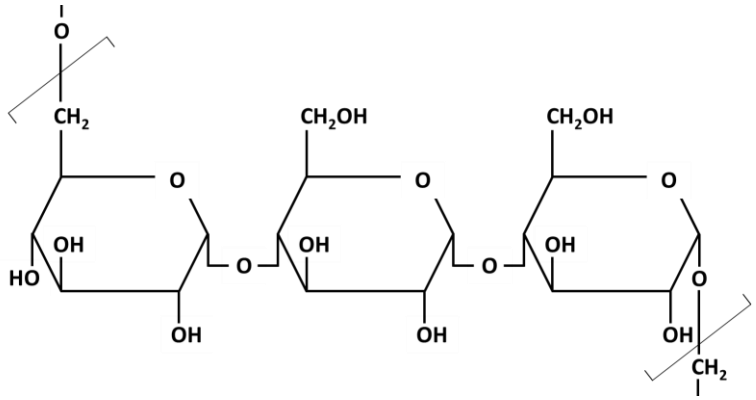

Fig. 3 Structure of the maltotriose unit in pullulan.

The great industrial potential of *A. pullulans* and its bioproducts call for an efficient genome editing strategy in order to insert genetic elements and understand pathways and regulatory regimes, allowing for the knowledge-based strain design of *A. pullulans* for industrial applications. In the last years, the engineered CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated) system from *Streptococcus pyogenes* has become a widely adopted tool for genome editing due to its modification efficiency, versatility, multiplexable targeting and ease of target programming. (Doudna and Charpentier, 2014; Sternberg and Doudna, 2015).

CRISPR loci can be found in the genomes of most bacteria and archaea and consist of a variable number of short repeats separated by variable spacer sequences. Together with the adjacent CRISPR-associated (*cas*) genes they form CRISPR/Cas systems that provide bacteria and archaea with adaptive immunity against invading genetic elements. CRISPR/Cas mediated immunity comprises three distinct stages: (1) adaptation or acquisition, (2) expression and processing of CRISPR and (3) interference (Fig. 4). During the adaptation stage, a short fragment of DNA (protospacer) from an invading phage or plasmid is incorporated in the CRISPR locus as a new spacer. The selection of a protospacer is usually determined by the presence of a protospacer adjacent motif (PAM). PAMs are mostly two to five nucleotides long and the sequence depends on the CRISPR/Cas subtype. The mechanism of spacer acquisition is poorly understood, but the most highly conserved Cas proteins, Cas1 and Cas2, are suspected to play central roles in the acquisition of new spacers. In the second stage the CRISPR locus is transcribed, generating long precursor CRISPR RNAs (pre-crRNAs) that are subsequently processed to mature crRNAs, each containing a single spacer. In the last stage the mature crRNA associates with one or more Cas proteins to form a ribonucleoprotein effector complex. The crRNA programs the complex to destroy any sequence that is complementary to the spacer and flanked by the PAM. Based on the presence of signature *cas* genes CRISPR/Cas systems can be classified into six types, which are additionally grouped into two classes based on the number of Cas subunits that form the effector complex. The CRISPR/Cas9 system only requires the Cas9 protein, along with a crRNA and a trans-activating crRNA (tracrRNA), to target and cleave complementary target-DNA sequences, if they are adjacent to the PAM (Fig. 4). The nuclease domains of Cas9 cleave both strands of the target, thus creating double-strand breaks

(DSBs) (Sorek *et al.*, 2008; van der Oost *et al.*, 2009; Wiedenheft *et al.*, 2012; Barrangou and Marraffini, 2014; Chylinski *et al.*, 2014; van der Oost *et al.*, 2014; Wright *et al.*, 2016; Makarova *et al.*, 2020).

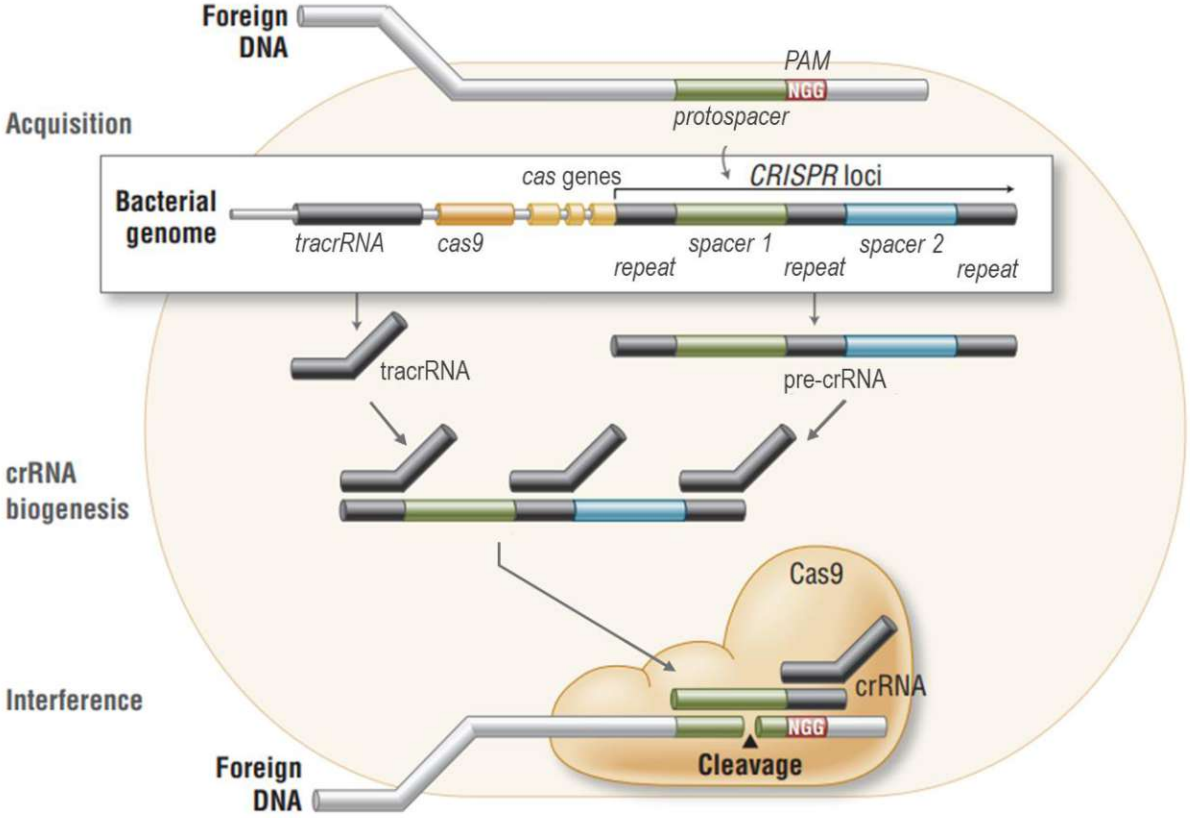

Fig. 4 *Stages of adaptive immunity mediated by type II CRISPR/Cas system from Streptococcus pyogenes. In the acquisition stage a short fragment (protospacer) from a foreign DNA molecule that is flanked by the PAM 5'-NGG-3' is incorporated in the CRISPR locus as a new spacer (green). crRNA biogenesis involves transcription of the CRISPR locus and procession into mature crRNAs. In the interference stage a crRNA:tracrRNA duplex guides Cas9 to the PAM-flanked target sequence that is subsequently cleaved by Cas9. Picture modified from NEB Expressions, Issue I 2014.*

DNA double-strand breaks trigger cellular repair mechanisms that can be utilised for targeted genome editing (Doudna and Charpentier, 2014; Sander and Joung, 2014) (Fig. 5). The non-homologous end joining (NHEJ) repair pathway ligates the ends of a DSB, often resulting in random insertion/deletion mutations (indels) at the site of junction that can abolish gene function. Precise edits can be achieved in the presence of an exogenous DNA template (donor or repair DNA) containing a sequence of interest with homologous flanks via the homology directed repair (HDR) pathway (Hsu *et al.*, 2014; Sander and Joung, 2014). The CRISPR/Cas9 system from *S. pyogenes* has been adapted for generating sequence specific DSBs in DNA targets. Cas9 is programmed using a single chimeric RNA molecule (single guide RNA, sgRNA) that combines tracrRNA and crRNA features. The first 20 nucleotides of the sgRNA define the target sequence. Since Cas9 from *S. pyogenes* requires the PAM 5'-NGG-3' any $(N)_{20}$-NGG sequence can be targeted by the CRISPR system for genome editing (Jinek *et al.*, 2012; Sander and Joung, 2014; Sternberg and Doudna, 2015). Delivery of CRISPR/Cas9 components into cells can be performed via ribonucleoproteins (RNPs), DNA or mRNA/RNA (Yip, 2020). Delivery of CRISPR/Cas9 via RNPs is a straight-forward technique that does not require construction of plasmids and does not

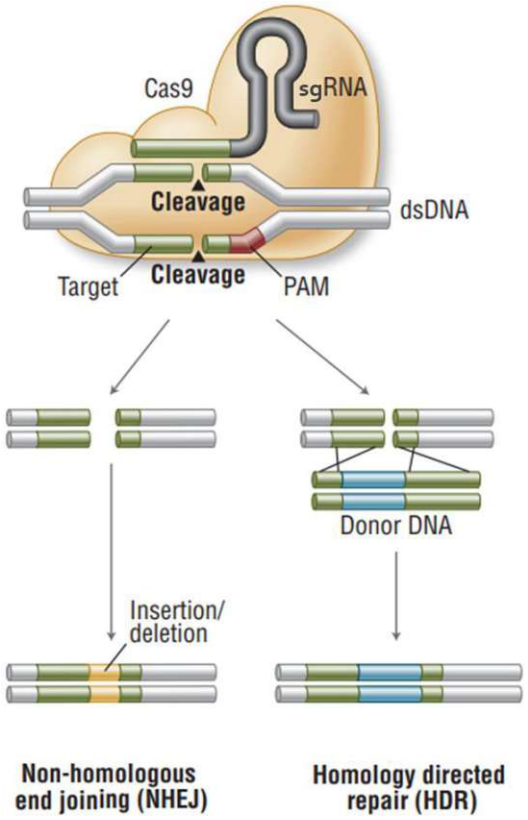

Fig. 5 *Genome editing with CRISPR/Cas9 via NHEJ or HDR. Picture modified from NEB Expressions, Issue I 2014.*

depend on in vivo transcription and translation. Furthermore, RNPs do not pose a risk of insertional mutagenesis. RNPs are assembled in vitro and can be delivered into cells using standard transformation protocols. They cleave the target sequence almost immediately after delivery into cells and are degraded rapidly, reducing off-target effects (Kim *et al.*, 2014; Yip, 2020).

Aims

The main aim of this work was to edit the genome of *Aureobasidium pullulans* (previously known as *Aureobasidium pullulans* var. *pullulans*) with Cas9-sgRNA RNPs. First, a transformation protocol for *A. pullulans* had to be established. For proof of concept of CRISPR/Cas9 mediated genome editing in *A. pullulans* with delivery of Cas9 and sgRNA as RNPs, the *ura3* gene (encoding for orotidine-5'-phosphate decarboxylase) was to be targeted. This gene can be used as a counter selectable selection marker and therefore was to be disrupted using Cas9 RNPs, exploiting the NHEJ repair pathway. Subsequently other genes were to be targeted. Since genes aside from *ura3* cannot be used for negative selection, the *ura3* gene was to be co-targeted along with the gene of interest in a multiplexing approach. Lastly, the HDR pathway was to be exploited to test if RNPs increase homologous recombination events in *A. pullulans* and to identify the optimal length of homologous flanks for high recombination rates.

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PART 1

PEG-mediated protoplast transformation of *Aureobasidium pullulans*

Summary

A PEG-mediated protoplast transformation protocol was developed for *Aureobasidium pullulans*. To test the protocol, the *A. pullulans* strains EXF-150 (reference strain), NBB 7.2.1 and ATCC 42023 were transformed to hygromycin resistance using plasmids pAN7-1 and pRLM$_{EX}$30 that contain the bacterial hygromycin B phosphotransferase gene (*hph*) flanked by promoter and terminator elements of *Aspergillus nidulans* and *Trichoderma reesei*, respectively. For the reference strain Southern hybridization of transformants revealed ectopic integration of plasmid pRLM$_{EX}$30 and presence of pAN7-1 extrachromosomally.

Introduction

Aureobasidium pullulans is a ubiquitous, polymorphic yeast-like fungus known for the production of pullulan and other bioproducts of biotechnological significance (Cooke, 1959; Chi *et al.*, 2009). The main research focus for *A. pullulans* lies in process and strain optimization and there is only little research on molecular biology. Therefore, a transformation protocol for *A. pullulans* was generated by modifying and optimizing the protocol for PEG-mediated protoplast transformation of filamentous fungi to allow for molecular biological research. The protocol was tested with two different plasmids carrying the *E. coli* hygromycin B phosphotransferase (*hph*) gene for hygromycin B resistance (HmB^R), $pRLM_{EX}30$ and pAN7-1. pAN7-1 contains the *hph* gene flanked by *gpdA* promoter elements (glyceraldehyde-3-phosphate dehydrogenase encoding gene) and terminator elements of the *trpC* gene (encoding for a multifunctional tryptophan biosynthesis) of *Aspergillus nidulans* (Punt *et al.*, 1987). $pRLM_{EX}30$ carries the hygromycin resistance gene flanked by promoter elements of the *pki1* gene (encoding for pyruvate kinase) and terminator elements of the *cbh2* gene (encoding for cellobiohydrolase II) of *Trichoderma reesei* (Mach *et al.*, 1994). Cullen et al. transformed *A. pullulans* strain Y117 to hygromycin resistance at low frequency (1 transformant per µg plasmid DNA) with plasmid pDH33, containing *hph* fused to the promoter from the glucoamylase gene (*glaA*) of *Aspergillus niger* (Cullen *et al.*, 1991). Thornewell et al. transformed *A. pullulans* strain R106 at higher frequency (4 transformants per µg plasmid DNA) with plasmids carrying the *hph* gene fused to the homologous promoter *TEF1p* from the gene encoding the *A. pulluIans* translation elongation factor 1-a (*TEF1*) (Thornewell *et al.*, 1995). Both used a similar PEG-mediated protoplast transformation protocol. In this study three different *A. pullulans* strains (EXF-150, ATCC 42023 and NBB 7.2.1) were transformed to hygromycin resistance with plasmids pAN7-1 and $pRLM_{EX}30$, respectively. Although strain ATCC 42023 was originally assigned to *A. pullulans* (Zajic and LeDuy, 1973), recent phylogenetic analyses suggest that it rather belongs to *A. pullulans* var. *melanogenum* / *A. melanogenum* (Zalar *et al.*, 2008; Rich *et al.*, 2016). For the *A. pullulans* reference strain EXF-150 six colonies were randomly selected and confirmed via PCR. Fluorescence Southern hybridization was carried out to investigate the fate of the transformed plasmids.

Results

The *E. coli* hygromycin B phosphotransferase gene *hph* was used as a selectable marker for the optimization of a transformation protocol for *A. pullulans* based on a fungal PEG-mediated protoplast transformation protocol. Colonies appeared after five to seven days of incubation at 24°C and the transformation efficiency was calculated as colony forming units (CFUs) per µg of transformed plasmid DNA. For the *A. pullulans* reference strain EXF-150 transformation of protoplasts yielded approx. 5 CFUs per µg pAN7-1 and pRLM$_{EX}$30, respectively (Table 1). Two other strains of *A. pullulans* (ATCC 42023 and NBB 7.2.1) were transformed to hygromycin resistance with lower efficiency. Addition of β-mercaptoethanol to protoplasts as described by Cullen et al. (Cullen *et al.*, 1991) lead to a slight increase in transformation efficiency. Transformation was also successful with frozen protoplasts.

Table 1 Transformation efficiency of three different *A. pullulans* strains.

	pAN7-1	**pRLM$_{EX}$30**
EXF-150	**5.4 CFUs/µg plasmid DNA*** *frozen protoplasts* **8.6 CFUs/µg plasmid DNA** *frozen protoplasts, 1% β-mercaptoethanol*	**5.2 CFUs/µg plasmid DNA*** *fresh protoplast* **5 CFUs/µg plasmid DNA** *frozen protoplasts*
NBB 7.2.1	1.6 CFUs/ µg plasmid DNA *frozen protoplasts*	1 CFU/5 µg plasmid DNA *frozen protoplasts* 2 CFUs/5 µg plasmid DNA *frozen protoplasts, 1% β-mercaptoethanol*
ATCC 42023	4.2 CFUs/µg plasmid DNA *frozen protoplasts*	3.4 CFUs/µg plasmid DNA *fresh protoplasts*

** six colonies were picked for PCR and Southern hybridization*

Six HmB^R colonies obtained after the transformation of *A. pullulans* EXF-150 with pAN7-1 and pRLM$_{EX}$-30, respectively, were randomly picked (Fig. 1A) and uptake of the corresponding plasmid was confirmed via diagnostic PCR (Fig. 1B and C). For pRLM$_{EX}$-30 candidate #4 the second fragment in the PCR analysis is missing, but growth on media containing hygromycin B was still possible. This could be explained with the design of the used primers; pRLMex2_fwd and pRLMex2_rev bind downstream of *hph,* and part of this segment was probably lost during transformation.

To determine mode of plasmid transformation and copy number of plasmids in *A. pullulans* EXF-150 HmB^R transformants, fluorescence Southern-blot analyses were carried out with undigested and EcoRI-digested DNA. Digested and undigested DNA were probed with a biotinylated 399 bp-long fragment of *hph*. For pAN7-1 the hybridization signals of undigested DNA from the transformants indicate that the transforming DNA did not integrate in the chromosomal DNA and is present extrachromosomally (Fig. 2A). EcoRI cuts within pAN7-1 twice, generating two fragments with a size of about 2500 bp and 4200 bp (contains *hph*), respectivley. For transformants #1, 2, 3 and 4 digestion with EcoRI (Fig. 2B) shows hybridization signals at around 4.5 kb, presumably corresponding to the 4200 bp-fragment. For transformants #2, 5 and 6 signals at lower molecular weight were obtained that might be explained by the loss of nucleotides and rearrangements. Transformant #2 shows two bands, suggesting the presence

of two copies of the *hph* gene. For *A. pullulans* EXF-150 wildtype no hybridization signals were obtained. For pRLM$_{EX}$-30 the high-molecular-weight bands of undigested DNA from the transformants #1, 2, 3, 5, and 6 indicate ectopic integration of the vector (Fig. 2C). Digestion with EcoRI (Fig. 2D) resulted in hybridization signals of the same size in the transformants #1, 2, 3, 5, and 6, suggesting integration at the same genomic location. For transformant #4 no hybridization signal was obtained despite its ability to grow on media containing hygromycin B. This suggests that hygromycin B resistance resulted from a random mutation rather than the uptake of the plasmid. As expected no hybridization signal was obtained for *A. pullulans* EXF-150 wildtype.

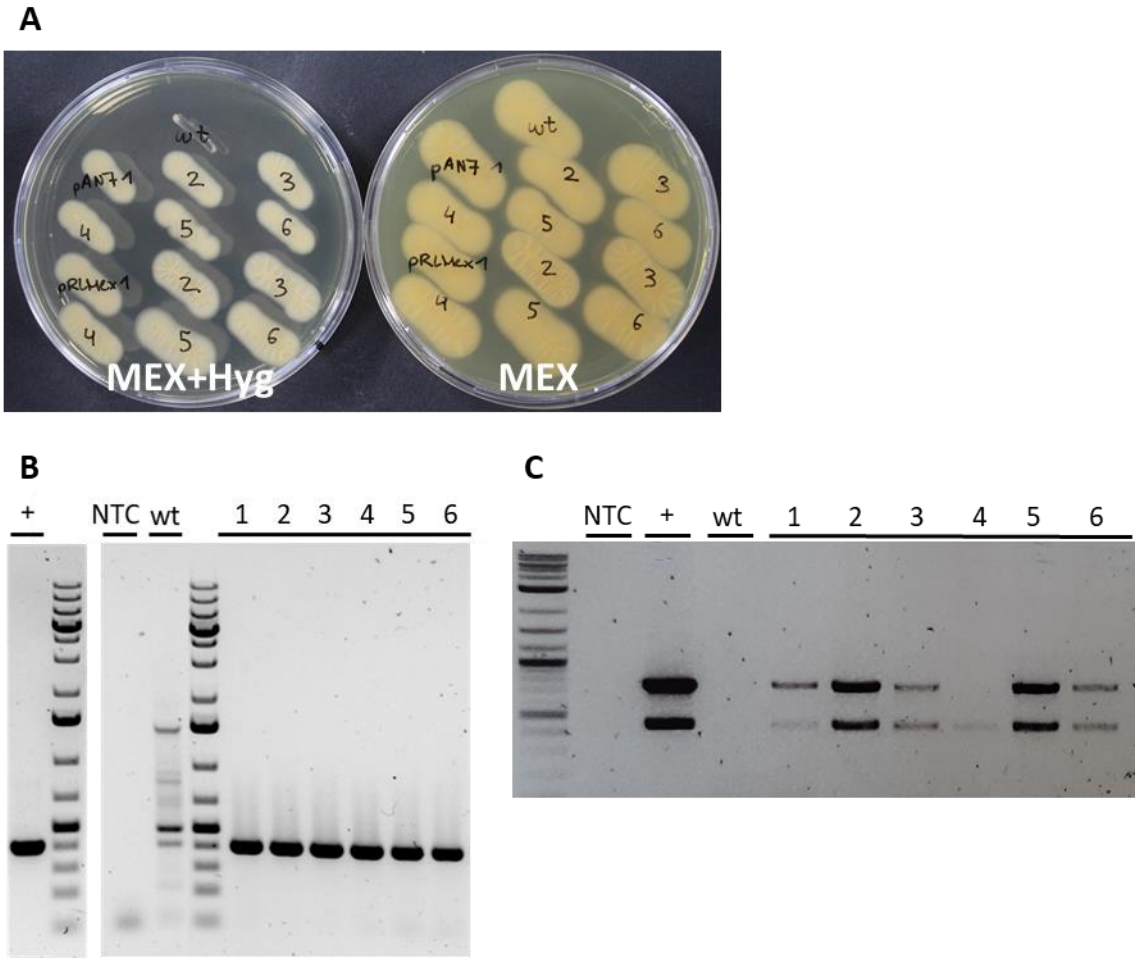

Fig. 1 *Transformation of A. pullulans EXF-150 to hygromycin resistance.*

A *Hygromycin B resistance of candidates (pAN7 #1-6, pRLM$_{EX}$ #1-6) and sensitivity of wildtype (left, malt extract agar supplemented with 113 U/ml hygromycin, MEX+Hyg). Right shows the control plate (MEX). Photos taken after 3 days incubation at 24°C.*

B *Agarose gel electrophoresis of the fragments obtained by PCR with primers pAN7_fwd and pAN7_rev using DNA of the parent strain (EXF-150 wildtype, wt) and six candidates (#1-6) obtained after the transformation with pAN7-1 as template. NTC, no template control; +, positive control pAN7-1.*
The wildtype shows many unspecific bands.

C *Agarose gel electrophoresis of the fragments obtained by PCR with primers pRLMex1_fwd and pRLMex1_rev, pRLMex2_fwd and pRLMex2_rev using chromosomal DNA of the parent strain (EXF-150 wildtype, wt) and six candidates (#1-6) obtained after the transformation with pAN7-1 as template. NTC, no template control; +, positive control pRLM$_{EX}$30. For transformant #4 the bigger fragment is missing.*

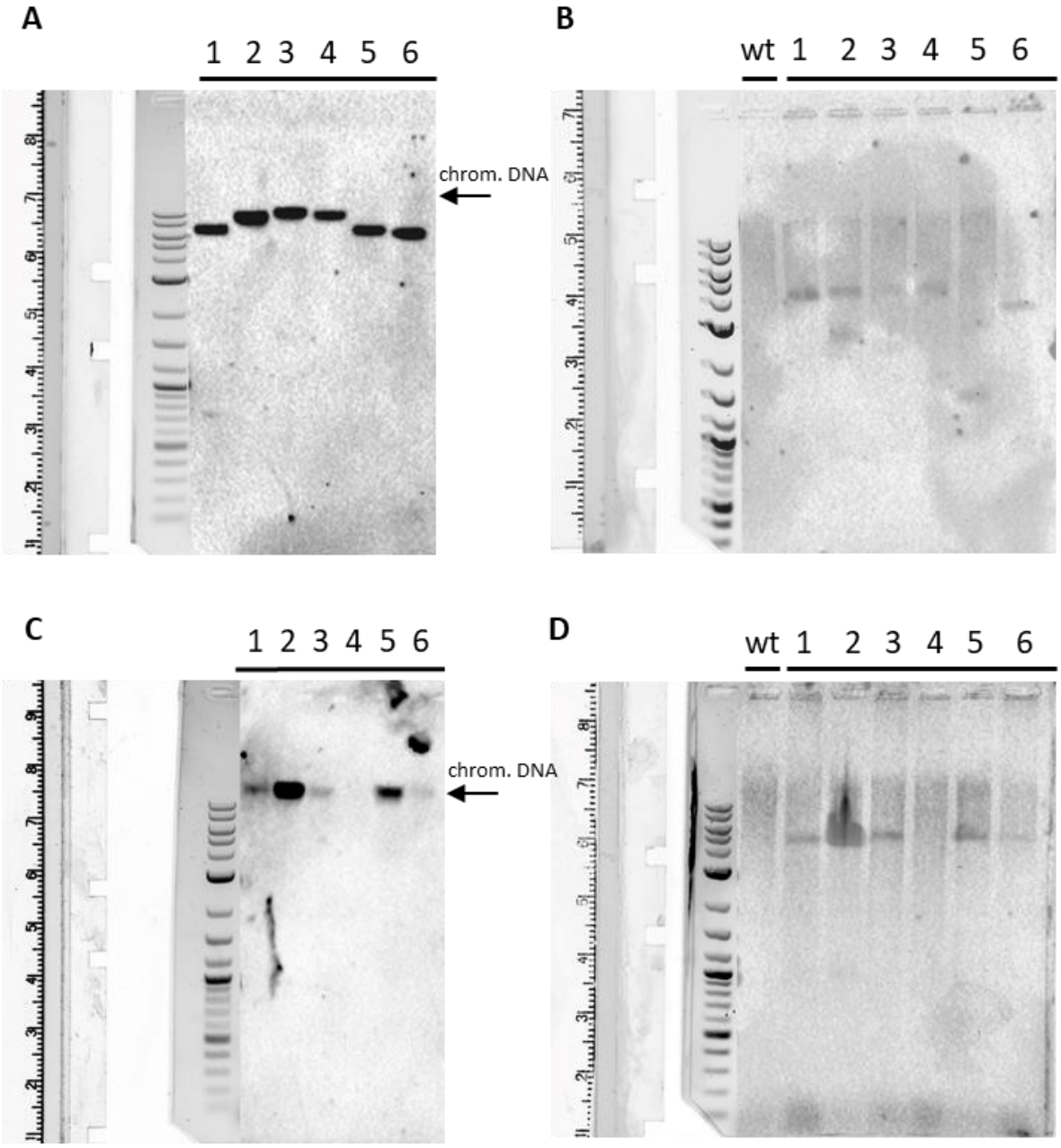

Fig. 2 *Southern blot analyses of A. pullulans EXF-150 HmB^R transformants.*

A *Southern blot analysis of undigested DNA from pAN7-1 transformants (#1-6) indicates presence of transforming DNA outside of the chromosomal DNA. The position of the undigested, high-molecular-weight chromosomal DNA (chrom. DNA) is indicated by a black arrow.*

B *Southern blot analysis of EcoRI-digested genomic DNA from pAN7-1 transformants (#1-6) and A. pullulans EXF-150 wildtype (wt). As expected, the wildtype shows no hybridization signal. Hybridization signals of transformants #1, 2, 3 and 4 at approximately 4,5 kb. Hybridization signals of transformants #2, 5 and 6 at lower molecular weights (#2: ca. 2,8 kb, #5: ca. 1,5 kb, #6: ca. 4 kb).*

C *Southern blot analysis of undigested DNA from pRLM$_{EX}$-30 transformants (#1-6) indicates ectopic integration of transforming plasmid DNA. The position of the undigested, high-molecular-weight chromosomal DNA (chrom. DNA) is indicated by a black arrow.*

D *Southern blot analysis of EcoRI-digested genomic DNA from pRLM$_{EX}$-30 transformants (#1-6) and A. pullulans EXF-150 wildtype (wt). As expected the wildtype shows no hybridization signal. Hybridization signals of transformants #1, 2, 3, 5 and 6 indicate at the same genomic location. Transformant #4 showed no hybridization signal despite its ability to grow on media containing hygromycin B.*

Discussion

A transformation protocol for *A. pullulans* was successfully developed by modifying and optimizing the protocol for PEG-mediated protoplast transformation of filamentous fungi. For the *A. pullulans* reference strain transformation of protoplasts yielded 5.4 CFUs per µg pAN7-1 and 5.2 CFUs per µg $pRLM_{EX}30$. This is comparable to the transformation frequency of 4 transformants per µg plasmid DNA reported by Thornewell et al. (Thornewell *et al.*, 1995). However, they used plasmids where the *hph* gene was fused to a promoter from a highly expressed gene in *A. pullulans*. The plasmids used in present study instead carry the *hph* gene flanked by promoter and terminator elements of *T. reesei* ($pRLM_{EX}30$) and *A. nidulans* (pAN7-1), respectively (Punt *et al.*, 1987; Mach *et al.*, 1994). Cullen et al. transformed *A. pullulans* to hygromycin resistance with a plasmid containing *hph* fused to a heterologous promoter as well, but at lower frequency (1 transformant per µg plasmid DNA). However, it is important to keep in mind that they worked with different plasmids and different *A. pullulans* strains, possibly explaining the differences in transformations efficiency. Positive transformants not only proof that the transformation protocol works but also that these heterologous regulatory elements are recognized by *A. pullulans*. Strains ATCC 42023 and NBB 7.2.1 were transformed to hygromycin resistance with lower efficiency. One possible explanation for this is that the heterologous regulatory sequences of pAN7-1 and $pRLM_{EX}30$ do not work as well as in strain EXF-150. Furthermore, strain ATCC 42023 is phylogenetically more similar to *A. melanogenum* and might need to be reassigned. It was observed for a strong and early melanin and pullulan production that might impair protoplast formation and DNA uptake. Addition of β-mercaptoethanol to protoplasts lead to a slight increase in transformation efficiency, possibly by destabilizing the cell membrane. Freezing of protoplasts did not negatively impact transformation efficiency, reducing time and effort for experiments considerably.

Southern blot hybridization analysis revealed ectopic integration of $pRLM_{EX}30$. Digestion with EcoRI resulted in hybridization signals of the same size in transformants, suggesting integration at the same genomic location. pAN7-1 did not integrate into the chromosomal DNA. However, hygromycin resistance was maintained despite cell division and proliferation, indicating the presence of a sequence that is recognized by *A. pullulans* as an ARS element (autonomously replicating sequence). Digestion of pAN7-1 with EcoRI generates two fragments with a size of about 2500 bp and 4200 bp (contains *hph*), respectivley. For four transformants hybridization signals at around 4.5 kb, presumably corresponding to the 4200 bp-fragment, were obtained. This implies the presence of the original plasmid in these transformants. For the three other transformants signals at lower molecular weight were obtained that might be explained by the loss of nucleotides and rearrangements in transforming DNA. Non-integrating DNA does not pose a risk of insertional mutagenesis and is easily transformed into and removed from cells, creating new approaches for molecular research in *A. pullulans*.

Experimental Procedures

Strains and cultivation conditions

A. pullulans strains EXF-150 (CBS 100280 (Gostinčar *et al.*, 2014)), ATCC 42023 (Zajic and LeDuy, 1973) and NBB 7.2.1 (CCOS1008, (Hilber-Bodmer *et al.*, 2017)) were maintained on malt extract (MEX) agar at 24°C. For liquid cultures, *A. pullulans* was grown in MEX medium at 24°C either in a volume of 20 mL in 100-mL Erlenmeyer flasks or 50 mL in 250-mL Erlenmeyer flasks on a rotary shaker at 220 rpm. If applicable, hygromycin B was added to a final concentration of 113 U ml^{-1}.

PEG-mediated protoplast transformation

For the generation of protoplasts 10 ml of an overnight liquid culture with an OD_{600} of approx. 1 was centrifuged at 6000 g for 5 min. The cell pellet was washed with 20 ml buffer A (100 mM KH_2PO_4, 1.2 M sorbit, pH 5.6) and resuspended in sterile-filtered lysing solution (15 ml buffer A containing 150 mg lysing enzymes from *T. harzianum* (Sigma-Aldrich, St. Louis, MO, USA, L1412) and 150 mg β-glucanase from T. *longibrachiatum* (Sigma-Aldrich, G4423)). This suspension was incubated at 24°C on a rotary shaker at 140 rpm until protoplasts formed (approx. 1 h). Protoplasts were recovered by the addition of 25 ml ice-cold 1.2 M sorbit and subsequent centrifugation at 4°C and 3000 g for 10 min. Protoplasts were washed once with 30 ml 1.2 M ice-cold sorbit and twice with 10 ml ice-cold buffer B (1 M sorbitol, 25 mM $CaCl_2$, 10 mM Tris.Cl pH 7.5). Finally, the protoplasts were resuspended in 1 ml ice-cold buffer B (volume adjusted to the OD_{600} of the overnight culture). For storage at -80°C, 1 ml of the protoplast suspension was mixed with 1 ml "20% PEG solution" (20% (w/v) PEG 4000, 0.67 M sorbit, 20 mM $CaCl_2$, 10 mM Tris.Cl pH 7.5) and 20 µl dimethyl sulfoxide. Aliquots of 200 µl were stored at -80°C. For transformation 100 µl of fresh protoplast suspension was mixed with 150 µl buffer B, 100 µl "20% PEG solution" and 2 µl β-mercaptoethanol. Alternatively, an aliquot of frozen protoplasts was thawed on ice and mixed with 150 µl buffer B and 2 µl β-mercaptoethanol. Typically 5 µg of undigested plasmid DNA was used for the transformation. The transformation mixture was incubated on ice for 30 minutes followed by a stepwise addition of 50, 200 and 500 µl of "60% PEG solution" (60% (w/v) PEG 4000, 10 mM $CaCl_2$, 10 mM Tris.Cl pH 7.5). After incubation for 20 minutes at room temperature buffer C (1 M sorbit, 10 mM Tris.Cl pH 7.5) was added stepwise (200, 400, 1000 and 2500 µl) to the mixture. Subsequently, 100, 300, 1000 and 3800 µl of the transformation reaction each was added to 20 mL of melted, 50°C warm selection medium, containing 1 M sucrose. These mixtures were poured into sterile petri dishes. The plates were incubated at 24°C for 5 to 7 days until colonies were visible.

PCR screening

For the extraction of DNA the colony PCR protocol described by Wu et al. (Wu *et al.*, 2017) was used: Cells from single colonies were picked up with a tooth pick and resuspended in 50 µl 20 mM NaOH, followed by 3 cycles of 99°C – 1 min / 4°C – 1 min in a thermocycler. Lastly, the cells were spun down on a table-top centrifuge. For diagnostic PCR, 2µL of the resulting crude DNA extract was used as the template in a 50-µl PCR with the OneTaq DNA polymerase (New England Biolabs, Inc., Ipswich, MA, USA) according to the manufacturer's instructions. For subsequent agarose gel electrophoresis of the DNA fragments, a GeneRuler 1-kb Plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) was applied to estimate the fragment size.

Southern hybridization analysis

For DNA extraction for Southern hybridization analysis a 50 ml overnight liquid culture was prepared. Cells were harvested by centrifugation at 4°C and 6000 g for 5 min. The pellet was washed with water once, followed by a final centrifugation step at 4°C and 9000 g for 5 minutes. DNA was isolated from biomass by grinding in liquid nitrogen, followed by a phenol-chloroform extraction. 0.1 g of biomass powder was mixed with 800 µl DNA extraction buffer (0.1 M Tris.Cl pH 8.0, 1.2 M NaCl, 5 mM EDTA). Subsequently, 800 µl of a phenol-chloroform-isoamyl alcohol mixture (25:24:1) was added, mixing until both phases blended to a homogenous emulsion. After incubation at room temperature for 10 minutes, the mixture was centrifuged at 4°C and 15 000 g for 10 minutes. The aqueous top phase was transferred to a new reaction tube and mixed with 1 ml chloroform until both phases blended to a homogenous emulsion. After centrifugation at 4°C and 15 000 g for 10 minutes, the top aqueous phase was again transferred to a new reaction tube. RNA was degraded by adding RNase A (Thermo Fisher Scientific) to a final concentration of 40 µg ml^{-1} and incubating at 37°C for 1 hour. A 0.6 volume of isopropanol was added and the tube was inverted until DNA precipitated. After centrifugation at 4°C and 20 000 g for 5 minutes, the DNA pellet was washed with 1 ml 70% ethanol and dissolved in 100 µl to 1 ml double-distilled water (ddH_2O), depending on pellet size, overnight at 4°C.

Aliquots (25 µg) of DNA were digested with 75 U of the indicated restriction enzyme overnight. The mixture was incubated at 70°C for 20 minutes to deactivate the enzyme and allow for better separation of DNA. After concentrating the reaction to a final volume of approx. 30 µl, the resulting DNA fragments were separated by electrophoresis (low voltage) on an 1% agarose gel. For analysis of undigested DNA, 7 µg of extracted DNA was directly loaded onto an 0.8% agarose gel for electrophoresis. For estimation of molecular weight a GeneRuler 1-kb Plus DNA ladder (New England Biolabs) was used. After electrophoresis, DNA was denatured by incubating the gel in 0.4 M NaOH, 0.6 M NaCl for 30 minutes followed by incubating in 0.5 M Tris-Cl pH 7.5, 1.5 M NaCl for 30 minutes. For undigested DNA, the gel was incubated in 0.25 M HCl for 10 minutes and washed with ddH_2O prior to denaturing. Subsequently, DNA was transferred onto a Biodyne B 0.45-µm-pore-size nylon membrane (Pall Corporation, Port Washington, NY) using 10x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.2) overnight. After marking the position of the gel on the membrane, the membrane was incubated

in 0.4 M NaOH and 0.2 M Tris.Cl pH 7.5 for 1 minute each. The membrane was put on a damp (0.2 M Tris.Cl pH 7.5) Whatman filter paper and DNA was cross-linked onto the membrane via UV light in the Bio-Rad GS Gene Linker (Bio-Rad Laboratories, Hercules, CA), using program C3. Blocking was performed by incubating the membrane in 20 ml Southern blot hybridization buffer (60 ml ddH_2O, 25 ml 20x SSC, 10 ml 50x Denhardt's Solution (10 g l^{-1} Ficoll 400, 10 g l^{-1} polyvinylpyrrolidone, 10 g l^{-1} BSA), 5 ml 1 M NaH_2PO_4, 1 ml 10% SDS, 200 µl 0.5 M EDTA pH 8.0, 0.5 g BSA) containing 100 µg ml^{-1} freshly denatured (95°C, 10 minutes) sheared, single stranded salmon sperm DNA (ssssDNA) at 60°C, rolling for 3 hours. The membrane was hybridized in 15 ml Southern blot hybridization buffer containing 100 µg ml^{-1} freshly denatured ssssDNA and 1 µg freshly denatured probe at 65°C, rolling overnight. Generation of the probe was performed via 50-µl PCR containing 25 µl Biotin PCR labelling mix (0.1 mM of dATP, dCTP and dGTP, 0.05 mM of dTTP and Biotin-dUTP), 10 µl 5x OneTaq buffer (New England Biolabs), 2 µl pAN7_fwd (10 µM), 2 µl pAN7_rev (10 µM), 2 ng plasmid DNA and 0.5 µl OneTaq DNA polymerase (New England Biolabs). The normal PCR program was used, but with longer elongation time (2 min/kb) and 35 cycles. The PCR product was checked on a gel and cleaned with the GeneJet PCR Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions (elute 2 times with 25 µl). After hybridization the membrane was washed twice in 50 ml 2x SSC, 0.1% SDS at room temperature for 5 minutes and twice in 50 ml 0.1x SSC, 0.1% SDS at 65°C for 15 minutes. Subsequently, the membrane was incubated in 50 ml Southern blot blocking solution (125 mM NaCl, 17 mM Na_2HPO_4, 8 mM NaH_2PO_4, 0.5% (v/v) SDS, pH 7.2) at room temperature for 10 minutes, followed by light-proof incubation with Pierce Streptavidin Poly-HRP (Thermo Fisher Scientific) at room temperature for 30 minutes. The membrane was washed thrice in 50 ml 1:10 diluted Southern blot blocking solution at room temperature and under light-proof conditions for 10 minutes each. Finally, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) under light-proof conditions for 30 minutes. Imaging was performed with the ChemiDoc MP imaging system (Bio-Rad Laboratories), using the SAM (signal accumulation mode) technique (5 pictures in 120 seconds, first picture after 20 seconds). For visualisation and subsequent interpretation, the graphics software GIMP was used to overlay the pictures of the Southern Blot membrane and gel.

Oligonucleotides

All oligonucleotides used in this study are listed in Table 2.

Table 2 Oligonucleotides.

Name	Sequence (5' – 3')
pAN7_fwd	GACCTGCCTGAAACCGAACTG
pAN7_rev	GAAGAAGATGTTGGCGACCTCG
pRLMex1_fwd	AGATAACGGTGAGACTAGCGGC
pRLMex1_rev	ATTAGGTGATGCTGCGCG
pRLMex2_fwd	GCTAGCTTTCGTGACCGGGC
pRLMex2_rev	ACTAGTGCTATTAACGTTTGGAAAGCCATC
Hybridization probe	GACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGA GGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGT TCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGT GATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACT GTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGA GCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGC ACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATA ACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATA CGAGGTCGCCAACATCTTCTTC

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PART 2

Fast and efficient CRISPR-mediated genome editing in *Aureobasidium pullulans* using Cas9 ribonucleoproteins

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Fast and efficient CRISPR-mediated genome editing in *Aureobasidium pullulans* using Cas9 ribonucleoproteins

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Fast and efficient CRISPR-mediated genome editing in *Aureobasidium pullulans* using Cas9 ribonucleoproteins

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Running Title: Genome Editing using RNPs in *A. pullulans*

Summary

Aureobasidium pullulans is a ubiquitous, polyextremotolerant, "yeast-like" ascomycete used for the industrial production of pullulan and other products and as biocontrol agent in the agriculture. Its application potential and its wide-spread occurrence make *A. pullulans* an interesting study object. The availability of a fast and efficient genome editing method is an obvious advantage for future basic and applied research on *A. pullulans*. In this study, we describe the development of a CRISPR/Cas9-based genome editing method using ribonucleoproteins (RNPs). We demonstrate that this method can be used for single and multiplex genome editing using only RNPs by targeting *ura3* (encoding for orotidine-5'-phosphate decarboxylase), *praics* (encoding for phosphoribosyl aminoimidazole-succinocarboxamide synthase) and *asl* (encoding for arginine succinate lyase). We demonstrate the applicability of *Trichoderma reesei pyr4* and *Aspergillus fumigatus pyrG* to complement the *ura3* deficiency. Further, we show that the usage of RNPs can boost the homologous recombination rate up to nearly 100%, even when using only 20bp long homologous flanks. Therefore, the repair cassettes can be constructed by a single PCR, abolishing the need for laborious and time-consuming cloning. The here presented method allows fast and efficient genome editing for gene deletions, modifications, and insertions in *A. pullulans*.

Introduction

Aureobasidium pullulans is a ubiquitous, black yeast-like ascomycete (*Dothideomycetes*, *Dothideales*), characterized by the production of melanin, phenotypic plasticity, polyextremotolerance and adaptability (Cooke, 1959; de Hoog, 1993; Schoch *et al.*, 2006; Gostinčar *et al.*, 2011). *A. pullulans* is used industrially for the production of pullulan (Bernier,

1958; Bender *et al.*, 1959; Leathers, 2003). Pullulan and its derivatives have a multitude of practical applications in the food, pharmaceutical, agricultural, and chemical industries (Leathers, 2003; Chi *et al.*, 2009). Further products of *A. pullulans* with potential industrial applications are other extracellular polysaccharides, enzymes, antimicrobial compounds, siderophores, heavy oils, poly(β-L-malic acid) (Chi *et al.*, 2009; Prasongsuk *et al.*, 2018). Further, *A. pullulans* can be used as a biocontrol agent in the agriculture sector (Sharma *et al.*, 2009). Based on the wide-spread occurrence and the application potential of *A. pullulans*, there is an obvious demand for an easy and efficient genome editing method.

The clustered regularly interspaced short palindromic repeat (CRISPR) system from *Streptococcus pyogenes* has been used for genome editing in various organisms due to the ease of target programming, modification efficiency, and multiplexing capacity (Doudna and Charpentier, 2014; Sternberg and Doudna, 2015). The modified system depends on a single multifunctional Cas protein (Cas9) and a single guide RNA (sgRNA) which programs Cas9 to introduce a double-strand break (DSB) in a 20 nt-target sequence upstream of a protospacer adjacent motif (PAM, 5'-NGG-3') (Jinek *et al.*, 2012; Sternberg and Doudna, 2015). Subsequent to the DSB, two main repair pathways i.e., the error-prone non-homologous end joining (NHEJ) and the homology directed repair (HDR) can be exploited for genome editing. The NHEJ repair pathway readily ligates DSBs but often causes insertion/deletion mutations at the target site that can lead to loss of gene function. The HDR pathway can be utilized to insert a defined sequence at the target site. A repair or donor DNA template must be provided to this end (Hsu *et al.*, 2014; Sander and Joung, 2014). There are different methods for delivery of Cas9 and sgRNA into cells available (Yip, 2020). DNA carrying the genes for Cas9 and sgRNA can be transformed. This is cost-effective but requires cloning steps and the plasmid DNA might be inserted at unwanted sites in the genome. Further, the prolonged expression of Cas9 increases the chance of off-target effects (Yip, 2020). Second, the mRNA for the Cas9 can be transformed together with the sgRNA. This minimizes the risk of unwanted integration and off-target effects

but is expensive. Third, ribonucleoproteins (RNPs) consisting of the Cas9 protein and the sgRNA can be assembled in vitro and inserted into the target cell. This is a fast and easy delivery technique of CRISPR components that does not require cloning or in vivo transcription and translation. RNPs enable immediate transient gene editing with reduced off-target effects (Kim *et al.*, 2014; Yip, 2020). Cas9-sgRNA RNPs have been shown to efficiently edit the genomes of human and animal cells (Cho *et al.*, 2013; Kim *et al.*, 2014; Chaverra-Rodriguez *et al.*, 2018; Chen *et al.*, 2019), plant cells (Park *et al.*, 2019; Lee *et al.*, 2020) and various fungi (Foster *et al.*, 2018; Zou *et al.*, 2020). In *A. pullulans*, CRISPR mediated genome editing was previously performed using plasmids (Zhang *et al.*, 2019) but RNPS have not yet been used.

In this study, we demonstrate that Cas9-sgRNA RNPs can be used for single and multiplex genome-editing of three *A. pullulans* strains (EXF-150, ATCC 42023 and NBB 7.2.1) by targeting the *ura3* (encoding for orotidine-5'-phosphate decarboxylase), *praics* (encoding for phosphoribosyl aminoimidazole-succinocarboxamide synthase) and *asl* (encoding for arginine succinate lyase) genes. Further, we complemented the uridine auxotrophy with *ura3* homologues from *Trichoderma reesei* and *Aspergillus fumigatus*. Lastly, we demonstrate that integration cassettes with flanks as short as 20 bp can be used for an HDR-mediated gene insertion with homologous integration rates of up to 100%.

Results and Discussion

CRISPR/Cas9 RNPs can be used for genome editing in *A. pullulans*

To test, whether Cas9 RNPs can be used in *A. pullulans*, we used the *ura3* gene as a target, because loss-of-function mutations in this gene results in a resistance against 5-fluoroorotic acid (5-FOA) (Rose *et al.*, 2000). We designed two sgRNAs (ura3_sgRNA1 and ura3_sgRNA2) targeting two sites in *ura3* (Fig. 1A). This strategy aimed to enhance the rate of loss-of-function, because the middle gene fragment is expected to get lost during the NHEJ repair. The initial RNP delivery experiments were conducted without sgRNA refolding or the

addition of β-mercaptoethanol. For the *A. pullulans* reference strain EXF-150, we obtained about 250 5-FOA resistant colonies after delivering approx. 0.084 nmol of Cas9 and sgRNA each (Fig. S1). To verify that the obtained *ura3* loss-of-function was indeed a result of the CRIPSR mediated DSBs, we sequenced the *ura3* locus of six random colonies. In four colonies (#1, 2, 4, and 5), we observed short deletions at the target site of ura3_sgRNA1 (Fig. 1B), which is a typical result of NHEJ repair mistakes after a DSB (Hsu *et al.*, 2014; Sander and Joung, 2014). In two colonies (#3 and 6), the 800bp-long fragment between the two target sites was deleted (Fig. 1C). Based on this outcome, we conclude that ura3_sgRNA1 is more effective than ura3_sgRNA2. This is in accordance with previous studies; choice of sgRNA affects efficiency and specificity of CRISPR/Cas9 genome editing (Chari, Mali, Moosburner, & Church, 2015; Doench et al., 2016; Doench et al., 2014; Wang, Wei, Sabatini, & Lander, 2014; Xu et al., 2015). However, neither of the six colonies could grow on medium lacking uridine (SC-URA) (Fig. 1D). The uridine auxotrophy could be complemented with the *ura3* homologues from *Trichoderma reesei* (*pyr4*) and *Aspergillus fumigatus* (*pyrG*) (Fig. S2). To this end, the auxotrophic mutant Δ*ura3* #6 was transformed with plasmids pJET-pyr4 (Derntl *et al.*, 2016) and pJET-pyrG, respectively.

Next, we tested the applicability of the RNPs in other *A. pullulans* strains. To this end, approx. 0.042 nmol of Cas9 and ura3_sgRNA1 each were delivered into the strains ATCC 42023 and NBB 7.2.1, yielding about 300 and two colonies, respectively (Fig. S3 and Fig. S4**Error! Reference source not found.**). Only ura3_sgRNA1 was used since it was more effective than ura3_sgRNA2 in EXF-150. Sequencing of the *ura3* locus of six random 5-FOA resistant ATCC 42023 colonies and the two 5-FOA resistant NBB 7.2.1 colonies confirmed deletion of nucleotides at the ura3_sgRNA1 target site (Fig. S5 and Fig. S6). Notably, ATCC 42023 was suggested to be *A. pullulans* var. *melanogenum* or *A. melanogenum* in recent studies (Zalar *et al.*, 2008; Rich *et al.*, 2016). Accordingly, we observed an enhanced melanin production in this strain compared to the strains EXF-150 and NBB 7.2.1, and a high sequence similarity of the

ura3 gene to *A. melanogenum* strain TN3-1 (Fig. S7). As the genome of ATCC 42023 is not sequenced, we used ura3_sgRNA1 at a venture and could obtain a relatively high number of colonies (Fig. S3) despite a mismatch in the sgRNA target site (Fig. S5). The low genome editing efficiency of *A. pullulans* NBB 7.2.1 (2 colonies, Fig. S4) could be improved by the addition of β-mercaptoethanol to the protoplasts (as described in (Cullen *et al.*, 1991) and by denaturing and refolding the sgRNA prior to the RNP assembly (as suggested in (Pohl *et al.*, 2018)). These modifications resulted in about 50 colonies for approx. 0.084 nmol Cas9 and sgRNA each (Fig. S8). Consequently, all following RNP delivery approaches were performed with sgRNA denaturing and refolding and β-mercaptoethanol addition.

Multiplex genome editing allows manipulation of not directly selectable genes

To test for the possibility of multiplex genome editing using Cas9 RNPs, we simultaneously targeted *ura3* and another gene; either the *praics* or the *asl* gene. Loss-of-function mutations in *praics* and *asl* cause adenine and arginine auxotrophy, respectively. For the co-delivery, the sgRNAs targeting *praics* or *asl* were mixed with the ura3_sgRNA1 in a ratio of 11:1 and delivered into *A. pullulans* EXF-150. We selected for *ura3* deficiency (5-FOA resistance) and then tested 24 randomly picked colonies for adenine and arginine auxotrophy, respectively. For *praics*, four out of 24 candidates were adenine auxotroph; they could not grow without adenine (Fig 2A), and turned red, due to the accumulation of the intermediate AIR (5'-phosphoribosyl-5-aminoimidazole) (Fig. 2B). These four candidates carry deletions at the sgRNA target site (Fig. S9). For *asl*, only one out of the tested 24 candidates was arginine auxotroph (Fig. 3), due to mutations at the sgRNA target site (Fig. S10). We speculate that the obtained low frequency of loss-of-function mutations in *praics* and *asl* might be a result of different effectivities of the used sgRNAs. The ura3_sgRNA1 appears to be highly effective. However, the obtained adenine and arginine auxotrophic strains might be used in future studies.

Cas9 RNPs can be used to increase the recombination frequency during HDR

Homologous recombination allows advanced genomic manipulations such as the insertion of point mutations, protein tags, and longer genetic material, or exchange of sequences. In many fungi, the NHEJ-pathway is the dominant repair mechanisms and recombination rate is very low (Krappmann, 2007). We were interested whether Cas9 induced DSBs enhance the recombination frequency. To this end, we constructed disruption cassettes and designed a sgRNA targeting the *dl4* gene (encoding DNA ligase IV involved in the NHEJ pathway). The disruption cassettes consisted of the *pyr4* gene from *T. reesei* as marker and homologous flanks of different lengths (20 bp or 500 bp) (Fig. 4). We transformed 3 µg of the disruption cassettes alone or together with dl4_sgRNA-Cas9 RNPs into *A. pullulans* EXF-150 Δ*ura3* #6 and selected for uridine prototrophy. 24 randomly selected colonies for each transformation reaction were tested for homologous integration at the target locus. Without the addition of RNPs, HR frequencies with the 20 bp and 500 bp long flanks were 50% and 83%, respectively (Fig. S11 and Fig. S12). Addition of RNPs increased the frequencies to 96% and 100%, respectively (Fig. S13 and Fig. S14). We verified the homologous recombination after transformation with the 20 bp-long flanks by sequencing (Fig. S15). Homologous recombination could be confirmed in 11 of 12 tested transformants. One transformant showed homologous recombination at the 3' site but an inconclusive sequencing result at the 5' site. Notably the corresponding PCR product is longer than expected (Fig. S13). We originally chose this gene with the aim to construct a NHEJ-deficient strain. This strategy has previously been used in a series of fungi to enhance the homologous recombination rate (Krappmann, 2007). To our pleasant surprise, this does not seem to be necessary in *A. pullulans* EXF-150, as the frequency of homologous recombination was already high (50% and 83% using 20 bp and 500 bp-long flanks, respectively). Remarkably, the recombination rate could be enhanced by the usage of RNPs (96% and 100%, respectively). Since 20 bp-long flanks in combination with Cas9 RNPs suffice for a high recombination rate in *A. pullulans* EXF-150, construction of

disruption/integration/deletion cassettes can easily be performed via PCR and primers with 20bp-long overhangs.

Experimental Procedures

Strains and cultivation conditions

A. pullulans strains EXF-150 (CBS 100280, (Gostinčar *et al.*, 2014)), ATCC 42023 (Zajic and LeDuy, 1973) and NBB 7.2.1 (CCOS1008, (Hilber-Bodmer *et al.*, 2017)) were maintained on malt extract (MEX) agar at 24°C. Defined medium without yeast extract (Ueda *et al.*, 1963) was used as a minimal medium for testing of adenine and arginine auxotrophy. SC-URA medium (1,71 g l^{-1} Yeast Nitrogen Base, 1,92 g l^{-1} Yeast Synthetic Drop-Out Medium Supplements without Uracil, 5 g l^{-1} $(NH_4)_2SO_4$ and 20 g l^{-1} glucose) was used as a uridine free medium. If applicable, uridine, 5-fluoroorotic acid (5-FOA), adenine and arginine were added to final concentrations of 5 mM, 2 g l^{-1}, 0.5 mM and 2.5 mM, respectively.

Cas9 protein and sgRNAs

For generation of sgRNAs, target-specific DNA oligonucleotides were designed in silico using the EnGen sgRNA Template Oligo Designer (New England Biolabs, Inc., Ipswich, MA, USA). Templates for sgRNA *in vitro* transcription were synthesized by hybridizing the target-specific oligo and the *S. pyogenes* Cas9 scaffold oligo and filling up with T4 DNA polymerase (New England Biolabs) according to the manufacturer's instructions. Using this DNA fragment as template, sgRNA was transcribed *in vitro* using the HiScribe Quick T7 High Yield RNA Synthesis Kit (New England Biolabs). The transcribed sgRNA was treated with DNaseI (Thermo Fisher Scientific) and purified using the RNA Cleanup Kit (New England Biolabs). Prior to RNP assembly, the sgRNA was denatured and refolded as described by Pohl et al. (Pohl *et al.*, 2018). RNPs were assembled in a 150 µl reaction in buffer B (1 M sorbitol, 25 mM $CaCl_2$, 10 mM Tris.Cl pH 7.5) containing 15 µl 10x Cas9 buffer (20 mM HEPES, 150 mM KCl, 8 mM

$MgSO_4 \cdot 7\ H_2O$, 0.1 mM EDTA, 0.5 mM dithiothreitol, pH 7.5), 4.25 µl EnGen Cas9-NLS (20 µM, New England Biolabs), 2.7 µg sgRNA at 37°C for 10 min.

RNP delivery and transformation

For the generation of protoplasts 10 ml of an overnight liquid culture with an OD_{600} of approx. 1 were centrifuged at 6000 g for 5 min. The cell pellet was washed with 20 ml buffer A (100 mM KH_2PO_4, 1.2 M sorbitol, pH = 5.6) and resuspended in lysing solution (15 ml buffer A containing 150 mg lysing enzymes from *T. harzianum* (Sigma-Aldrich, St. Louis, MO, USA, L1412) and 150 mg β-glucanase from T. *longibrachiatum* (Sigma-Aldrich, G4423). This suspension was incubated at 24°C on a rotary shaker at 140 rpm until protoplasts formed (approx. 1 h). Protoplasts were recovered by the addition of 25 ml ice-cold 1.2 M sorbitol and centrifugation at 4°C and 3000 g for 10 min. Protoplasts were washed once with 30 ml 1.2 M ice-cold sorbitol and twice with 10 ml ice-cold buffer B and then resuspended in 1 ml ice-cold buffer B (volume adjusted to the OD_{600} of the overnight culture). For transformation, 100 µl of the protoplast suspension were mixed with 100 µl "20% PEG solution" (20% (w/v) PEG 4000, 0.67 M sorbitol, 20 mM CaCl2, 10 mM Tris pH = 7.5) and 2 µl β-mercaptoethanol added. Next, 150 µl of the RNP mix or 150 µl Buffer B were added. For transformation of DNA, 5 µg of undigested plasmid DNA or 3 µg of linear donor DNA were used, respectively. The reactions were incubated on ice for 30 minutes and 750 µl "60% PEG solution" (60% (w/v) PEG 4000, 10 mM CaCl2, 10 mM Tris pH = 7.5) added stepwise. After 20 minutes at 23°C, 4.1 ml of buffer C (1 M sorbitol, 10 mM Tris.Cl pH= 7.5) were added stepwise. Different amounts of the transformation mix were added to 20 mL of melted, 50°C warm selection medium, containing 1 M sucrose. This mixture was poured into sterile petri dishes. The plates were incubated at 24°C for 6 to 14 days until colonies were visible.

Construction of pJET-pyrG

The *pyrG* gene of *A. fumigatus* was amplified by PCR using the Q5 DNA Polymerase (New England Biolabs), the primers pyrG_fwd-AflII-NsiI and pyrG_rev-EcoRI-AatII, and the

plasmid pFC330 (Nødvig *et al.*, 2015) as template. The PCR was inserted into pJET1.2 using the CloneJET PCR Cloning Kit (Thermo Scientific) according to the manufacturer's instructions. The sequence was verified by Sanger sequencing (at Microsynth AG, Balgach, Switzerland) (Fig. S16 and Fig. S17).

Construction of disruption cassettes

For the construction of pUC18_Apdp4 (Fig. S18) a gene assembly strategy using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs) was followed. The *pyr4* gene was amplified using the primers pyr4dl4_20bp_rev and pyr4dl4_20bp_fwd and the plasmid pJET-pyr4 (Derntl *et al.*, 2016) as template. The two 500 bp-long homology flanks of the *dl4* gene were amplified using the primers dl4_5Overlap500_fwd and dl4_5Overlap_rev or dl4_3Overlap_fwd and dl4_3Overlap500_rev and genomic DNA of *A. pullulans* EXF-150 as template. The plasmid pUC18 was amplified with the primers pUC18_fwd and pUC18_rev. The sequence of pUC18_Apdp4 was verified by Sanger sequencing (at Microsynth AG). Linear disruption cassettes (Fig. S19) for transformation were amplified using the primers dl4_20bpover_fwd and dl4_20bpover_rev or dl4_500bpover_fwd and dl4_500bpover_rev and pUC18_Apdp4 as template. The PCR products were purified with the GeneJet PCR Purification Kit (Thermo Scientific) according to the manufacturer's instructions. Several PCR reactions were pooled to obtain enough DNA for transformation. The DNA was concentrated by precipitation with sodium acetate and ethanol and dissolved in double-distilled water (ddH_2O).

Genotyping

For the extraction of chromosomal DNA, we used the colony PCR protocol described by Wu et al. (Wu *et al.*, 2017). For diagnostic PCR, 2µL of the resulting crude DNA extract was used as the template in a 50-µl PCR with the OneTaq DNA polymerase (New England Biolabs) according to the manufacturer's instructions. For subsequent agarose gel electrophoresis of the DNA fragments, a GeneRuler 1-kb Plus DNA ladder (New England Biolabs) was applied to estimate the fragment size. Loci to be sequenced were amplified by PCR with the Q5 DNA

polymerase (New England Biolabs) according to the manufacturer's instructions and sequenced at Microsynth AG.

Oligonucleotides

All oligonucleotides used in this study are listed in Table S1.

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Figure legends

Fig. 1 CRISPR/Cas9 mediated manipulation of *ura3* in *A. pullulans* EXF-150 with RNP delivery.

A Two different sgRNAs (green, ura3_sgRNA1; yellow, ura3_sgRNA2) direct the Cas9 to two distinct target sites in the *ura3* coding region. Introns are indicated in dark red.

B Partial sequences of *ura3* coding region of the *A. pullulans* EXF-150 wildtype (wt) and six 5-FOA resistant colonies (#1-6) obtained after the delivery of Cas9 RNPs. Exon sequences are bold, the corresponding amino acid sequence is given above the genomic sequence. Target site of ura3_sgRNA1 and ura3_sgRNA2 are highlighted in green and yellow, respectively. PAM sites are underlined. In four colonies (#1, 2, 4 and 5) deletions occurred at the sgRNA1 target site. In #2, 4 and 5, this resulted in a frame shift. In two colonies (#3 and 6) the entire gene fragment between the two target sites (#3: 843 bp segment, #6: 821 bp segment) was lost.

C The *A. pullulans* EXF-150 wildtype (wt) and six 5-FOA resistant colonies (#1-6) were cultivated on medium lacking uridine (left, SC-URA) and medium containing 5 mM uridine (right, SC+Uri) for 7 days at 24°C.

Fig. 2 Auxotrophy testing of colonies resulting from a co-delivery of RNPs targeting the *ura3* and the *praic* genes.

A The *A. pullulans* EXF-150 wildtype (wt), the uridine auxotrophic strain Δura3 #6 (Δ), and 24 randomly selected 5-FOA resistant colonies resulting from the co-delivery of RNPs targeting the *ura3* and the *praics* genes were cultivated on minimal medium lacking adenine (left, MM+Uri-Ade) and on medium containing 0.05 mM adenine (right, MM+Uri+Ade) for 7 days at 24°C. Colonies #10, 13, 17 and 21 were not able to grow on medium without adenine (red arrows).

B The same strains were cultivated malt extract (MEX) plates supplemented with uridine and adenine (MEX+Uri+Ade) for 7 days at 24°C. Adenine auxotrophic mutants produce a red pigment due to the accumulation of an intermediate in purine biosynthesis.

Fig. 3 Auxotrophy testing of colonies resulting from a co-delivery of RNPs targeting the *ura3* and the *asl* genes.

The *A. pullulans* EXF-150 wildtype (wt), the uridine auxotrophic strain Δura3 #6 (Δ), and 24 randomly selected 5-FOA resistant colonies resulting from the co-delivery of RNPs targeting the *ura3* and the *asl* genes were cultivated on minimal medium lacking arginine (left, MM+Uri-Arg) and medium containing 2.5 mM arginine (right, MM+Uri+Arg) for 7 days at 24°C. Colony #21 was not able to grow on medium without arginine (red arrow).

Fig. 4 Schematic representation of disruption of *dl4* with *pyr4* disruption cassettes via homologous recombination.

The uridine auxotrophic strain (EXF-150 Δura3 #6) was transformed with *pyr4* integration cassettes, in order to complement uridine auxotrophy and disrupt *dl4* (red arrow) with the marker gene *pyr4* (grey arrow) via homologous recombination. The yellow frames represent 5′- and 3′-flanks for the homologous recombination. 20 and 500 bp-long segments in vicinity to the dl4_sgRNA target site were chosen as flanks. The white arrow indicates the recombination event. Primers (black arrows) used for PCR are depicted: 1, 500_flank_fwd; 2, 20_flank_fwd; 3, pyr4_flank_rev; 4, 500_flank_rev; 5, 20_flank_rev; 6, pyr4_flank_fwd. Transformation of donor DNA was carried out with and without delivery of dl4_sgRNA-Cas9 RNPs to assess whether CRISPR/Cas9 RNPs increase HR frequency in *A. pullulans*.

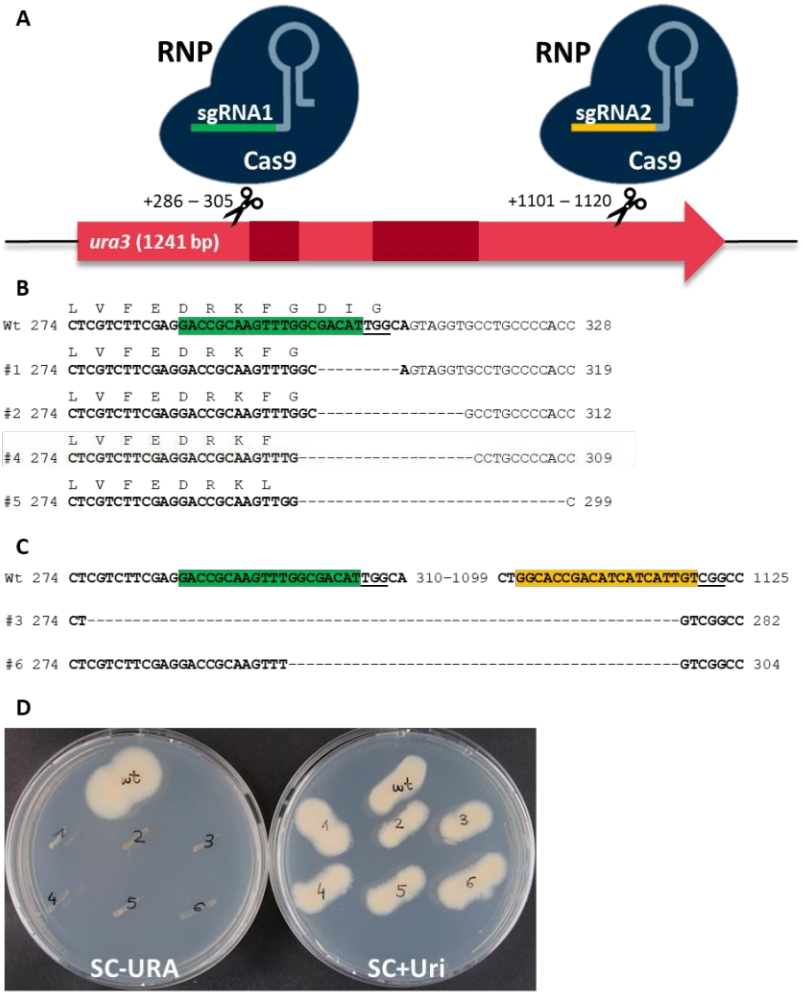

Figure 1 CRISPR/Cas9 mediated manipulation of ura3 in *A. pullulans* EXF-150 with RNP delivery. **A** Two different sgRNAs (green, ura3_sgRNA1; yellow, ura3_sgRNA2) direct the Cas9 to two distinct target sites in the *ura3* coding region. Introns are indicated in dark red. **B** Partial sequences of *ura3* coding region of the *A. pullulans* EXF-150 wildtype (wt) and six 5-FOA resistant colonies (#1-6) obtained after the delivery of Cas9 RNPs. Exon sequences are bold, the corresponding amino acid sequence is given above the genomic sequence. Target site of ura3_sgRNA1 and ura3_sgRNA2 are highlighted in green and yellow, respectively. PAM sites are underlined. In four colonies (#1, 2, 4 and 5) deletions occurred at the sgRNA1 target site. In #2, 4 and 5, this resulted in a frame shift. In two colonies (#3 and 6) the entire gene fragment between the two target sites (#3: 843 bp segment, #6: 821 bp segment) was lost. **C** The *A. pullulans<(i> EXF-150 wildtype (wt) and six 5-FOA resistant colonies (#1-6) were cultivated on medium lacking uridine (left, SC-URA) and medium containing 5 mM uridine (right, SC+Uri) for 7 days at 24°C.*

464x521mm (59 x 59 DPI)

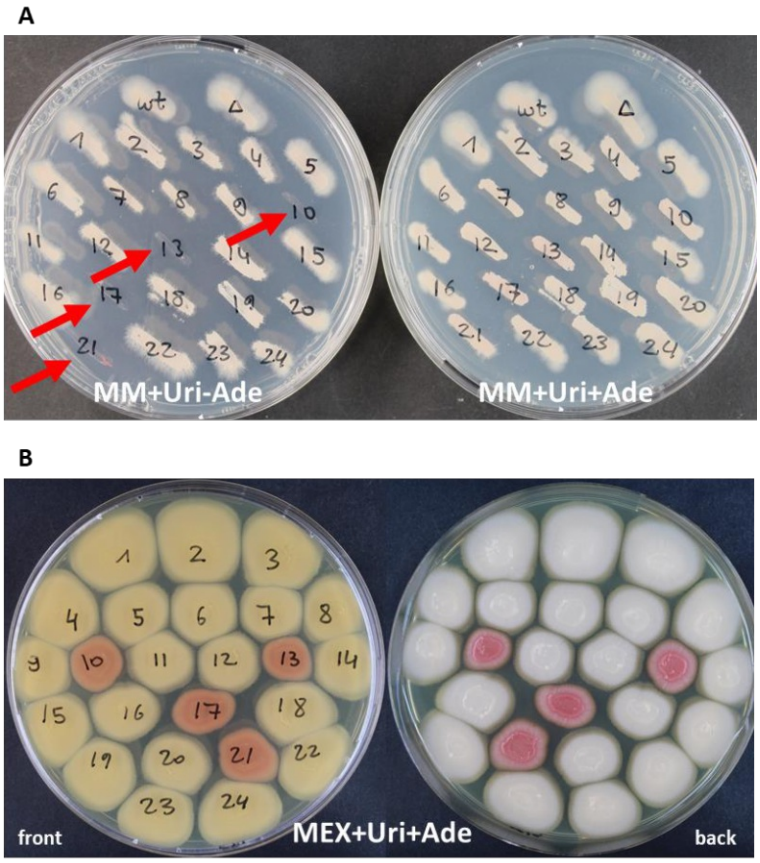

Figure 2 Auxotrophy testing of colonies resulting from a co-delivery of RNPs targeting the *ura3* and the *praic* genes. **A** The *A. pullulans* EXF-150 wildtype (wt), the uridine auxotrophic strain Δura3 #6 (Δ), and 24 randomly selected 5-FOA resistant colonies resulting from the co-delivery of RNPs targeting the *ura3* and the *praics* genes were cultivated on minimal medium lacking adenine (left, MM+Uri-Ade) and on medium containing 0.05 mM adenine (right, MM+Uri+Ade) for 7 days at 24°C. Colonies #10, 13, 17 and 21 were not able to grow on medium without adenine (red arrows). **B** The same strains were cultivated malt extract (MEX) plates supplemented with uridine and adenine (MEX+Uri+Ade) for 7 days at 24°C. Adenine auxotrophic mutants produce a red pigment due to the accumulation of an intermediate in purine biosynthesis.

358x410mm (59 x 59 DPI)

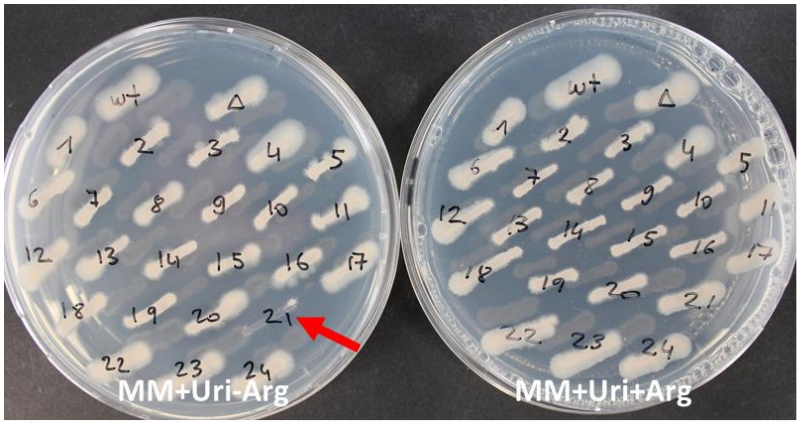

Figure 3 Auxotrophy testing of colonies resulting from a co-delivery of RNPs targeting the *ura3* and the *asl* genes. The *A. pullulans* EXF-150 wildtype (wt), the uridine auxotrophic strain Δura3 #6 (Δ), and 24 randomly selected 5-FOA resistant colonies resulting from the co-delivery of RNPs targeting the *ura3* and the *asl* genes were cultivated on minimal medium lacking arginine (left, MM+Uri-Arg) and medium containing 2.5 mM arginine (right, MM+Uri+Arg) for 7 days at 24°C. Colony #21 was not able to grow on medium without arginine (red arrow).

359x184mm (59 x 59 DPI)

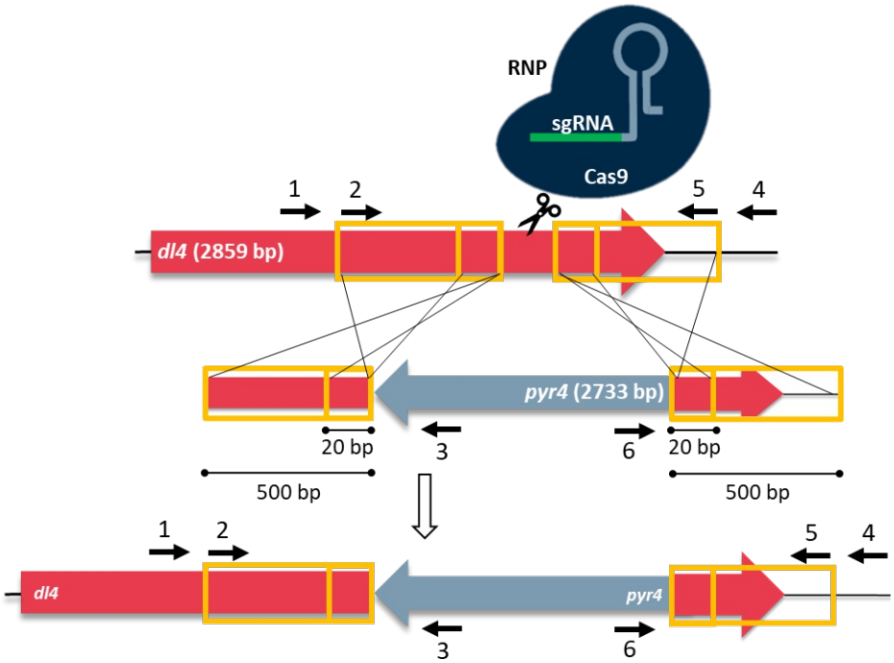

Figure 4 Schematic representation of disruption of *dl4* with *pyr4* disruption cassettes via homologous recombination. The uridine auxotrophic strain (EXF-150 Δura3 #6) was transformed with *pyr4* integration cassettes, in order to complement uridine auxotrophy and disrupt *dl4* (red arrow) with the marker gene *pyr4* (grey arrow) via homologous recombination. The yellow frames represent 5′- and 3′-flanks for the homologous recombination. 20 and 500 bp-long segments in vicinity to the dl4_sgRNA target site were chosen as flanks. The white arrow indicates the recombination event. Primers (black arrows) used for PCR are depicted: 1, 500_flank_fwd; 2, 20_flank_fwd; 3, pyr4_flank_rev; 4, 500_flank_rev; 5, 20_flank_rev; 6, pyr4_flank_fwd. Transformation of donor DNA was carried out with and without delivery of dl4_sgRNA-Cas9 RNPs to assess whether CRISPR/Cas9 RNPs increase HR frequency in *A. pullulans*.

482x329mm (59 x 59 DPI)

Supplemental Material for

Fast and efficient CRISPR-mediated genome editing in *Aureobasidium pullulans* using Cas9 ribonucleoproteins

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* Corresponding author: christian.derntl@tuwien.ac.at

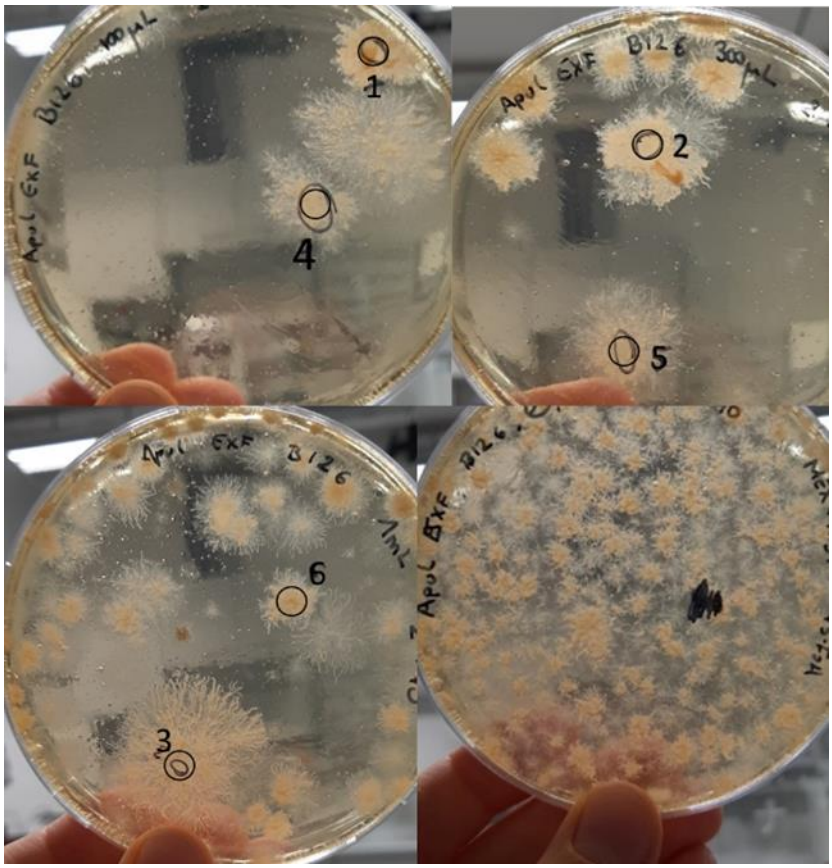

Fig. S1 Selection plates (MEX + Uri + 5-FOA) resulting from the delivery of ura3_sgRNA1 and ura3_sgRNA2-Cas9 RNPs into *A. pullulans* EXF-150 after 14 days. About 250 CFUs were obtained after the delivery of approx. 0.084 nmol Cas9 and sgRNA each.

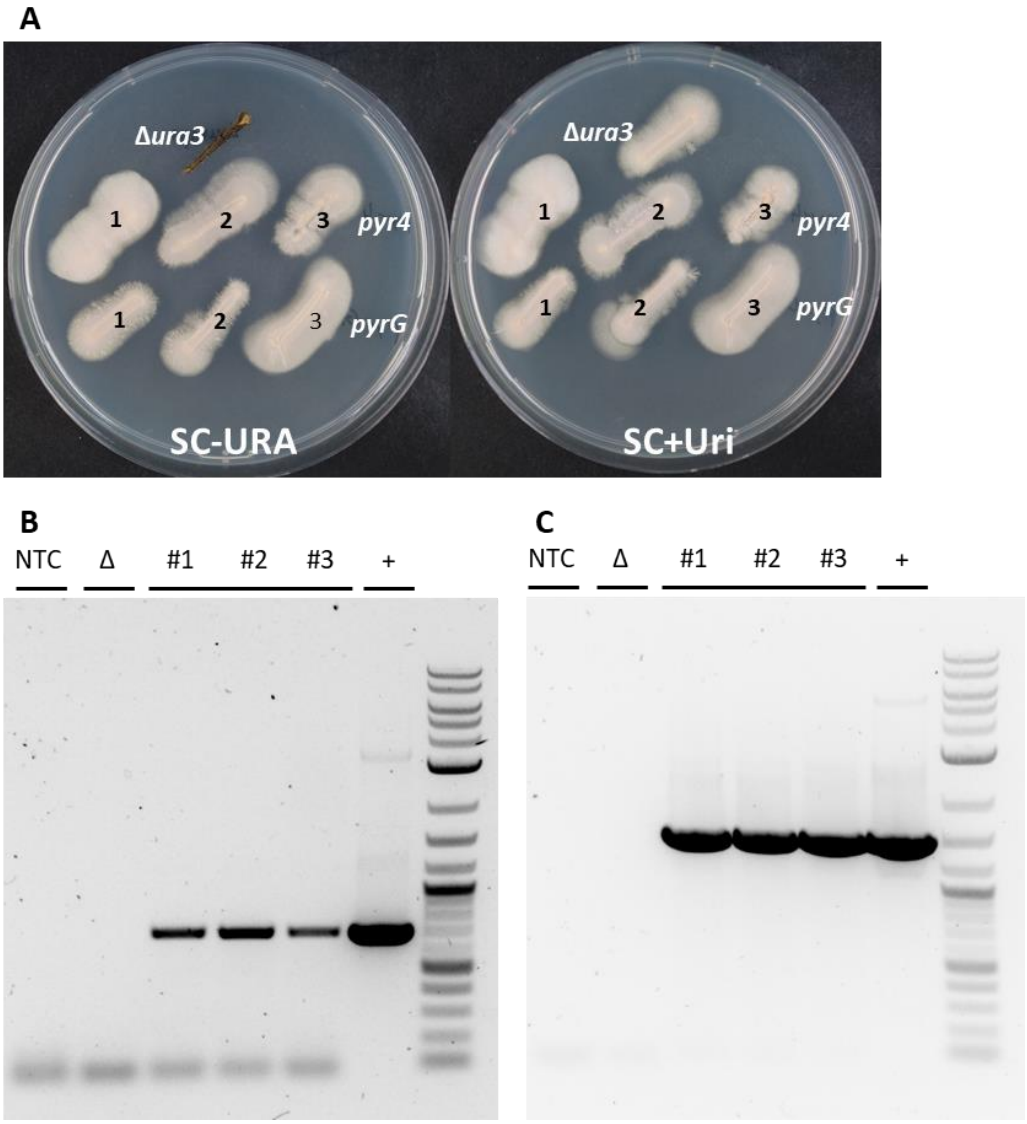

Fig. S2 Complementation of uridine auxotrophy in *A. pullulans EXF*-150 uridine auxotrophic mutant #6 (Δura3 #6) with *pyr4* (*T. reesei*) and *pyrG* (*A. fumigatus*), respectively.

A The parent strain Δura3 #6 cannot grow on medium lacking uridine (SC-URA) (left). Growth is only possible after the addition of 5 mM uridine (SC+Uri) (right). Colonies resulting from the transformation with pJET-pyr4 / pJET-pyrG are able to grow on SC-URA. Pictures taken after 7 days incubation at 24°C.

B Complementation of uridine auxotrophy with *pyr4*: Agarose gel electrophoresis of the fragments obtained by PCR with primers pyr4_fwd and pyr4_rev using chromosomal DNA of the parent strain (Δura3 #6, Δ) and candidates (pyr4 #1-3) as template. NTC, no template control; +, positive control pJET-pyr4.

C Complementation of uridine auxotrophy with *pyrG*: Agarose gel electrophoresis of the fragments obtained by PCR with primers pyrG_fwd and pyrG_rev using chromosomal DNA of the parent strain (Δura3 #6, Δ) and candidates (pyrG #1-3) as template. NTC, no template control; +, positive control pJET-pyrG.

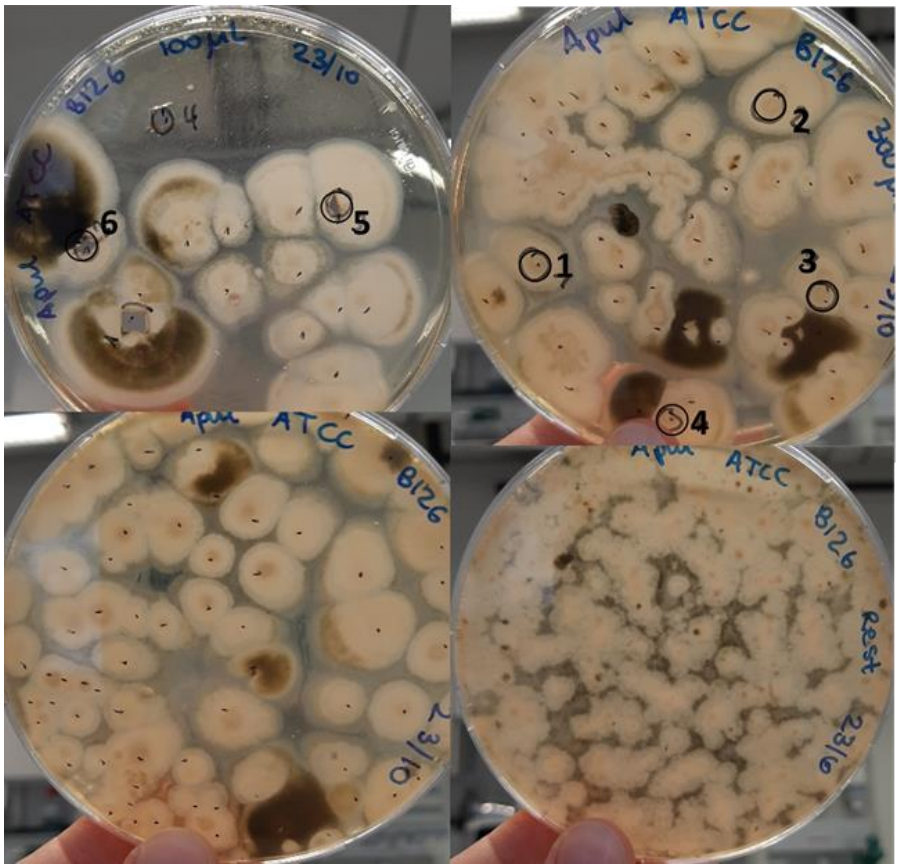

Fig. S3 Selection plates (MEX + Uri + 5-FOA) resulting from the delivery of ura3_sgRNA1-Cas9 RNPs into *A. pullulans* ATCC 42023 after 11 days. About 300 CFUs were obtained after the delivery of approx. 0.042 nmol Cas9 and sgRNA each.

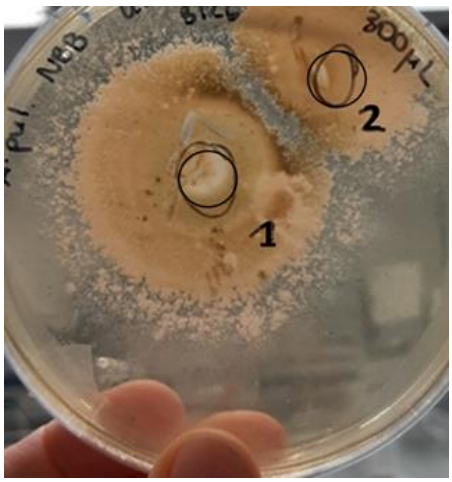

Fig. S4 Selection plate (MEX + Uri + 5-FOA) resulting from the delivery of ura3_sgRNA1-Cas9 RNPs into *A. pullulans* NBB 7.2.1 after 28 days. Two CFUs were obtained after the delivery of approx. 0.042 nmol Cas9 and sgRNA each.

A

```
Wt      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTTGGCGACATTGGCAGTATGTTCATGCCCCACT
#1      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTT--------------TATGTTCATGCCCCACT
#2      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTT--------------TATGTTCATGCCCCACT
#3      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTT---------------ATGTTCATGCCCCACT
#4      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCCCATT----------GGCAGTATGTTCATGCCCCACT
#5      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTTG------------GAATGTTCATGCCCCACT
#6      GCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTTGGCG----------TATGTTCATGCCCCACT
```

B

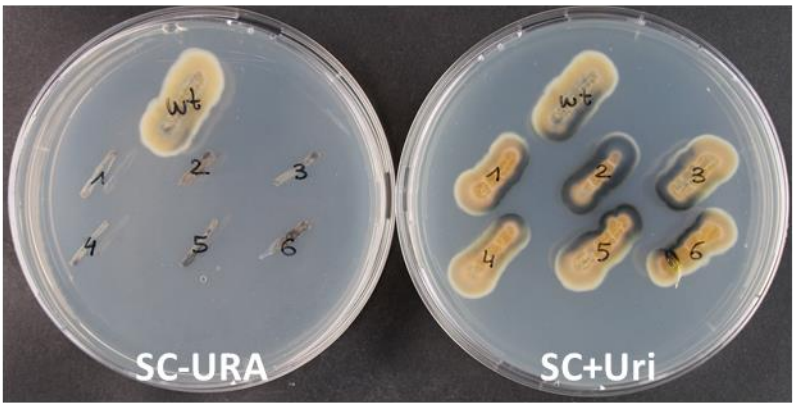

Fig. S5 CRISPR/Cas9 mediated manipulation of *ura3* in *A. pullulans* ATCC 42023 with RNP delivery.

A Partial sequences of *ura3* coding region of the *A. pullulans* ATCC 42023 wildtype (wt) and six 5-FOA resistant colonies (#1-6) obtained after the delivery of Cas9 RNPs. Target site of ura3_sgRNA1 is highlighted in green. Mismatch against the sgRNA is indicated in bold. PAM site is underlined. In all transformants deletions occurred at the sgRNA target site, resulting in a frame shift in colonies #1, 2, 4 and 6.

B The *A. pullulans* ATCC 42023 wildtype (wt) and the six 5-FOA resistant colonies (#1-6) were cultivated on medium lacking uridine (left, SC-URA) and medium containing 5 mM uridine (right, SC+Uri) for 7 days at 24°C.

A

```
Wt      GCCAAGAAGCACTTCCTCGTCTTCGAGGACCGCAAGTTTGGCGACATTGGCAGTAGGTGCCTGCCCCACC

#1      GCCAAGAAGCACTTCCTCGTCTTCGAGGACCGCAAGTTTG----------------------CCCCACC

#2      GCCAAGAAGCACTTCCTCGTCTTCGAGGACCGCAAGTTTGGCGA-ATTGGCAGTAGGTGCCTGCCCCACC
```

B

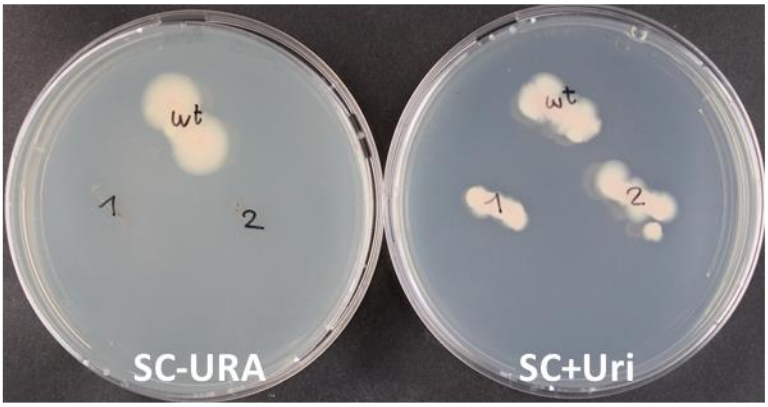

Fig S6 CRISPR/Cas9 mediated manipulation of *ura3* in *A. pullulans* NBB 7.2.1 with RNP delivery.

A Partial sequences of *ura3* coding region of the *A. pullulans* NBB 7.2.1 wildtype (wt) and two 5-FOA resistant colonies (#1 and 2) obtained after the delivery of Cas9 RNPs. Target site of ura3_sgRNA1 is highlighted in green. PAM site is underlined. In all candidates deletions occurred at the sgRNA target site, resulting in a frame shift.

B The *A. pullulans* NBB 7.2.1 wildtype (wt) and the two 5-FOA resistant colonies (#1 and 2) were cultivated on medium lacking uridine (left, SC-URA) and medium containing 5 mM uridine (right, SC+Uri) for 7 days at 24°C.

GGCGACTCAATATGCATGCTCAAGACACACGCAGACATTATCAATGACTTTGGCCCTCGCACCATTCAAGGCCTG
AAAGAGATTGCCGCCAAAAAACACTTCCTCGTTTTCGAGGATCGCAAGTTTGGCGACATTGGCAGTATGTTCATG
CCCCACTATCGTCTTGCTCGAACCACACACTGACAGCTGCTCGCAGGCACGGTGCAGAAGCAGTTCACCGCTGGG
CCTCTGCAAATCGTCCGCTGGGCAAACATTATCAACGCTCACATCTTTCCTGGCCCTGCCATCATTACTGCTCTC
TCGCAAGCTGCCCACGACGCCGTTACTTCGCTCAATACCGCAGTGACCACTTCCATCTCAGCCTCACCCGTTCCT
TCGTACATGGATGACAGCGATGAGGTCGACTCTCCTGCCTTGTCTCACCACGACGATTCGGATGACCTTGACAGG
ATGTCCAGCGACGAGGATACCAACCAGCTGTCTACATACAACGACCCTACCGGTCGCAAGCCCAGTGTCGTTTCC
GTCTCTACCACCATCAGCACAAAGACAGAAAGCATCTCACCTCAACCCACACCGAACCACCTTGGTGGTCCTTCC
GACTCCATCTCGGCTATCAGTGCAGGCTCGGCTAGTCAAGAATCCTCTGCTTTGGCCCGCCTCGGCGAACCTCCT
CTCCTTCGAAGCTTGCTCATTCTTGCAGAGATGAGCAGCGCGGGTAACTTGATGACTGGTGCATATACGGAACAA
TGTGTAGTTGAAGCACGCAAAAACCCGGAGTTTGTCATGGGCTTCAT

Fig. S7 Sequence of part of the *ura3* gene in *A. pullulans* ATCC 42023. Primer ura3_2_fwd is indicated in blue, primer ura3_2_rev is indicated in green and ura3_sgRNA1 target site is indicated in yellow (mismatched base is indicated in bold).

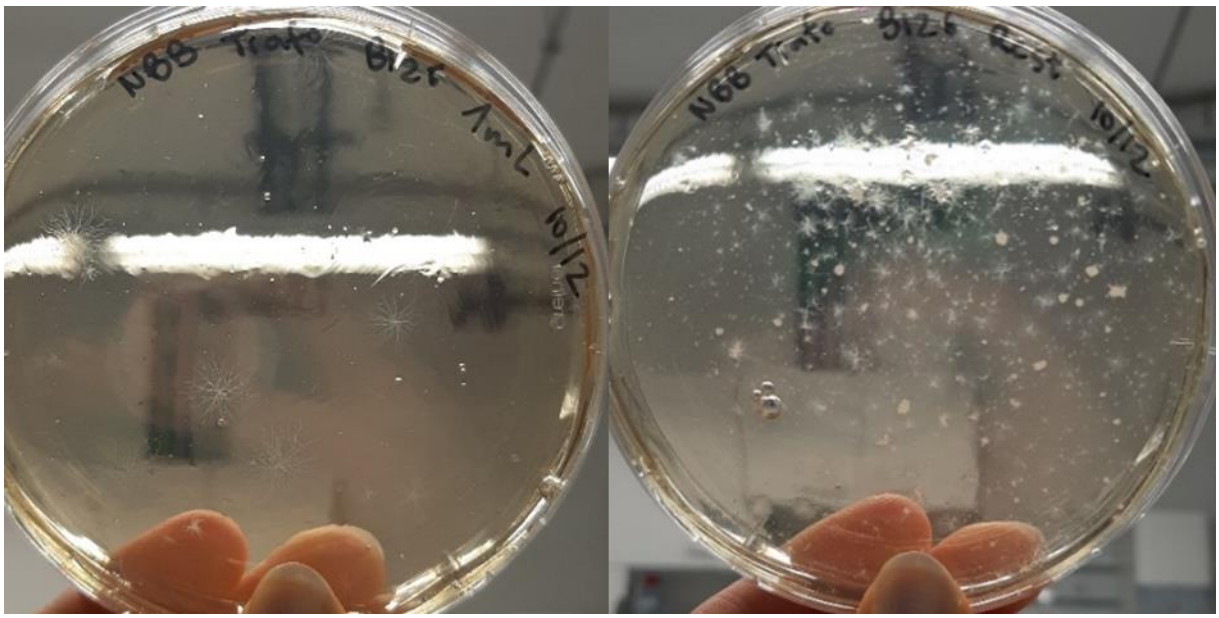

Fig. S8 Selection plate (MEX + Uri + 5-FOA) resulting from the delivery of ura3_sgRNA1-Cas9 RNPs into *A. pullulans* NBB 7.2.1 with addition of β-mercaptoethanol to protoplasts and sgRNA denaturing and refolding before RNP assembly, after 12 days. About 50 CFUs were obtained after the delivery of 0.084 nmol Cas9 and sgRNA each.

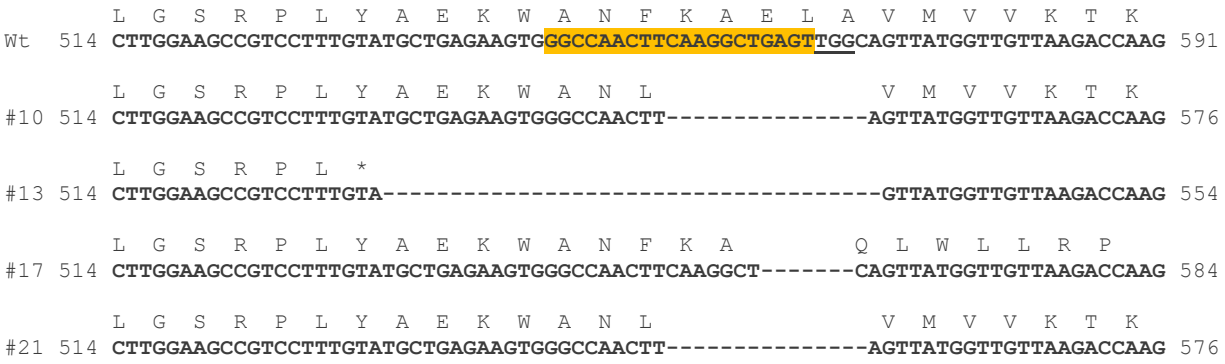

Fig S9 CRISPR/Cas9 mediated manipulation of *praics* in *A. pullulans* EXF-150 via co-delivery of RNPs targeting the *ura3* and the *praics* genes.

Partial sequences of *praics* coding region of the *A. pullulans* EXF-150 wildtype (wt) and the four adenine auxotrophic mutants (#10, 13, 17 and 21) obtained after the delivery of Cas9 RNPs. The corresponding amino acid sequence is given above the genomic sequence. Target site of praics_sgRNA is highlighted in yellow. PAM site is underlined. In all mutants deletions occurred at the sgRNA target site. In mutant #13 this lead to an early stop codon (*). In mutant #21 deletion of nucleotides resulted in a frame shift.

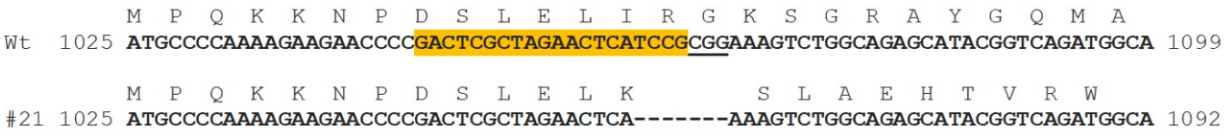

Fig. S10 CRISPR/Cas9 mediated manipulation of *asl* in *A. pullulans* EXF-150 via co-delivery of RNPs targeting the *ura3* and the *asl* genes.

Partial sequences of *asl* coding region of the *A. pullulans* EXF-150 wildtype (wt) and the arginine auxotrophic mutant (#21) obtained after the delivery of Cas9 RNPs. The corresponding amino acid sequence is given above the genomic sequence. Target site of asl_sgRNA is highlighted in yellow. PAM site is underlined. Deletions occurred at the sgRNA target site, resulting in a frame shift.

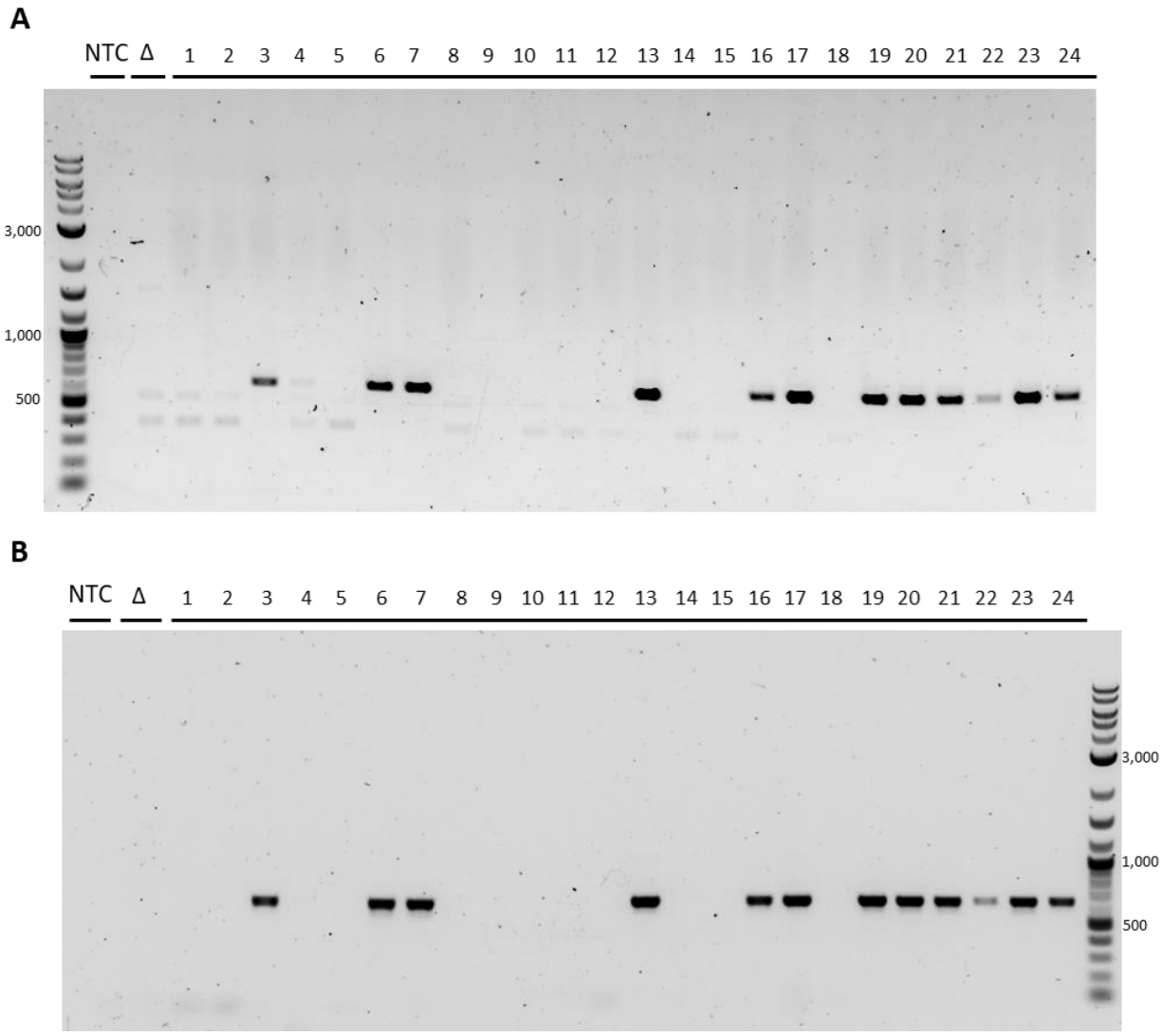

Fig. S11 Agarose gel electrophoresis of the fragments obtained by PCR to test for homologous integration at the target locus in 24 candidates (#1-24) obtained after transformation of 3µg donor DNA with 20 bp-long homology flanks, without sgRNA-Cas9 RNPs. HR frequency = 12/24 = 50%.

A Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_20test_fwd (2) and pyr4_flank_rev (3) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 24 candidates as template. NTC, no template control.

PCR product of candidates # 3, 6, 7, 13, 16, 17, 19-24 is of expected length (618 bp).

B Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_20test_rev (5) and pyr4_flank_rev (6) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 24 candidates as template. NTC, no template control.

PCR product of candidates # 3, 6, 7, 13, 16, 17, 19-24 is of expected length (638 bp).

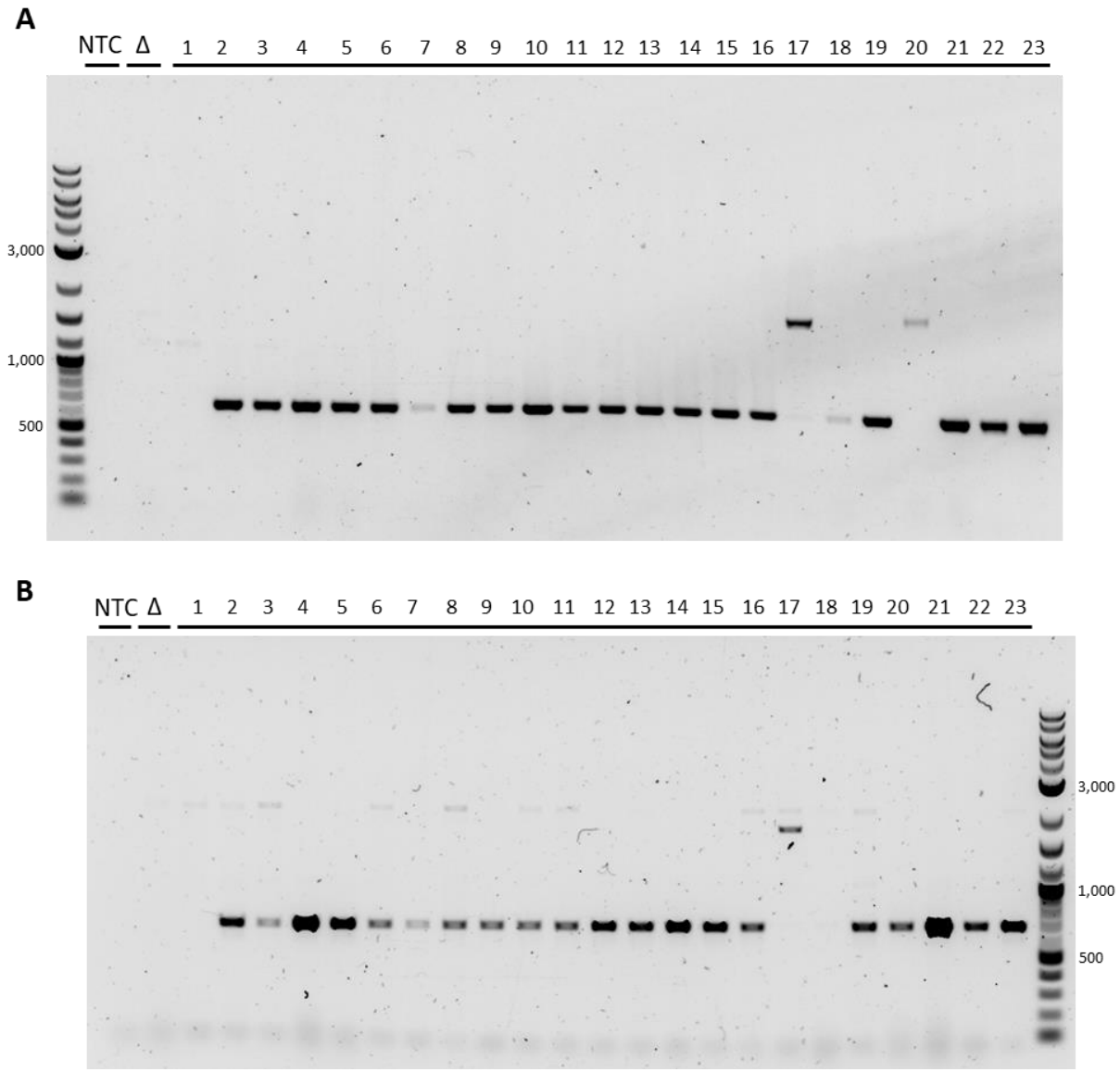

Fig. S12 Agarose gel electrophoresis of the fragments obtained by PCR to test for homologous integration at the target locus in 23 candidates (#1-23) obtained after transformation of 3µg donor DNA with 500 bp-long homology flanks, without sgRNA-Cas9 RNPs. HR frequency = 19/23 =83%.

A Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_500test_fwd (1) and pyr4_flank_rev (3) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 23 candidates as template. NTC, no template control.

PCR product of candidates # 2-16, 18, 19, 21-23 is of expected length (643 bp).

B Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_500test_rev (4) and pyr4_flank_rev (6) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 23 candidates as template. NTC, no template control.

PCR product of candidates # 2-16, 19-23 is of expected length (667 bp).

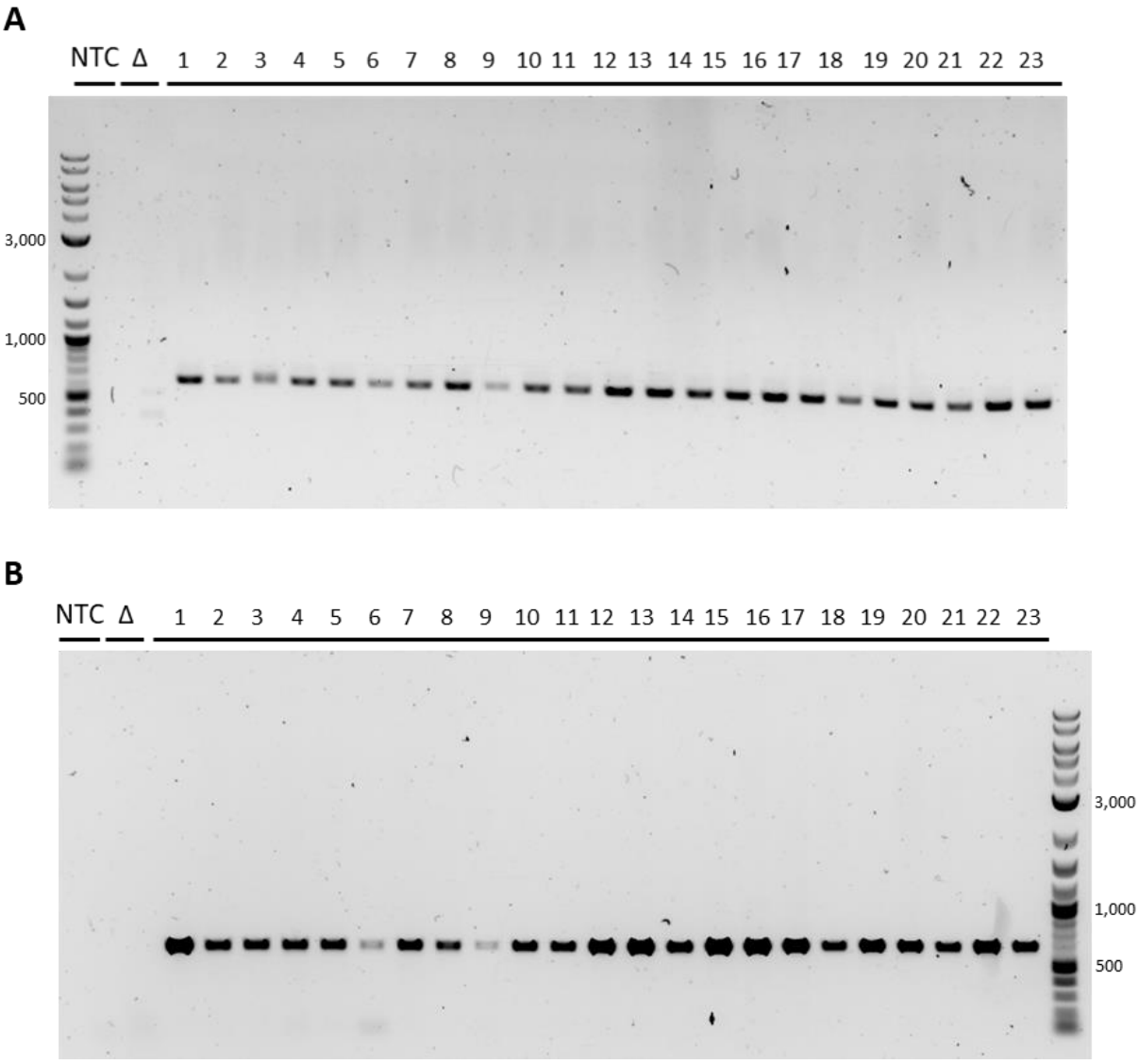

Fig. S13 Agarose gel electrophoresis of the fragments obtained by PCR to test for homologous integration at the target locus in 23 candidates (#1-23) obtained after transformation of 3µg donor DNA with 20 bp-long homology flanks, with sgRNA-Cas9 RNPs. HR frequency = 22/23 = 96%.

A Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_20test_fwd (2) and pyr4_flank_rev (3) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 23 candidates as template. NTC, no template control.

PCR product of candidates # 1, 2, 4-23 is of expected length (618 bp). PCR product of #3 is slightly longer than expected.

B Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_20test_rev (5) and pyr4_flank_rev (6) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 23 candidates as template. NTC, no template control.

PCR product of all candidates is of expected length (638 bp).

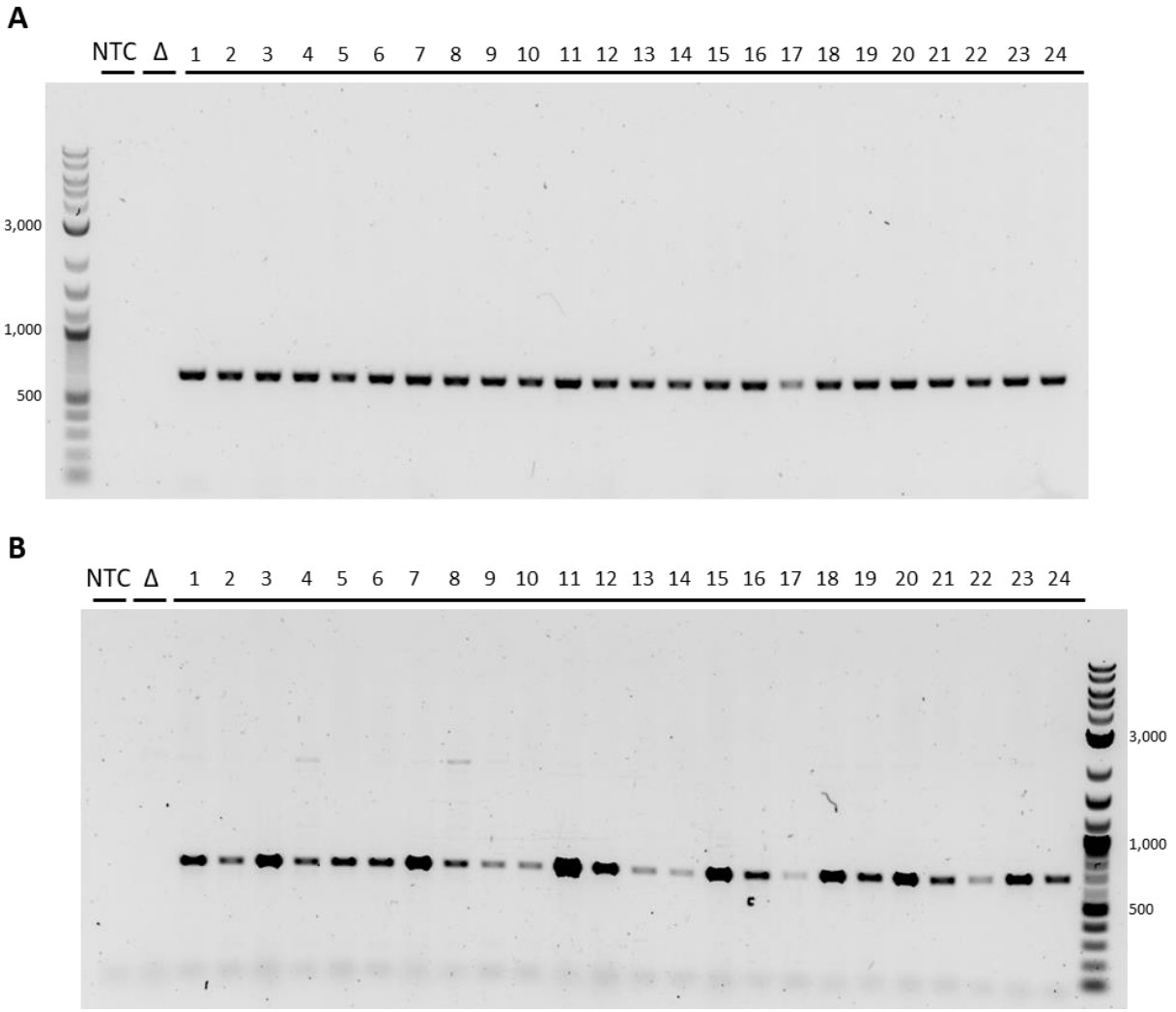

Fig. S14 Agarose gel electrophoresis of the fragments obtained by PCR to test for homologous integration at the target locus in 24 candidates (#1-24) obtained after transformation of 3µg donor DNA with 500 bp-long homology flanks, with sgRNA-Cas9 RNPs. HR frequency = 24/24 = 100%.

A Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_500test_fwd (1) and pyr4_flank_rev (3) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 24 candidates as template. NTC, no template control.

PCR product of all candidates is of expected length (643 bp).

B Agarose gel electrophoresis of the fragments obtained by PCR with primers dl4_500test_rev (4) and pyr4_flank_rev (6) (Fig. 4) using chromosomal DNA of the parent strain (Δura3 #6, Δ) and 24 candidates as template. NTC, no template control.

PCR product of all candidates is of expected length (667 bp).

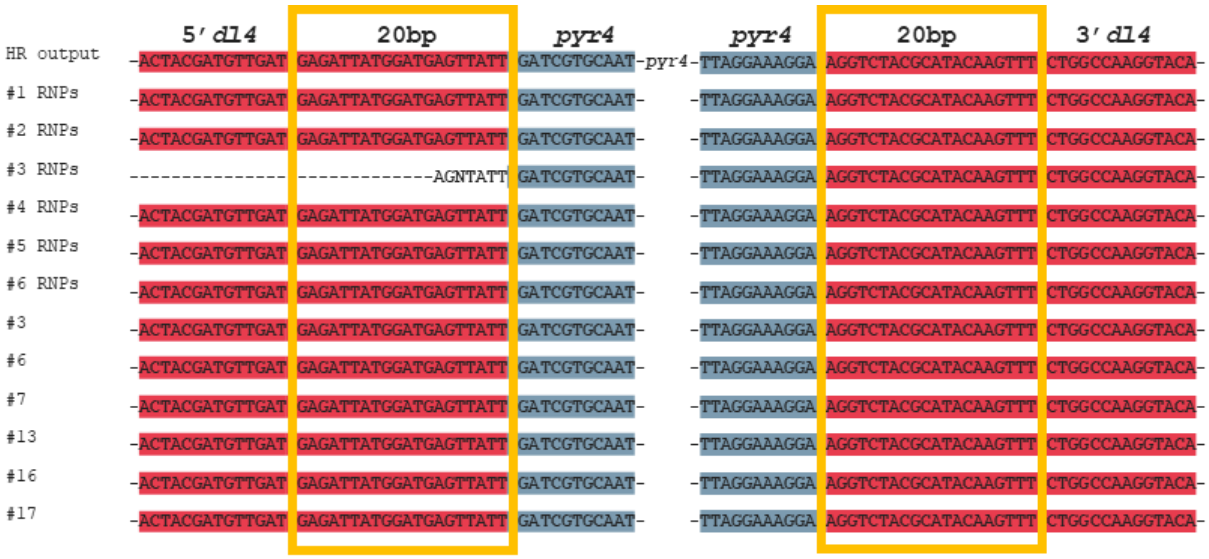

Fig. S15 Sequence of the *dl4* insertion site in the theoretical HR output and 12 colonies obtained after transformation of donor DNA with 20 bp-long homology flanks (six with RNPs, #1, 2, 3, 4, 5 and 6; six without RNPs, #3, 6, 7, 13, 16 and 17). Homologous recombination could be confirmed in 11 transformants. One transformant (#3 RNPs) showed homologous recombination at the 3'flank but an inconclusive sequencing result at the 5' site. Notably the corresponding PCR product is slightly longer than expected (Fig. S12). Red indicates *dl4* sequence; blue indicates *pyr4* sequence; yellow frame indicates 20bp-long homologous flank.

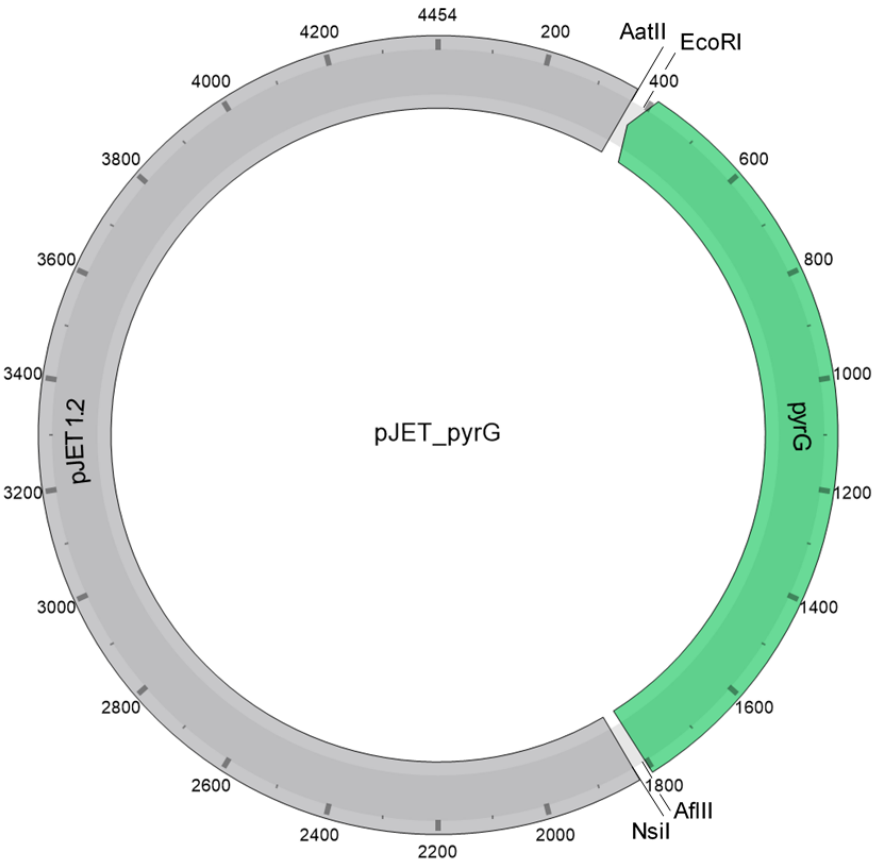

Fig. S16 Vector map of pJET_pyrG

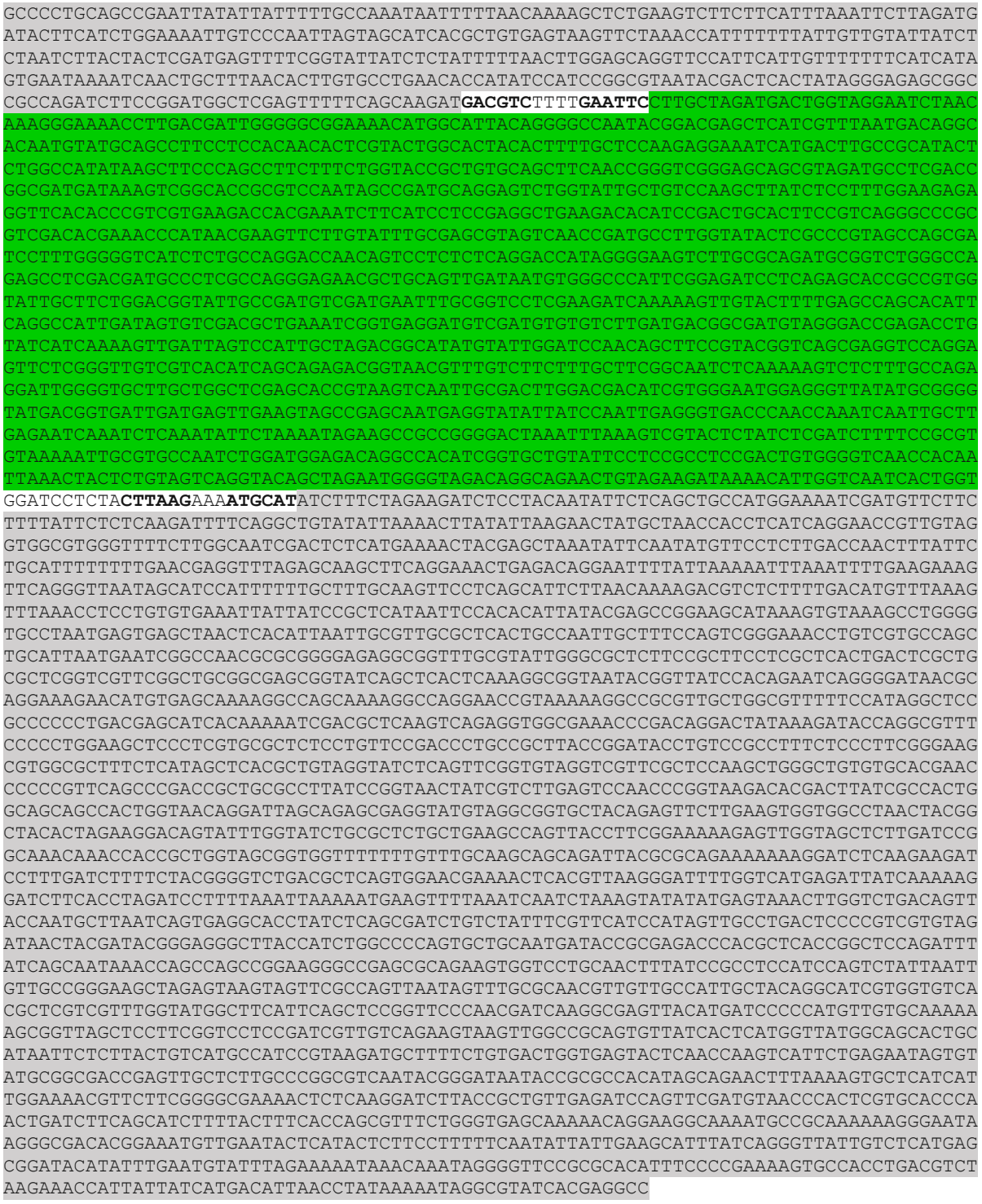

Fig. S17 Sequence of pJET_pyrG

The *pyrG* gene is indicated in green and the pJET backbone is indicated in grey. Bold letters indicate cut sites of restriction enzymes AatII, EcoRI, AflII and NsiI, respectively.

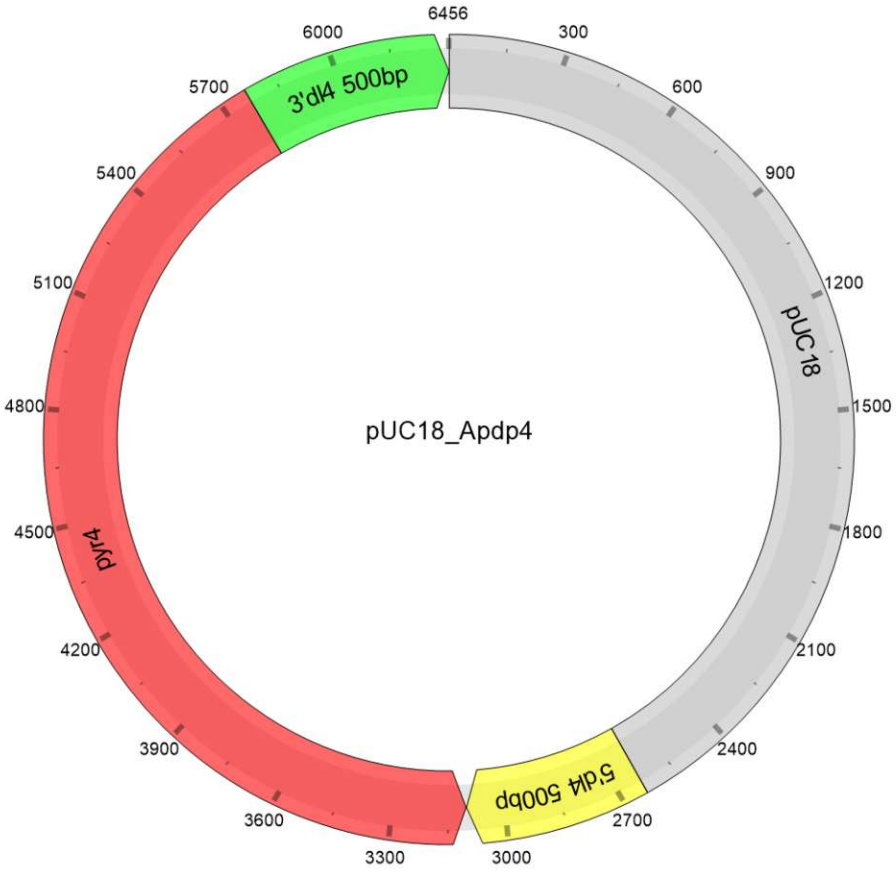

Fig. S18 Vector map of pUC18_Apdp4

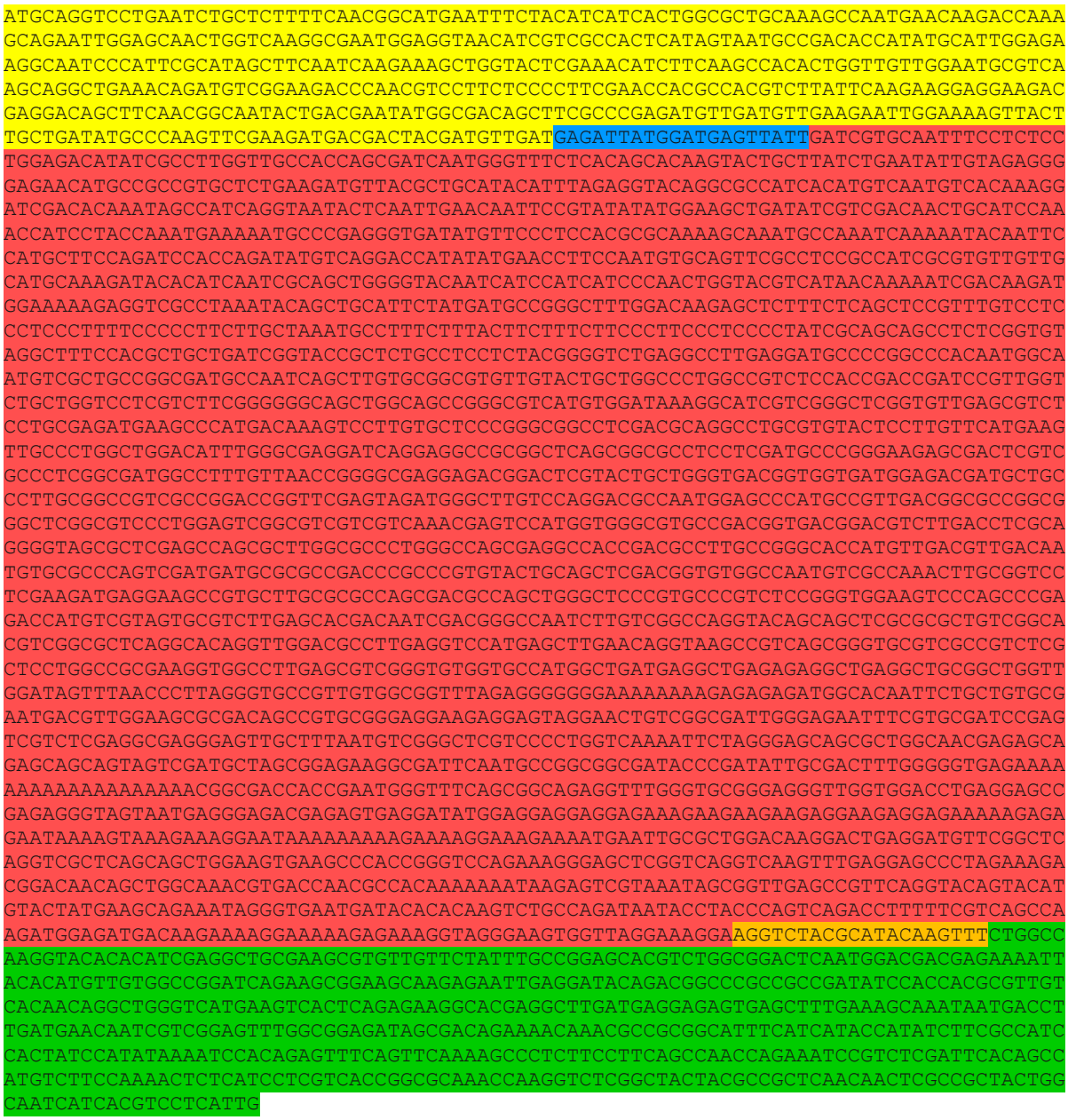

Fig. S19 Sequence of linear disruption cassettes.

The 5’ 500 bp-long homology flank is indicated in yellow and the 5’ 20 bp-long homology flank is indicated in blue. The *pyr4* gene is indicated in red. The 3’ 20 bp-long homology flank is indicated in orange and the 3’ 500 bp-long homology flank is indicated in green.

Table S1: Oligonucleotides

Name	Sequence (5' – 3')
CRISPR/Cas9 genome editing	
S. pyogenes Cas9 scaffold oligo	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
ura3 specific DNA oligo 1	TTCTAATACGACTCACTATAGACCGCAAGTTTGGCGACATGTTTTAGAGCTAGA
ura3_sgRNA1	GACCGCAAGUUUGGCGACAUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
ura3 specific DNA oligo 2	TTCTAATACGACTCACTATAGGCACCGACATCATCATTGTGTTTTAGAGCTAGA
ura3_sgRNA2	GGCACCGACAUCAUCAUUGUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
praics specific DNA oligo	TTCTAATACGACTCACTATAGGCCAACTTCAAGGCTGAGTGTTTTAGAGCTAGA
praics_sgRNA	GGCCAACUUCAAGGCUGAGUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
asl specific DNA oligo	TTCTAATACGACTCACTATAGACTCGCTAGAACTCATCCGGTTTTAGAGCTAGA
asl_sgRNA	GACUCGCUAGAACUCAUCCGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dl4 specific DNA oligo	TTCTAATACGACTCACTATAGGACCAGGATGTATGTTCAGGTTTTAGAGCTAGA
dl4_sgRNA	GGACCAGGAUGUAUGUUCAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
Sequencing of *ura3* in EXF-150	
ura3_fwd	ATGTCCCGTCACGCAACAATC
ura3_rev	TCACCTGCTACCTTTTGTTGC
Sequencing of *ura3* in ATCC 42023 and NBB 7.2.1	
ura3_2_fwd	GGCGACTCAATATGC
ura3_2_rev	ATGAAGCCCATGAC
Sequencing of *praics*	
praics_fwd	GTCACGCACAGAGGCATAC
praics_rev	GACGTCGTCGGTGATGTCG
Sequencing of *asl*	
asl_fwd	CTCAACCTCTAGAGATACTG
asl_rev	GGGTGATCATCTAACATAAC
diagnostic PCR	
pyrG_fwd	CTTGCTAGATGACTGGTAGG
pyrG_rev	ATGCATTTTTAGAGGATC
pyr4_fwd	ATATGAATTCGATCGTGCAATTTCCTCTCCTGG
pyr4_rev	ACTAGTGAGAGGCTGCTGCGATAGG

Cloning	
pyrG_fwd-AflII-NsiI	ATGCATTTTCTTAAGTAGAGGATCCACCAGTGATTG
pyrG_rev-EcoRI-AatII	GACGTCTTTTGAATTCCTTGCTAGATGACTGGTAGG
pyr4dl4_20bp_rev	AAACTTGTATGCGTAGACCTTCCTTTCCTAACCACTTCCCTACC
pyr4dl4_20bp_fwd	GAGATTATGGATGAGTTATTGATCGTGCAATTTCCTCTCCTGG
dl4_5Overlap_rev	AATAACTCATCCATAATCTCA
dl4_5Overlap500_fwd	GAGCTCGAATTCGTAATCATGATGCAGGTCCTGAATC
dl4_3Overlap_fwd	AGGTCTACGCATACAAGTTT
dl4_3Overlap500_rev	ACACAGGAAACAGCTATGACCAATGAGGACGTGATGATTG
pUC18_rev	CATGATTACGAATTCGAGCTC
pUC18_fwd	GTCATAGCTGTTTCCTGTGTGA
preparative PCR	
dl4_20bpover_rev	AAACTTGTATGCGTAGACCT
dl4_20bpover_fwd	GAGATTATGGATGAGTTATTGATC
dl4_500bpover_rev	CAATGAGGACGTGATGATTG
dl4_500bpover_fwd	ATGCAGGTCCTGAATC
diagnostic PCR + sequencing	
dl4_20test_fwd	ATGCAGGTCCTGAATC
dl4_500test_fwd	GCAGACGAACAGGTCAAAACG
pyr4_flank_rev	GCAGCGTAACATCTTCAGAGC
dl4_20test_rev	CAATGAGGACGTGATGATTG
dl4_500test_rev	CTTCTTAGCCTTGTTGACGTC
pyr4_flank_fwd	GATAATACCTACCCAGTCAGACC

Conclusion

A transformation protocol for *A. pullulans* was successfully developed by modifying and optimizing the protocol for PEG-mediated protoplast transformation of filamentous fungi. Furthermore, this work successfully demonstrates that Cas9-sgRNA RNPs can be used to disrupt one or several genes simultaneously without the introduction of foreign DNA and increase homologous recombination frequencies in *A. pullulans*, even with short homologous flanks. The genome of *A. pullulans* has already been successfully edited with CRISPR/Cas9 using the plasmid delivery strategy. However, the big advantages of using RNPs for introduction of Cas9 and sgRNA(s) in comparison to other CRISPR/Cas9 delivery strategies are little effort for generation of RNPs and limited off-target effects. Together with the developed transformation protocol the genome of *A. pullulans* can be edited fast and efficiently with Cas9 RNPs. This allows and facilitates elucidation of gene function and regulatory regimes and insertion of genetic elements, necessary for further research and the generation of powerful strains for application in industry.