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An Improved Transformation-Associated Recombination Cloning Approach for Direct Capturing of Natural Product Biosynthetic Gene Clusters

Olena Kurylenko[1,2] | Anja Palusczak[3] | Andriy Luzhetskyy[3] | Yuriy Rebets[1,2]

[1]Explogen LLC, Lviv, Ukraine | [2]German-Ukrainian Core of Excellence in Natural Products Research (CENtR), Lviv, Ukraine | [3]Pharmazeutische Biotechnologie, Universität des Saarlandes, Saarbrücken, Germany

Correspondence: Yuriy Rebets (info@explogen.com.ua)

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ABSTRACT

The phylum Actinomycetota and genus *Streptomyces* in particular are the major source for discovery of natural products with diverse chemical structures and a variety of biological activities. Genes encoding biosynthetic pathways for bacterial natural products are grouped together into biosynthetic gene clusters (BGCs). The size of a typical actinobacterial BGC may range from 10 kb to 200 kb, which makes their cloning for heterologous expression a challenging task. Various DNA cloning and assembly methods have been established for capturing BGCs. Among them, the transformation-associated recombination (TAR) in *Saccharomyces cerevisiae* remains one of the most cost-effective, accessible, customisable and precise approaches. However, the drawback of TAR cloning is a need for intensive screening of clones in order to identify one carrying the BGC. In this study, we report a further development of the TAR cloning approach by introducing the direct selection of colonies with BGC of interest based on the yeast killer phenomenon. For this, a new TAR cloning vector system was constructed and the strategy was validated by successful cloning of chelocardin (35 kb) BGC from *Amycolatopsis sulphurea* and daptomycin BGC (67 kb) from *Streptomyces filamentosus*. Both BGCs were functionally expressed in a heterologous host, resulting in the production of the corresponding antibiotics. The proposed approach could be widely applied for precise direct cloning of BGCs from the representatives of phylum Actinomycetota and easily adopted for other bacteria.

1 | Introduction

Despite the obvious progress in medicinal chemistry, the natural products and their derivatives still remain the leading players on the pharmaceutical market, especially among approved anticancer and antibacterial drugs (Newman and Cragg 2020). Actinomycetes, a group of phylum Actinomycetota, are regarded as one of the richest sources for discovery of diverse bioactive natural products, mainly due to the structural novelty, diversity and complexity of compounds produced as an outcome of their secondary metabolism (Wan, Ma, and Yuan 2023). The genes responsible for biosynthesis, self-resistance, regulation and transport of bacterial natural products are generally grouped together forming biosynthetic gene clusters (BGCs). With the significant development of DNA sequencing techniques and bioinformatic tools, the genome mining approach is widely applicable to identify potentially valuable BGCs within the actinobacterial genomes. However, the majority of these BGCs are

silent (cryptic) in their native hosts under laboratory conditions. One of the suitable strategies for activation of transcriptionally silent BGCs is their cloning and heterologous expression in well-characterised and genetically amenable hosts (Nah et al. 2017).

Despite significant progress of the bioinformatic tools for BGC identification and classification in the last 20 years, the cloning of large regions of the chromosome remains the main bottleneck in accessing the chemical diversity of bacterial secondary metabolism. The desired BGC should be isolated from genomic DNA, assembled with a suitable vector and subsequently transferred into a heterologous host for expression (Wang, Zheng, and Lu 2021). Several types of high-capacity vectors are available for cloning BGCs, including cosmids and artificial chromosomes. However, the application of cosmids is restricted by their cloning capacity (up to 45 kb). *Escherichia coli—Streptomyces* shuttle bacterial artificial chromosomal (BAC) vectors have been developed to carry the large-sized BGCs such as pStreptoBAC and pSBAC (Miao et al. 2005; Liu et al. 2009; Nah et al. 2017). However, the construction of BAC and cosmid libraries is laborious and expensive and results in cloning of random parts of the genome. This approach is more suited for massive cloning of BGCs from the genome rather than capturing the particular BGC of interest. Several in vitro or in vivo DNA assembly strategies have been developed recently to directly capture the microbial BGCs. Since the traditional restriction cutting and ligation methods have low efficiency and are enzyme site-dependent, recombination-based approaches are more attractive (Wang, Zheng, and Lu 2021). A DNA assembly method named CATCH utilises Cas9 digestion of genomic DNA in agarose-plaques followed by Gibson assembly with a linearised vector. However, this approach was not effective for cloning large BGCs (more than 50 kb) with high GC content (Jiang et al. 2015).

Currently, the most commonly used direct cloning and in vivo assembly approaches are based on a homologous recombination ability of yeasts or *E. coli* expressing Red/ET system (Du et al. 2015; Jiang et al. 2015; Lee, Larionov, and Kouprina 2015; Wang et al. 2018). Methods based on phage-recombinase mediated homologous recombination cloning in *E. coli* are represented by linear-linear homologous recombination (LLHR), linear-circular homologous recombination (LCHR) (Fu et al. 2012) and exonuclease combined with Red/ET (ExoCET) (Wang et al. 2018). Recently, a robust method named CAPTURE (Cas12a-assisted precise targeted cloning using in vivo Cre-*loxP* recombination) was developed by combining in vitro Cas12a-based treatment of genome and in vivo Cre-l*oxP* recombination in *E. coli* (Enghiad et al. 2021).

The transformation-associated recombination (TAR) cloning is employing the highly efficient recombination capacity of yeast *Saccharomyces cerevisiae* (Figure 1; Wang, Zheng, and Lu 2021). It was originally developed for isolation of large regions of mammalian chromosomes carrying ARS-like sequences (Larionov et al. 1997). TAR cloning was adapted for capturing microbial BGCs by constructing the yeast/*E. coli*/*Streptomyces* shuttle vector pTARa and pCAP01 (Kim et al. 2010; Yamanaka et al. 2014). The approach is based on cloning of 1-kb targeting sequences (hooks) homologous to the flanking regions of desired BGCs into the dedicated vectors, followed by co-transformation of linearised construct together with the genomic DNA into yeast cells. The approach was proven successful by cloning and expressing several different BGCs (Bonet et al. 2015; Jordan and Moore 2016; Wu et al. 2017; Rebets et al. 2019; Voitsekhovskaia et al. 2020). However, the efficiency of the TAR approach is 0.1%–2% due to vector recircularisation by non-homologous end joining (NHEJ) (Wang, Zheng, and Lu 2021). This means that the

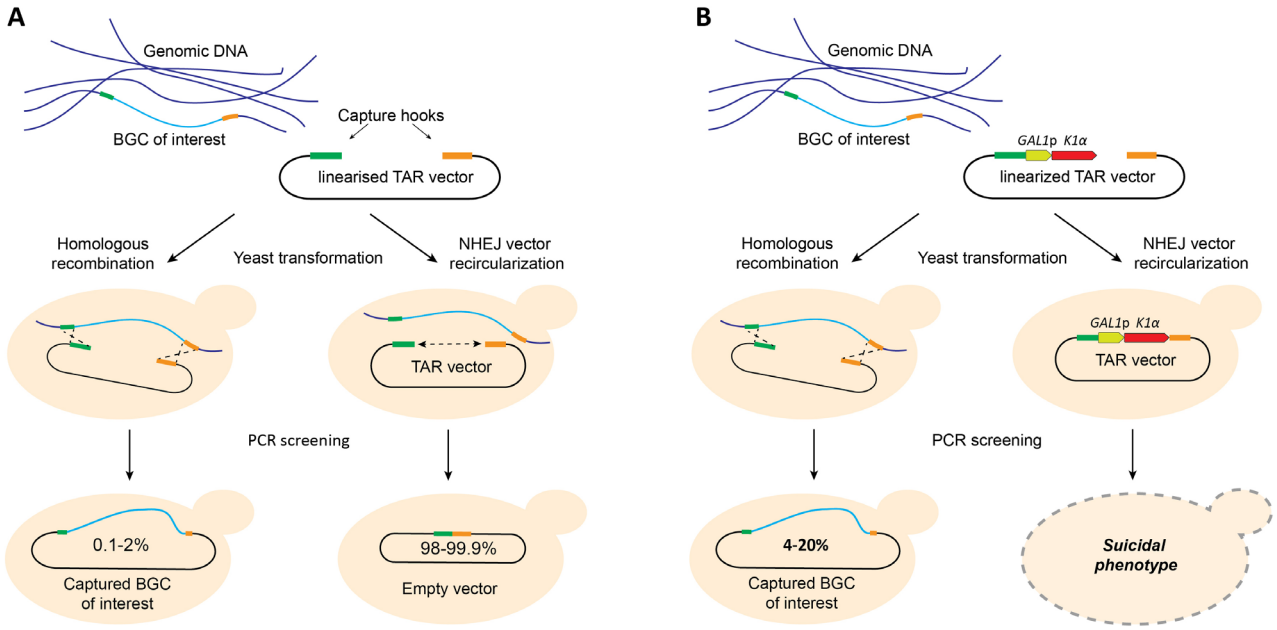

FIGURE 1 | The schematic representation of the TAR cloning procedure. The cloning of the desired region of genomic DNA is facilitated by homologous recombination in yeast cells. The TAR plasmid with two capture hooks is linearised with a restriction nuclease and co-transformed into *S. cerevisiae* together with the genomic DNA. At this step, two parallel processes take place: (1) homologous recombination, leading to cloning of the desired region of the genome, and (2) NHEJ repair of the linearised TAR plasmid; (A) TAR cloning involving a polymerase chain reaction (PCR)-based screening procedure. (B) TAR cloning with direct selection of BGC carrying clones based on the application of a counterselectable marker.

time-consuming and laborious screening of hundreds or even thousands of clones is needed in order to identify one carrying BGC of interest. To eliminate the empty vector background, the pCAP01 was modified by adding an *URA3* counterselectable marker (sensitivity to 5-fluoroorotic acid [5-FOA]), resulting in the pCAP03 (Tang et al. 2015). In pCAP03, the capture hooks are placed between the TATA box of the *ADH1* promoter and the transcription initiation site of *URA3*. This limits the size of capture sequences to 130 bp totally. The larger hooks cause inactivation of the *URA3* gene and consequent resistance to 5-FOA. On the other hand, the limited size of capture hooks (~60 bp each) resulted in low recombination efficiency. Huang et al. (2023) reported unsuccessful attempts to clone 23 and 25 kbp BGCs for nonribosomal peptides eponemycin and TMC-86A using pCAP03 with 50 bp hooks. Till date, *URA3* is the only reported counterselectable marker used in the TAR cloning (Wang, Zheng, and Lu 2021; Kouprina and Larionov 2023).

Some strains of yeast are known to exert the so-called killer phenomenon. It is based on the ability of killer strains to kill sensitive yeast cells by producing and secreting extracellular protein toxins (Schmitt and Breinig 2006). The phenomenon was discovered in *S. cerevisiae* infected with the M1 double-stranded RNA 'killer' virus, which makes them produce one of the best studied killer toxins named K1 (Gier et al. 2020). K1 is produced as precursor preprotoxin, which after processing is secreted as a functional heterodimeric protein consisting of K1α and K1β subunits. The K1α acts as an ionophore leading to the disruption of membrane integrity by forming cation-selective channels and finally causing cell death (Gier, Schmitt, and Breinig 2017). The K1β subunit facilitates the toxin binding to the target cell and the α subunit exerts the toxic effect. The intracellular expression of K1α in *S. cerevisiae* mimics the action of externally applied toxin resulting in suicidal phenotype.

Herein, we report the development of the TAR cloning approach employing the α subunit of K1 toxin as a counterselectable marker (Figure 1B). It enables direct cloning of large BGCs in *S. cerevisiae*, stably maintaining and manipulating the cloned BGC in *E. coli*, and heterologous expression in *Streptomyces* hosts.

2 | Experimental Procedures

2.1 | Strains, Media and Growth Conditions

A. sulphurea NRRL 2822 and *S. filamentosus* NRRL 15998 were used as a source of DNA for cloning chelocardin (CHD) BGC and daptomycin (DPT) BGC, respectively. *S. albus* Del14 (Myronovskyi et al. 2018) were used as heterologous hosts for BGC expression. *E. coli* GB2005 was used for standard cloning procedures (Gene Bridges, Germany). *E. coli* WM6026 served as a donor in intergeneric conjugation (Circello et al. 2010). *S. cerevisiae* BY4742 ΔKu80 (Dr. M. Lopatniuk, personal communications) was used as a host for TAR cloning. *S. cerevisiae* was grown in liquid YPD medium (1% yeast extract, 1% peptone, 2% D-glucose) prior to transformation. Yeast transformants were selected on YNB (6.7 g/L Yeast Nitrogen Base without amino acids, 1.6 g/L yeast synthetic drop out medium supplements without leucine, 20 g/L glucose or galactose) (Sigma-Aldrich, USA). Actinobacterial strains were grown on mannitol soy flour agar (MS agar) (Kieser et al. 2000) and in a liquid tryptic soy broth (TSB) medium (Sigma-Aldrich, USA). For daptomycin and chelocardin production, solid MS agar medium was used. *E. coli* strains were cultivated in liquid or on solid LB medium (Sigma-Aldrich, USA) at 37°C. Yeast and actinobacteria were grown at 30°C. Antibiotics fosfomycin (50 μg/mL), ampicillin (100 μg/mL), kanamycin (50 μg/mL) and apramycin (50 μg/mL) were used when required (Carl Roth, Germany, Sigma-Aldrich, USA).

2.2 | Isolation and Manipulation of DNA

DNA isolation and manipulation, *E. coli* transformation and *E. coli*/*Streptomyces* intergeneric conjugation were performed according to standard protocols (Kieser et al. 2000; Sambrook, Fritsch, and Maniatis 2001). Dream Taq polymerase (Thermo Fisher Scientific, USA) was used in cloning experiments and for PCR-based colony screening and final constructs verification. DNA fragments were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Germany). Total DNA from *S. cerevisiae* was isolated by the standard protocol with lyticase treatment (Sambrook, Fritsch, and Maniatis 2001). Restriction enzymes and ligase were used according to manufacturer recommendations (New England Biolabs, USA). All primers used in this study are listed in Table S1 (Genewiz, Germany).

2.3 | Construction of the Capture Vectors

The fragment consisting of replication origin ARSH4/CEN6, *LEU2* auxotrophic marker and origin of DNA transfer (oriT) was retrieved from the yeast centromeric plasmid pCLY10 by *Bam*HI/*Pst*I (Bilyk et al. 2016). The *E. coli* replication origin ori15A, the *aac(3)IV* gene (apramycin resistance) and *Streptomyces* elements consisting of the *φC31* integrase gene (*int*) with its attachment site (attP) were retrieved from the cos15A_AmInt plasmid (prof. A. Luzhetskyy, personal communications) by *Bam*HI/*Pst*I digestion. Both fragments were assembled by T4 DNA ligase resulting in a vector pEXG1. The K1α toxin under control of the galactose inducible *GAL1* promoter was designed in silico and synthesised by Genewiz GmbH (Germany) with standard cloning into the *Eco*RV site of pUC57 (resulting plasmid pK1). The gene, conferring resistance to kanamycin, was retrieved from the plasmid cos15A_KanInt (Dr. I. Ostash, Explogen, unpublished data) by digesting with *Bam*HI and cloned into *Bgl*II-linearised plasmid pK1. The resulting plasmid was named pK1-Kan. The *Eco*RI/*Hind*III-fragment containing K1α toxin cassette was isolated from the plasmid pK1-Kan and cloned into the *Bam*HI-linearised vector pEXG1 after blunting with T4 DNA polymerase. The resulting plasmid was named pEXG1-K1 and used for validation of TAR cloning strategy.

The BGC-specific capture hooks (around 1 kb) were amplified from the genomic DNA of *A. sulphurea* NRRL 2822 and *S. filamentosus* NRRL 15998 using appropriate primers (Table S1). In both cases, *Eco*RI/*Xba*I-digested left hooks and *Bam*HI/*Hind*III-digested right hooks were subcloned into corresponding sites of pK1-Kan. Resulting plasmids pK1-CHD-Kan and pK1-DPT-Kan

were *EcoRI*/*Hind*III digested and fragments carrying hooks flanking the K1α toxin cassette were end-blunted with T4 DNA polymerase and cloned into the *Bam*HI-linearised and end-blunted vector pEXG1. The resulting constructs for capturing the chelocardin and daptomycin BGCs were named pEXG1-K1-CHD and pEXG1-K1-DPT, respectively. Alternatively, the vector for capturing the CHD BGC lacking K1α toxin cassette was constructed. For this, pEXG1-K1-CHD was digested by *Pvu*II and self-ligated to remove the K1α toxin cassette, resulting in a plasmid pEXG1-CHD.

2.4 | Direct Capture of the CHD and DPT BGCs

Genomic DNA was isolated from cells of *A. sulphurea* NRRL 2822 and *S. filamentosus* NRRL 15998 by a standard salting out protocol (Kieser et al. 2000). Briefly, strains were grown at 30°C in 15 mL of TSB medium (Condalab, Spain) using 100 mL flasks containing glass beads until the late exponential growth phase. The cultures were centrifuged at 3000×*g* for 10 min. After removing the supernatant, the cells were resuspended in 10 mL of buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris–HCl pH 7.5) containing 20 mg of lysozyme and 0.5 mg of RNAse A. The mixture was incubated at 37°C for 1 h. Following this, 1 mL of 10% sodium dodecyl sulphate (SDS) and 6 mg of proteinase K was added, gently mixed and incubated at 55°C for 1 h until the solution became clear. Recovery of genomic DNA from cell lysate was performed by chloroform extraction. A total of 11 mL of chloroform was added to the cell lysate. The mixture was placed on a rocker to allow for gentle mixing without creating emulsion. Once the aqueous phase became completely white, the sample was centrifuged at 10,000×*g* for 10 min at room temperature. Without disturbing the precipitated proteins, the aqueous phase was aliquoted into 2 mL centrifuge tubes and the DNA was recovered using isopropanol precipitation. The DNA pellets were washed with 70% ethanol (v/v) and air dried. The DNA was rehydrated in MQ water and incubated at 37°C until fully dissolved and stored at 4°C.

Prior to TAR cloning, the capture constructs pEXG1-K1-CHD, pEXG1-CHD and pEXG1-K1-DPT were isolated using the Plasmid Miniprep Kit (Biomiga, USA) and linearised by *Bam*HI or *Bgl*II. The isolated genomic DNA and capture constructs were co-transformed into *S. cerevisiae* BY4742 ΔKu80 using a standard LiAc/ss carrier DNA/PEG yeast transformation protocol (Gietz and Schiestl 2007). Briefly, *S. cerevisiae* BY4727 ΔKu80 was grown in 50 mL of YPD medium at 30°C with shaking until an OD600 of 0.7–1.0 was reached. The cells were harvested, washed twice with water and resuspended in a transformation mix containing 36 μL of 1 M LiAc solution, 50 μL of 2 mg/mL carrier DNA (salmon sperm DNA) solution, 240 μL of 50% (w/v) PEG 3350 solution and 34 μL of Tris-EDTA containing approximately 1 μg of capture plasmid and 2 μg of genomic DNA. Transformation mix was incubated at 42°C for 40 min. Cells were then collected by centrifugation, resuspended in 100 μL of water and plated on YNB without leucine agar plates with glucose or galactose as a carbon source. The plates were incubated at 30°C until colonies appeared. The total DNA was isolated from yeast colonies and analysed by PCR. The total DNA from PCR-positive yeast clones which included the plasmids carrying BGCs of interest was transferred into *E. coli* GB2005 by electroporation. Plasmids carrying desired BGCs were recovered from *E. coli* GB2005 after selection on apramycin containing medium and verified by PCR and endonuclease restriction analysis.

2.5 | Heterologous Expression of CHD and DPT BGCs

Constructs pEXG1_CHD and pEXG1_DPT carrying the CHD and DPT BGCs, respectively, and empty control vector pEXG1 were transformed into *E. coli* WM6026, which was used as a donor strain for intergeneric conjugation with *S. albus* Del14. The conjugation-mediated transfer of constructs was conducted according to the standard protocol (Kieser et al. 2000). MS agar medium supplemented with 10 mM MgCl2 was used for the conjugation. Plates were overlaid with apramycin (50 μg/mL) and fosfomycin (50 μg/mL) after 7 hours of incubation in order to select for exconjugants and kill the *E. coli* donor strain. The exconjugants were plated by patches onto MS agar containing apramycin, followed by CHD and DPT production analysis.

2.6 | Antibacterial Assay

The antimicrobial activities of the obtained exconjugants were assessed using an agar blocks diffusion method. The bioassay was performed using *Kocuria rhizoplila* DSM 348 as an indicator organism. The *S. albus* Del14 strains carrying pEXG1_CHD and pEXG1_DPT constructs were grown on MS agar plates for 5 days at 30°C. A 30 μL of the overnight culture of *K. rhizoplila* was mixed with 20 mL of LB agar and poured into the Petri dish. After solidification of the medium, 8 mm diameter agar blocks with actinobacterial strains were placed onto LB plates carrying *K. rhizoplila*. Plates were incubated for 24 h at 30°C, and the diameter of the inhibition zone surrounding the agar blocks, resulting from the diffusion of compounds, was measured.

2.7 | Analysis of Secondary Metabolites Production

S. albus Del14 strains carrying pEXG1_CHD and pEXG1_DPT were cultured on MS agar plates for 5 days as described in Antibacterial assays. Medium was cut into slices and packed into 50 mL centrifuge tubes, and metabolites were extracted with 15 mL of methanol. The methanol was evaporated on Eppendorf Concentrator Plus (Eppendorf, Germany), and the pellet was resuspended in 200 μL of methanol. The LC-HRMS data were collected on a Dionex Ultimate 3000 RSLC system (Thermo Fisher Scientific, USA) with the maXis 4G hr-ToF (Bruker Daltonics, Germany). Samples were separated on a BEH C18, 100×2.1 mm, 1.7 μm dp column (Waters, Germany). Separation of the 1 μL sample was achieved by a linear gradient of solvent B (acetonitrile with 0.1% of formic acid) against solvent A (water with 0.1% of formic acid) at a flow rate of 600 μL/min and 45°C. The gradient started by a 0.5 min isocratic step at 5% B and then increased to 95% B over 18 min to end up with a 2 min step at 95% B before re-equilibration under the initial conditions. UV spectra were acquired by a DAD detector in the range of 200–600 nm. The mass spectrometry data were collected on a maXis 4G hr-ToF

ultrahigh resolution mass spectrometer (Bruker Daltonics, Germany) using the Apollo II ESI source. Mass spectra were acquired in centroid mode ranging from 200 to 2500 m/z at a 2 Hz scan rate.

Data were collected and analysed with the Bruker Compass Data Analysis software, version 4.1 (Bruker, Billerica, USA). The identification of DPT and CHD was performed using the Dictionary of Natural Products database version 6.1 (CRC Press, Boca Raton, USA) and PubChem database (NCBI), using the accurate molecular mass and absorption spectra as criteria. Compounds were considered to be similar when the difference in accurate mass was <3 ppm and absorption spectra were identical.

3 | Results

3.1 | Construction and Verification of the TAR Cloning System

TAR cloning is a promising approach for direct recovery of large regions of the genomic DNA for further manipulations (Figure 1). The success of the approach depends on two main factors: the high quality and integrity of genomic DNA and availability of an appropriate vector system capable of carrying large-size BGCs and providing their maintenance in *E. coli*, *S. cerevisiae* and actinomycetes (Kouprina and Larionov 2023). The bottleneck of this approach is caused by the activity of the NHEJ phenomenon in yeast, which results in a high rate of empty clones. The use of *URA3* as a counterselectable marker simplified the identification of positive clones; however, the design of the vector resulted in limited overall efficiency of the system (Tang et al. 2015).

In order to improve the application of the TAR cloning for actinobacterial BGC recovery, the new TAR vector system was constructed with the use of yeast killer K1 toxin as a counterselectable marker (Figure 2). The system consists of two plasmids, pEXG1 and pK1. The pEXG1 is a capture vector, which has a medium copy number in *E. coli* due to use of ori15A for stable maintenance of large DNA fragments and carries the apramycin resistance gene (*aac(3)IV*) for selection in both *E. coli* and *Streptomyces* (Figure 2B). For maintenance in yeast, the vector is equipped with an ARS element (ARSH4) and a positive-selectable marker (*LEU2*). The pEXG1 contains origin of transfer (*oriT* from RK2) and φC31 actinophage integration elements for site-specific integration into chromosomes of actinobacterial host. The pK1 is an assembly vector allowing the assembly of capture construct with the K1α toxin counterselectable marker. The gene coding for the toxic α-subunit of K1 toxin (105 amino acids) under transcriptional control of the galactose inducible *GAL1* promoter and *CYC1* terminator was designed in silico and synthesised (Figure 2). The cassette was cloned into the *Eco*RV site of pUC57, giving the pK1. To simplify the transfer of capture construct into pEXG1, the kanamycin resistance gene was introduced into the K1α cassette. The final plasmid pK1-kan is built in a way to allow for cloning of BGC-specific capture hooks on both sides from the K1α cassette. The recombination between the 'hooks' and homologous regions of genomic DNA will result in loss of K1α cassette, providing the survival of yeast cells in the presence of galactose. The clones carrying recircularised plasmid as a result of NHEJ activity will have the K1α function restored and thus will be eliminated in the presence of the inducer.

In order to test the functionality of the K1α counterselectable marker, the cassette was subcloned into pEXG1, giving pEXG1-K1. Both the parental vector and pEXG1-K1 were introduced into *S. cerevisiae* BY4742 ΔKu80 (Figure 2C). The transformants were directly plated on YNB with either glucose (2%), galactose (2%) or a mixture of both sugars (0.05% glucose with 2% galactose). The ability of recombinant strains to grow in the presence of these carbon sources was compared. Transformants carrying pEXG1 were able to grow in all tested conditions. In contrast, strain carrying pEXG1-K1 was growing well under non-inducing conditions (glucose as a carbon source). However, the expression of K1α toxin resulted in a strong suicidal phenotype, indicated by inability to grow on the medium with galactose or galactose and glucose (Figure 2C).

3.2 | Validation of the Efficiency of a Developed TAR Cloning System

The efficiency of the developed TAR system was tested by cloning BGC for CHD (35 kb) from *A. sulphurea* and DPT (67 kb) from *S. filamentosus* (Choi et al. 2019; Lukezic et al. 2020). In both cases, gene-specific targeting sequences (around 1 kb) were cloned in a way to flank the K1α toxin cassette. As a result, recombinant plasmids pEXG1-K1-CHD and pEXG1-K1-DPT were constructed. In addition, the CHD BGC capture plasmid pEXG1-CHD, the pEXG1-K1-CHD derivative lacking the K1α cassette, was constructed in order to compare the efficiency of selection-based and screening-based approaches. The plasmids were linearised by *Bam*HI (cutting between the right capture hook and K1α toxin cassette) or *Bgl*II (cutting between the K1α gene and kanamycin resistance gene) (Figure 2A) and co-transformed into *S. cerevisiae* BY4742 ΔKu80 together with genomic DNA of the strain, carrying the BGC of interest. The selection of yeast transformants was performed on a medium containing 2% galactose and 0.05% glucose. The glucose was added to support the recovery of yeast cells after transformation. Obtained yeast transformants were screened by PCR using three pairs of primers for each particular BGC. Two sets of primers anneal to the capture hooks and adjacent region of the respective BGC (Figure S1). The third set was designed for the unique internal region sequences of the target BGC. In the case of CHD, BGC 2-positive clones were identified after screening of 15 yeast transformants (13% positives). In contrast, the conventional approach without the counterselection step resulted in 0.26% (1 positive clone out of 380 colonies tested) efficiency of cloning of the CHD BGC. In the case of DPT BGC, the efficiency of cloning was slightly lower (4%), with two positive clones identified after screening of 47 transformants. The total DNA was isolated from positive yeast clones and re-transformed into *E. coli*. Plasmids were recovered from *E. coli* and the cloning of CHD and DPT BGCs was confirmed by restriction analysis (Figures S2 and S3). The resulting plasmids carrying CHD or DPT BGC were named pEXG1_CHD and pEXG1_DPT, respectively.

At the same time, the plasmid DNA from one negative clone from each experiment were also recovered and sequenced from

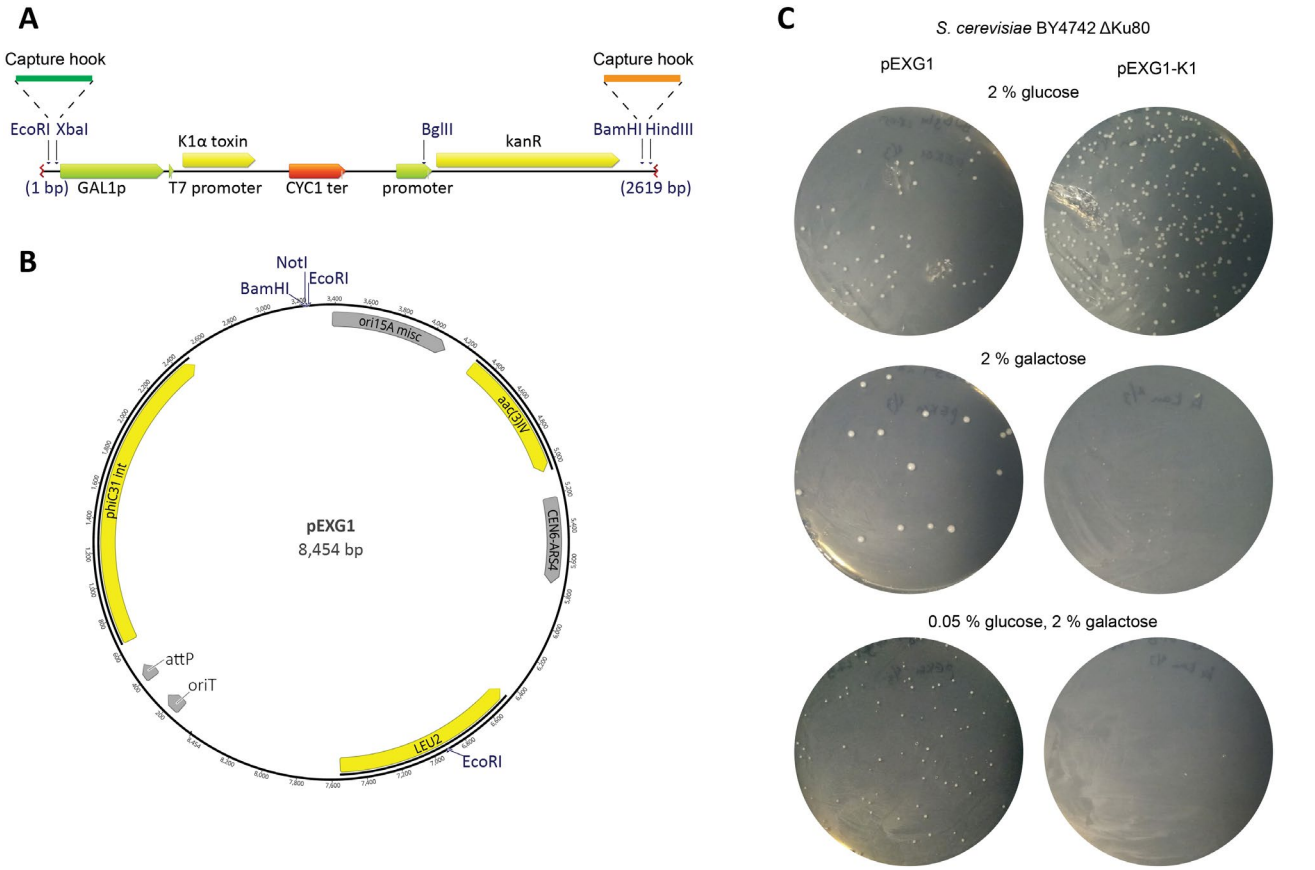

FIGURE 2 | TAR cloning system based on a K1α toxin counterselectable marker. (A) Schematic representation of the K1α toxin cassette. Coding sequences are highlighted in yellow, promoters—green, transcriptional terminator—red. (B) Schematic representation of the pEXG1 TAR cloning vector. (C) Growth of transformants of *S. cerevisiae* BY4742 ΔKu80 carrying pEXG1-K1 plasmid under non-inducing conditions (glucose as a carbon source) and inability to grow on the medium with galactose due to expression of K1α toxin. As a control pEXG1 plasmid without the K1α toxin cassette was used.

primers annealing either to the ends of capture hooks or *GAL1* promoter. In a case of DPT experiment, the entire K1α toxin cassette was deleted (Figure S4). At the same time, in case of survival plasmid from CHD BGC cloning experiment, the *E. coli* IS4 family element was found in place of the K1α coding sequence, while the other parts of K1 toxin cassette were preserved (Figure S5). In both cases, the structural perturbations in the K1α cassette were observed, which led to the loss of its function. These changes most probably are caused by the activity of NHEJ system, which is either random and uncontrollable, even in the case of deletion of the Ku protein encoding gene from the chromosome of *S. cerevisiae*.

3.3 | Heterologous Expression of Cloned BGCs and Production Analysis

The design of pEXG1 capture vectors allows for direct expression of cloned BGCs in the actinobacterial heterologous host. The cloned BGCs were introduced into *S. albus* Del14. The strain is lacking its own secondary metabolism due to the deletion of 14 BGCs (Myronovskyi et al. 2018). The use of such a host strain simplifies the identification of heterologously produced metabolites either by detection of specific activity or by detecting corresponding compounds with the analytical methods. Two constructs carrying CHD BGC (CHD-1 and CHD2) and two constructs with DPT BGC (DPT-1 and DPT-2) were transferred into *S. albus* Del14. Exconjugants were cultivated on solid MS medium for 5 days, and the antimicrobial activity was tested by the agar blocks diffusion method using *K. rhizophila* as a test culture (Figure 3). Both DPT and CHD are known to be active against Gram-positive bacteria and the use of *S. albus* Del14 significantly simplify the detection of compounds. The activity was estimated by the diameter of the growth inhibition zone caused by local confrontation between actinomycetes and test bacteria. In both cases, the BGCs were functionally expressed resulting in accumulation of corresponding compounds, when judged by the antimicrobial activity (Figure 3). However, the DPT-2 construct was found to be inactive. Restriction analysis revealed significant difference between of DPT-1 and DPT-2 constructs. The major part of DPT BGC seems to be missed in DPT-2 construct (Figure S3). It is hard to say the reasons for this without sequencing of the entire construct, but it is possible to predict that the recombination event(s) might have occurred within such large NRPS BGC like DPT, leading to its malfunctioning.

In order to confirm the heterologous production of both antibiotics, the extracts from the cultures used in the antimicrobial assay were prepared and analysed by high resolution LC–MS. In case of *S. albus* Del14 carrying CHD BGC, a peak with the RT of 7.2 and m/z 410.1250 $[M\text{-}H]^-$ was identified (Figure 4). This corresponds to the m/z of CHD in negative mode with the mass error below 3 ppm (CID 71402, calculated m/z 410.1245 $[M\text{-}H]^-$).

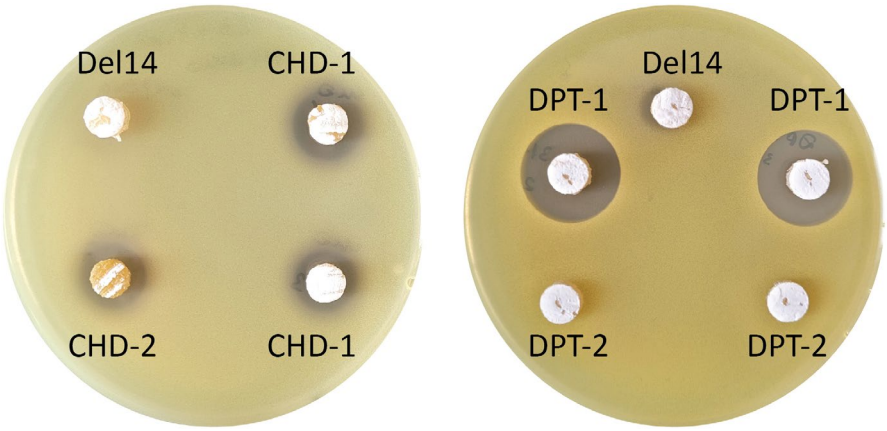

FIGURE 3 | Antimicrobial activity of recombinant strains of *S. albus* Del14 harbouring cloned chelocardin (CHD-1 and CHD-2) and daptomycin (DPT-1 and DPT-2) BGCs. *S. albus* Del14 carrying empty pEXG1 vector (Del14) was used as a control.

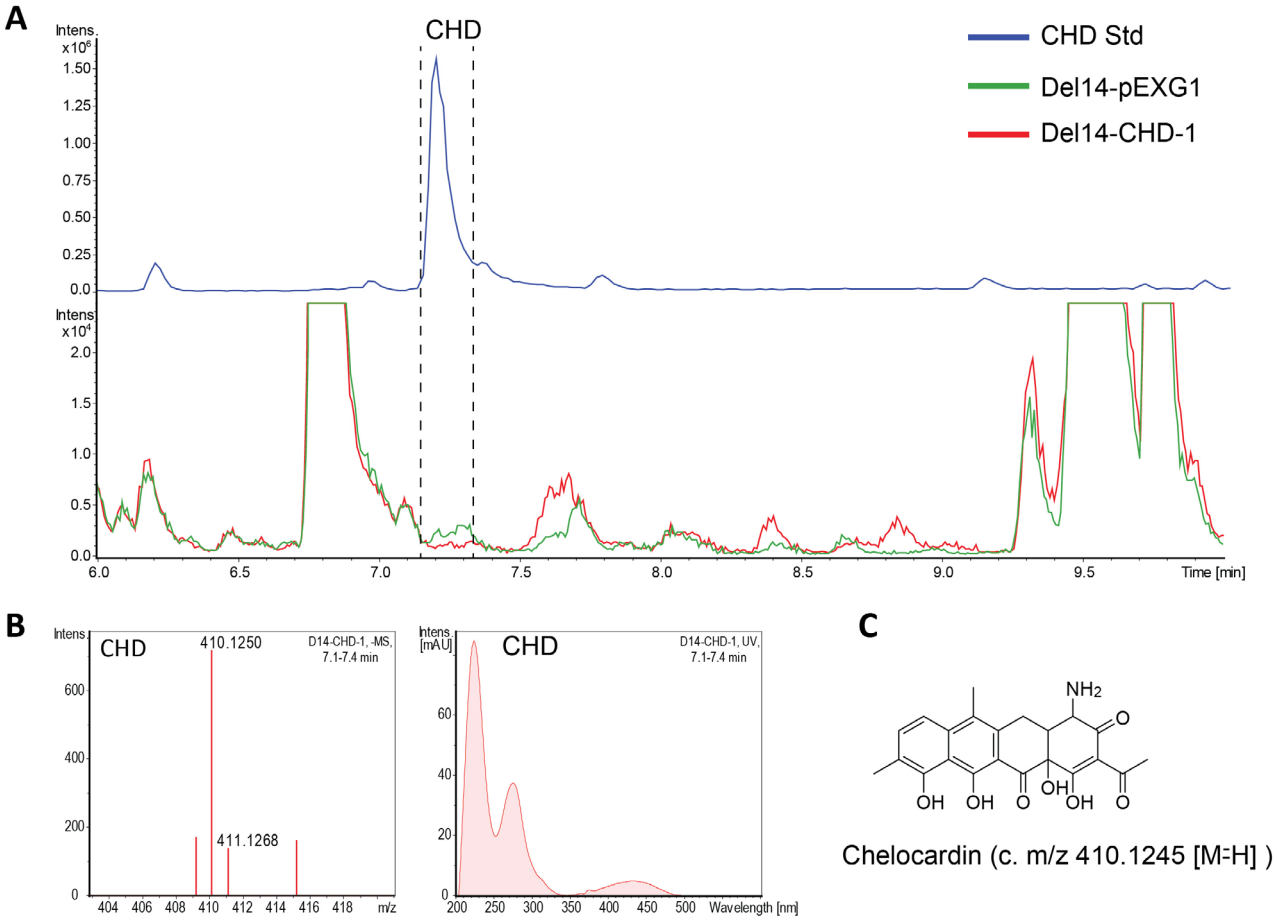

FIGURE 4 | LC–MS based identification of chelocardin production by *S. albus* Del14 carrying cloned CHD BGC (CHD-1). (A) Base peak chromatogram of pure chelocardin sample (blue) and extracts of metabolites from *S. albus* Del14 cultures with pEXG1 vector (red) and pEXG1_CHD construct with cloned CHD BGC (green). Peak with RT of 7.2 min corresponds to chelocardin. (B) Mass spectra and UV absorption spectra of peak with RT of 7.2 min from the extract of *S. albus* Del14 pEXG1_CHD (CHD-1). (C) Structure and monoisotopic mass of chelocardin (PubChem CID 71402).

In the case of DPT producer *S. albus* DPT-1, several peaks were identified, which are absent in the parental strain (Figure 5). One of them, with RT of 9 min and m/z 1618.70 $[M\text{-}H]^-$, corresponds to daptomycin (CID 16134395, calculated m/z in negative ionisation 1618.7030 $[M\text{-}H]^-$, mass error 2.9 ppm). However, the main products of the strain were compounds with m/z 1632.72, 1646.73 and 1660.75 $[M\text{-}H]^-$. These metabolites were identified as a daptomycin derivatives known as A21978C1 (CID 16131420, calculated m/z 1632.7187 $[M\text{-}H]^-$), A21978C2 (CID 16132068, calculated m/z 1646.7343 $[M\text{-}H]^-$) and A21978C3 (CID 16132067, calculated m/z 1660.7500 $[M\text{-}H]^-$). Besides that, several other peaks with characteristic large masses were present in the extract of *S. albus* Del14 carrying DPT BGC, which are absent in the extract of the parental

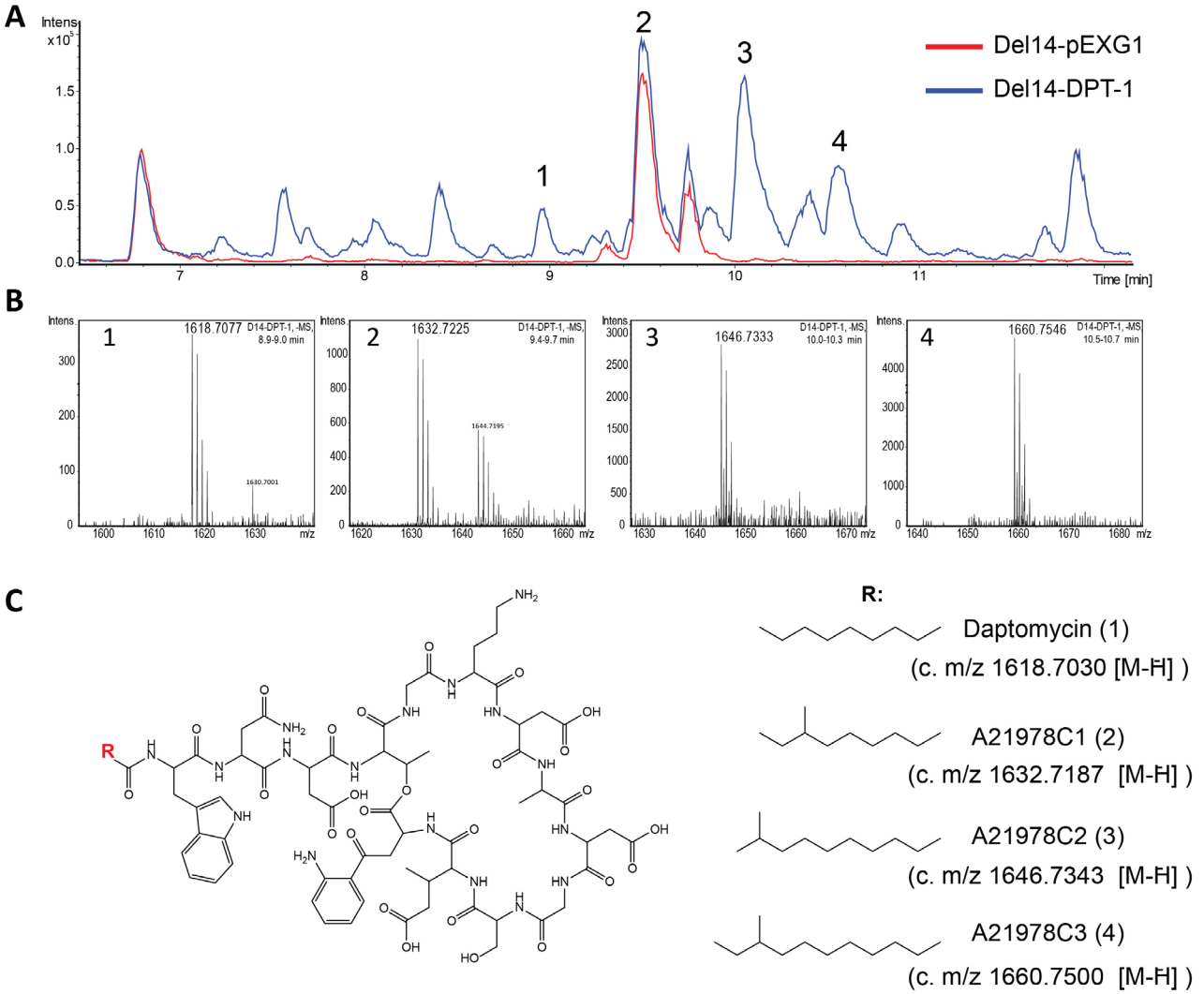

FIGURE 5 | LC–MS based identification of daptomycin and its derivatives production by *S. albus* Del14 caring cloned DPT BGC (DPT-1). (A) Base peak chromatogram of metabolites extracts from *S. albus* Del14 cultures with pEXG1 vector (red) and pEXG1_DPT construct with cloned DPT BGC (blue). Peaks which correspond to DPT and its derivatives are labelled as 1–4. (B) Mass spectra of peaks 1–4 from the extract of *S. albus* Del14 pEXG1_DPT (DPT-1). (C) Structures and monoisotopic masses of DPT (1) and its derivatives compounds A21978C1-3 (2–4) identified in the extract of *S. albus* Del14 pEXG1_DPT (DPT-1).

strain. However, these compounds could not be identified by LC–MS data deconvolution despite their obvious origin from the DPT biosynthetic pathway.

4 | Discussion

TAR cloning is a cost-effective approach allowing for direct cloning of large regions of the genome. It is used in diverse applications including cloning and expression of bacterial natural products BGCs (Kouprina and Larionov 2023). For the latter one, the dedicated shuttle vectors allowing for the migration of the cloned BGC from yeast through *E. coli* to actinomycetes were constructed and widely used (Kim et al. 2010; Yamanaka et al. 2014). Despite the obvious advantages of the method, it has a significant drawback—the efficiency of cloning is very low, ranging from 0.1% to 2% (Figure 1A; Wang, Zheng, and Lu 2021). This is why intensive screening is required in order to identify the clone(s) carrying the BGC of interest. Sometimes several thousands of clones have to be analysed. In this study, an improved strategy for direct and precise cloning of BGCs from actinobacterial genomes is described. The strategy is based on application of yeast K1 toxin as a counterselectable marker in the TAR cloning approach. The use of K1α toxin allowed switching from screening to selection, which significantly simplified and sped up the identification of clones carrying the BGC of interest (Figure 1B). The strategy was validated by cloning and expressing the CHD BGC from *A. sulphurea* NRRL 2822 (35 kb) and the DPT BGC from *S. filamentosus* NRRL 15998 (67 kb). CHD belongs to the tetracycline group of aromatic polyketides and exhibits a broad-spectrum antibiotic activity. Both BGCs were previously cloned with the use of laborious and costly methods. The CHD BGC (35 kb) was cloned by cosmid library construction and successfully expressed in *S. albus* (Lukežič et al. 2020). The DPT BGC (65 kb) was isolated from *S. roseosporus* ATCC31568 using a *Streptomyces* artificial chromosome vector system (Choi et al. 2019). The TAR approach described in this work is faster, less laborious and more cost-efficient than library-based

capturing of BGCs. TAR cloning in combination with direct clones' selection is a viable alternative to massive cloning approaches when a single specific BGC is the focus of research.

The phenomenon of killer (K) toxin production and immunity to the own toxin is widely spread among yeasts and is not restricted to the genus *Saccharomyces*. The most studied are K1, K2 and K28 toxins from *S. cerevisiae* strains infected with M-dsRNA satellite virus (Schmitt and Breinig 2002). Toxin-producing killer yeasts provide not only a convenient model system for studying the host–virus interactions, but have found their applications in environmental biotechnology (for biological control of plant pathogens), medicine (the development of novel antimycotics for the treatment of human and animal fungal infections), food and fermentation industries (to combat contamination in the production of cheese and wine) and in the field of recombinant DNA technology (Serviene and Serva 2023). However, somehow this phenomenon was neglected as a possible marker for genetic manipulations in yeast.

To our knowledge, this is the first report of successful application of K1 toxin as a yeast counterselectable marker in general and in TAR cloning of bacterial natural product BGCs in particular. The application of K1α in cloning of CHD and DPT BGCs significantly increased the efficiency of the procedure and helped avoid the screening step. In fact, the commonly used yeast marker *URA3* conferring resistance to 5-FOA was also previously adapted for TAR cloning applications. However, the original design with very short capture hooks, despite being very effective in eliminating empty clones, has limited cloning capabilities due to low efficiency of recombination. The nonribosomal peptides eponemycin (23 kb) and TMC-86A (25 kb) BGCs were cloned only after changing the strategy and increasing the size of capture sequences to 1 kb (Huang et al. 2023). Even so, the application of *URA3* is limited due the use of 5-FOA, which is regarded as highly toxic towards humans and mammals (PubChem CID 69711) (Boeke et al. 1987).

The K1α toxin in contrast does not require any supplements to be used. Furthermore, the proposed design when the K1α encoding gene is under transcriptional control of the *GAL1* promoter allows to fine-tune the system performance (Weinhandl et al. 2014). In fact, we did not observe any toxic effect of K1α when the yeast strains carrying the K1α cassette were grown on glucose containing media. Furthermore, galactose could be directly used both as an inducer of the K1α cassette expression and as a carbon source to support yeast growth. We also noticed that the general efficiency of transformation as well as TAR cloning could be improved when 0.1% or 0.05% of glucose is added to the selection media (Figure 2C). In such a case, the galactose transport into the cell as well as activity of catabolic repression dependent promoters, including *GAL1*p, are temporally attenuated, allowing yeast cells to recover after transformation procedure before the onset of K1α toxin production (Flick and Johnston 1990). On the other hand, the delayed accumulation of toxin could lead to an increased number of negative clones. However, even in such a case, the efficiency of the system is significantly higher when compared with the traditional screening-based approach.

The main cause of empty clones is the activity of the NHEJ system leading to recircularisation of the capture plasmid (Figure 1; Wang, Zheng, and Lu 2021; Wan, Ma, and Yuan 2023). NHEJ is a part of DNA repair mechanism allowing cells to survive the DNA double-strand breaks (Emerson and Bertuch 2016). The system recognises linear capture plasmid as an alteration in DNA structure and performs its cyclisation. At the same time, part of the plasmid close to the break is removed. The process is unpredictable and not controllable. In fact, the deletion of the Ku encoding gene from the genome of *S. cerevisiae* did not improve the situation. This is why, even with the use of counterselectable markers, the 100% efficiency of TAR cloning is hard or even impossible to achieve. We found that recircularised plasmids have structural changes in the region of K1α toxin cassette.

Furthermore, the TAR cloning efficiency also depends on the size of the targeted BGC. In the case of smaller size BGCs, like eponemycin and TMC-86A, the application of direct selection by a counterselectable marker decreased the plasmid recircularisation to 0%–33% (66%–100% efficiency) (Huang et al. 2023). At the same time, the efficiency of cloning of larger CHD BGC (35 kb) and DPT BGC (67 kb) was 13% and 4%, respectively. It is still significantly higher than 0.26% observed for CHD BGC when cloned by the screening rather than selection procedure. However, it is obvious that with the increase in size of the targeted genomic region, the rate of negative clones increases. The quality of genomic DNA is crucial for cloning large-size genomic fragments. At the same time, the recombination frequency was improved when the genomic DNA was enzymatically pre-treated with restriction endonucleases (Hu et al. 2018). The homologous recombination is much more efficient between capture hooks of the TAR plasmid and linearised fragments of genomic DNA located closer to the free ends. As an alternative, the CRISPR/Cas9-mediated DNA cleavage was used to introduce double-strand breaks when cloning large (> 100 kb) BGC since it is practically impossible to select suitable restriction endonuclease in such a case (Lee, Larionov, and Kouprina 2015; Zhang et al. 2023).

Past decades of bacterial natural products research led to the change in paradigm of the entire field. With the development of genomics, bioinformatics, synthetic biology and metabolic engineering tools and instruments, the strategy 'from compound to gene' is replaced with the 'from gene to compound'. However, such a shift to reverse genetics approach requires efficient tools and methods for BGC cloning and manipulating. Different technologies for cloning natural products BGCs have been established (Wang, Zheng, and Lu 2021). Among them, the TAR technology is one of the most potent for capturing the particular BGC. With the introduction of K1α toxin-based selection, the approach efficiency is significantly improved, solving its major drawback and expanding the range of applications in cloning of BGCs not only from microorganisms, but also potentially from environmental DNA samples.

Author Contributions

Olena Kurylenko: methodology, data curation, validation, investigation, visualization, writing – original draft. **Anja Palusczak:** methodology, resources. **Andriy Luzhetskyy:** data curation, validation, formal analysis, resources, writing – review and editing. **Yuriy Rebets:** conceptualization, data curation, supervision, writing – review and editing, formal analysis, visualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.