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Genetic approaches to improve clorobiocin production in *Streptomyces roseochromogenes* NRRL 3504

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Abstract

Streptomyces roseochromogenes NRRL 3504 is best known as a producer of clorobiocin, a DNA replication inhibitor from the aminocoumarin family of antibiotics. This natural product currently draws attention as a promising adjuvant for co-application with other antibiotics against Gram-negative multidrug-resistant pathogens. Herein, we expand the genetic toolkit for NRRL 3504 by showing that a set of integrative and replicative vectors, not tested previously for this strain, could be conjugally transferred at high frequency from *Escherichia coli* to NRRL 3504. Using this approach, we leverage a cumate-inducible expression of cluster-situated regulatory gene *novG* to increase clorobiocin titers by 30-fold (up to approximately 200 mg/L). To our best knowledge, this is the highest level of clorobiocin production reported so far. Our findings set a working ground for further improvement of clorobiocin production as well as for the application of genetic methods to illuminate the cryptic secondary metabolome of NRRL 3504.

Key Points

- *Efficient system for conjugative transfer of plasmids into NRRL 3504 was developed.*
- *Expression of regulatory genes in NRRL 3504 led to increase in clorobiocin titer.*
- *Secondary metabolome of NRRL 3504 becomes an accessible target for genetic manipulations using the expanded vector set and improved intergeneric conjugation protocol.*

Keywords *Streptomyces roseochromogenes* NRRL 3504 · Clorobiocin · Antibiotics · Cluster-situated regulatory genes · Conjugative transfer · Integrative plasmids

Introduction

Streptomyces roseochromogenes was first described in the 1970s as a producer of a unique natural product clorobiocin (Clo) (Mancy et al. 1974). Clo belongs to the aminocoumarin family of antibiotics (Fig. 1a) which, via inhibition of DNA gyrases (Flatman et al. 2006), interfere with DNA replication in many Gram-positive bacteria, including multidrug-resistant cocci (Galm et al. 2004). However, clorobiocin has not reached clinical application, unlike its structurally related counterpart novobiocin (Nov). The latter was an FDA-approved drug to treat infections caused by *Staphylococus aureus* (Walsh et al. 1993; Raad et al. 1995), until the withdrawal in 2011. Clo, however, is a more potent in vitro inhibitor of DNA gyrase than Nov (IC_{50} against *Escherichia coli* GyrB are 0.08 and 0.9 μM for Clo and Nov, respectively). Moreover, Clo and Nov (as well as their derivatives) also inhibit topoisomerase IV (Flatman et al. 2006). The clinical relevance of Nov fueled much interest in the family of aminocoumarin antibiotics. Biosynthetic gene clusters (BGCs) for Clo (*clo*) and Nov (*nov*) biosynthesis were sequenced and annotated (Fig. 1b) (Steffensky et al. 2000; Pojer et al. 2002; Li and Heide 2006). Later on, the whole genome sequences were reported for NRRL 3504 and

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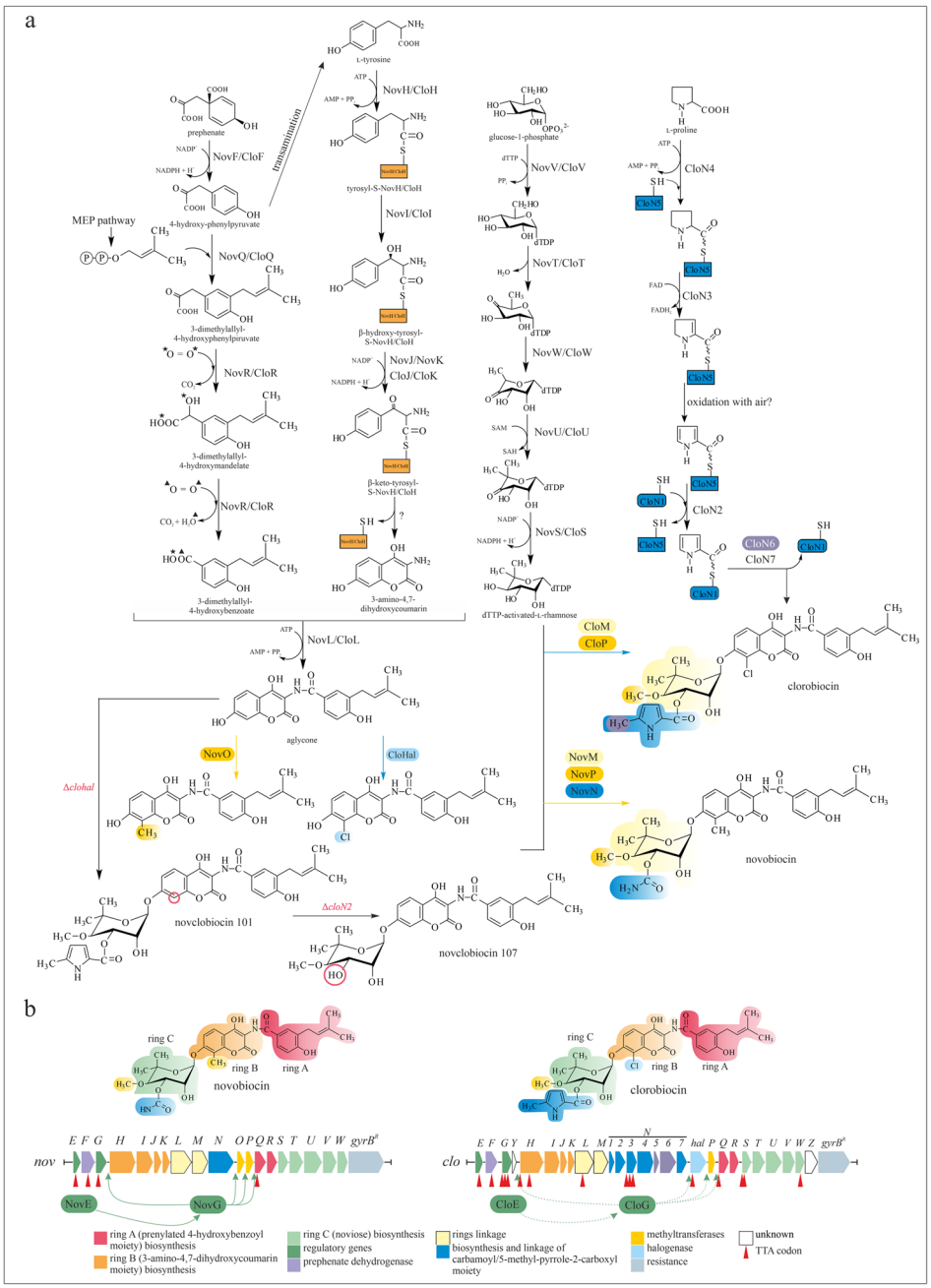

Fig. 1 Biosynthetic pathways of clorobiocin (Clo) and novobiocin (Nov). a) Biosynthetic pathways for both Nov and Clo differ by certain tailoring enzymes, encoded within *nov* and *clo* BGCs (b). Different portions of clorobiocin and novobiocin and the enzymes responsible for their assembly are color-coded appropriately. Putative regulatory schemes for *nov* and *clo* gene expression are shown; green arrows point to the promoters that are/expected to be targeted by the cluster-encoded transcriptional activators NovG and CloG

novobiocin producer *Streptomyces niveus* NCIMB 11,891 (Flinspach et al. 2014; Rückert et al. 2014).

Early identification of *clo* and *nov* BGCs allowed investigating the precise functions of almost all structural genes in a series of genetic and biochemical experiments (see extensive review in Heide 2009 and Pojer et al. 2003a, b). In fact, biosynthetic routes leading to Clo and Nov remain among the most studied secondary metabolic pathways (Fig. 1a). Some investigations were performed in the native producer (Eustáquio et al. 2003; Westrich et al. 2003; Xu et al. 2003), yet the most of our current understanding of aminocoumarin assembly comes from the studies of the heterologous hosts—*Streptomyces coelicolor* derivatives carrying *clo* BGC on a cosmid (Freitag et al. 2005a, b, 2006; Wolpert et al. 2007). The native host was manipulated via either protoplast transformation or by *E. coli—Streptomyces* conjugation to achieve both gene knockouts (Pojer et al. 2002) and knockout complementations (Eustaquio et al. 2003). Plasmids based on φC31 phage integrase or pIJ101 replicon were used for these experiments. Nevertheless, genetic manipulations of NRRL 3504 appeared to be a challenge grave enough to shift most of the work to heterologous hosts (Eustaquio et al. 2005).

More recently, both Nov and Clo were shown to enhance lipopolysaccharide transport in *E. coli* and now might be considered promising adjuvants for polymyxins and colistins to overcome multiple antibiotic resistance in Gram-negative pathogens (May et al. 2017; Mandler et al. 2018; Mattingly et al. 2020). This makes Clo an interesting target for further investigations, demanding significant amounts of the compound. Purification of Clo from fermentation broths of streptomycetes is currently the only feasible and scalable way to produce this compound. Several strains of *S. roseochromogenes* have been reported in the literature, some of which resulted from conventional selection for increased Clo accumulation, such as *S. roseochromogenes* subsp. *oscitans* DS 12.976, known also as NRRL 3504 (Mancy 1974). The latter was reported to produce 25 mg/L of Clo in an optimized fermentation medium (Eustaquio et al. 2005). Heterologous expression of *clo* BGC in the engineered *S. coelicolor* strains is another viable approach (Flinspach et al. 2010). Here, it was possible to reach up to cumulative 160 mg/L of Clo (16%) and its derivatives (Flinspach et al. 2010). While simplifying genetic studies of Clo biosynthesis, heterologous hosts do not offer ample advantages for Clo production as compared to the native strain; moreover, they may suffer from the instability of integrated cosmids. There are no works aimed at improving the titers of Clo in NRRL 3504, which might be naturally best adapted for Clo overproduction. We set out to explore the use of integrative and replicative plasmids for the expression of different regulatory genes in NRRL 3504, which would increase Clo production rates in the native host. An extended toolkit for the manipulation of NRRL 3504 would also facilitate the investigations of the other secondary metabolic pathways in this strain. Here, we show for the first time that one replicative (pSG5-based) and two integrative (actinophage-based) vectors (in addition to φC31-vectors reported previously) can be conjugally transferred and stably maintained in NRRL 3504. We also show that insertional inactivation of genes in NRRL 3504 is possible using non-replicative plasmid pKC1132. The introduction of different regulatory genes into this strain has been achieved, and some of them, particularly *novG* and *adpA*, led to quantitative and qualitative changes in aminocoumarin production. This report sets the working ground for further use of genetic tools to manipulate antibiotic production in NRRL 3504.

Materials and methods

Bacterial strains and growth media

S. roseochromogenes NRRL 3504 was used throughout the study. *Streptomyces sphaeroides* (= *niveus*) NRRL 2449 served as a source of genomic DNA to clone gene *novG*. *Micrococcus luteus* ATCC 4698 was used as a Clo-sensitive test culture in bioassays. *E. coli* DH5α was used for routine cloning procedures. *E. coli* ET12567 (pUZ8002) was used as a donor strain for intergeneric conjugations. *E. coli* strains were grown at 37 °C in liquid or agar LB medium, supplemented with 100 µg/mL of apramycin sulfate, 50 µg/mL of kanamycin sulfate, and 25 µg/mL of chloramphenicol when appropriate (Sambrook and Russell 2001). *Streptomyces* strains were routinely maintained on SFM agar (Kieser et al. 2000) at 30 °C, while ISP3 (Koshla et al. 2017) was the medium of choice to obtain sporulating lawns of *S. roseochromogenes*. To reveal endogenous antibiotic activity, *S. roseochromogenes* strains were grown in liquid and solid GYM (Koshla et al. 2017) medium for up to 120 h at 30 °C. For genomic DNA isolation, *S. roseochromogenes* and *S. sphaeroides,* strains were cultivated in TSB for 48–96 h. Recombinant *Streptomyces* strains were cultivated in presence of 50 µg/mL of apramycin sulfate, when appropriate; cumate (9 µg/mL) and thiostrepton (5 µg/mL) were used to induce *novG*/*cloG* expression from plasmids pGCymRP21 and pIJ6902, respectively. See Electronic Supplementary Materials (ESM) for compositions of all media used in this study.

Generation of recombinant plasmids

All vectors and plasmids, used in this study, are listed in Table 1. Oligonucleotide primers used for the recombinant plasmids generation are given in Table 2. Genomic DNA for cloning purposes was isolated according to salting out procedure no.4 or Kirby procedure as described in (Hopwood et al. 1985; Pospiech and Neumann 1995; Kieser et al. 2000). All recombinant vectors were verified with restriction mapping and sequencing.

Recombinant plasmids for *novG*/*cloG* expression were generated according to a single scheme. First, coding sequences (along with the putative RBS) of *novG* (1,012 bp) and *cloG* (1,022 bp) were amplified from the chromosomal DNA of *S. sphaeroides* and *S. roseochromogenes* using novG_XbaI_up/novG_EcoRI_rev and cloG_XbaI_up/cloG_EcoRI_RP primer pairs (Table 2), respectively. The amplicons were digested with *Eco*RI and *Xba*I restriction endonucleases, and cloned into the following integrative expression vectors: pmoeE5script (via *Eco*RI/*Spe*I sites), to obtain pNOVG101 and pCLOG101 plasmids (the *novG* and *cloG* would be under the control of promoter *moeEp*); pTES (via *Eco*RI/*Xba*I sites), yielding pNOVG102 and pCLOG102 (where *novG* and *cloG* would be under the control of *ermEp*); pGCymRP21 (via *Eco*RI/*Spe*I sites), to give pNOVG103 and pCLOG103 (the *novG* and *cloG* would be under the control of cumate-inducible P21 promoter); pIJ6902 (via *Eco*RI/*Xba*I sites) generating pNOVG104 and pCLOG104 plasmids (the *novG* and *cloG* would be under the control of thiostrepton-inducible promoter *tipAp*). Finally,

Table 1 Plasmids and bacterial strains and used in this work

Name	Characteristic	Reference
Plasmids		
pTES	Am^r; φC31-based *Streptomyces* integrative vector; expression of cloned gene from *ermEp*	(Herrmann et al. 2012)
pIJ6902	Am^r; φC31-based *Streptomyces* integrative vector; expression of cloned gene from thiostrepton-inducible promoter *tipAp*	(Huang et al. 2005)
pRT801	Am^r; φBT1-based *Streptomyces* integrative vector	(Gregory et al. 2003)
pKC1139	Am^r; pSG5-based *Streptomyces* replicative vector	(Bierman et al. 1992)
pOOB83e	Am^r; pKC1139E derivative carrying *Streptomyces ghanaensis* gene *moeH5* under the control of *ermEp*	(Ostash et al. 2013)
pTOSbldA	Am^r; pTOS (Herrmann et al. 2012) derivative carrying *bldA* gene from *Streptomyces albidoflavus* J1074 under the control of its native promoter	(Koshla et al. 2017)
pGCymRP21	Am^r; pGUS (Myronovskyi et al. 2011) derivative carrying *gusA* under the control of CymR operator fused with *P21p*;	(Horbal et al. 2014)
pmoeE5script	Am^r; pGUS (Myronovskyi et al. 2011) derivative carrying *gusA* under the control of *S. ghanaensis* promoter *moeE5p*	(Makitrynskyy et al. 2013)
pSAGA	Am^r; pSET152 (Kieser et al. 2000) derivative carrying *gusA* under the control of *aac(3)IVp*	(Koshla et al. 2019)
pRAGA1	Am^r; pRT801 derivative carrying *gusA* under the control of *aac(3)IVp*	(Yushchuk et al. 2020)
pKAGA1	Am^r; pKC1139 derivative carrying *gusA* under the control of *aac(3)IVp*	(Yushchuk et al. 2020)
pSoAGA2	Am^r; pSOK804 (Sekurova et al. 2004) derivative carrying *gusA* under the control of *aac(3)IVp*	(Yushchuk et al. 2020)
pKC1132	Am^r; suicide vector	(Bierman et al. 1992)
pKCBA	Am^r; pKC1139 derivative, carrying *bldA* gene from *S. albidoflavus* J1074 under the control of its native promoter	This work
pGM4181	Am^r; pmoeE5script derivative carrying *adpA* gene of *S. albidoflavus* J1074 under the control of promoter *moeE5p*	(Yushchuk et al. 2018)
pMoRT4181	Am^r; pRT801 derivative, carrying *moeE5p-adpA* fusion cloned from pGM4181	This work
pKOClo	Am^r; pKC1132 derivative, carrying 2,300 bp internal fragment of *cloH-I*	This work
pNOVG101	Am^r; pmoeE5script derivative carrying *novG* under the control of *moeE5p*	This work
pCLOG101	Am^r; pmoeE5script derivative carrying *cloG* under the control of *moeE5p*	This work
pNOVG102	Am^r; pTES derivative carrying *novG* under the control of *ermEp*	This work
pCLOG102	Am^r; pTES derivative carrying *cloG* under the control of *ermEp*	This work
pNOVG103	Am^r; pGCymRP21 derivative carrying *novG* under the control of *P21p*	This work
pCLOG103	Am^r; pGCymRP21 derivative carrying *cloG* under the control of *P21p*	This work
pNOVG104	Am^r; pIJ6902 derivative carrying *novG* under the control of *tipAp*	This work
pCLOG104	Am^r; pIJ6902 derivative carrying *cloG* under the control of *tipAp*	This work
pNOVG105	Am^r; pKC1139E derivative carrying *novG* under the control of *ermEp*	This work
pCLOG105	Am^r; pKC1139E derivative carrying *cloG* under the control of *ermEp*	This work
Bacterial strains		
E. coli DH5α	General cloning host	MBI Fermentas
E. coli ET12567 (pUZ8002)	(*dam*-13::Tn9 *dcm*-6), (pUZ8002), (Δ*oriT*), a donor strain for conjugative transfer of vector DNA	(Kieser et al. 2000)

Table 1 (continued)

Name	Characteristic	Reference
M. luteus ATCC 4698	Gram-positive bacterium, test strain for the clorobiocin antimicrobial activity assays	American Type Culture Collection
S. sphaeroides (=niveus) NRRL 2449	Wild type, novobiocin producer	ARS Culture Collection (NRRL)
S. roseochromogenes NRRL 3504	Wild type, clorobiocin producer	ARS Culture Collection (NRRL)
S. roseochromogenes pMoRT4181$^+$	NRRL 3504 derivative carrying pMoRT4181	This work
S. roseochromogenes pKCBA$^+$	NRRL 3504 derivative carrying pKCBA	This work
S. roseochromogenes pSAGA$^+$	NRRL 3504 derivative carrying pSAGA	This work
S. roseochromogenes pKAGA1$^+$	NRRL 3504 derivative carrying pKAGA1	This work
S. roseochromogenes pRAGA1$^+$	NRRL 3504 derivative carrying pRAGA1	This work
S. roseochromogenes pSoAGA2$^+$	NRRL 3504 derivative carrying pSoAGA2	This work
S. roseochromogenes pNOVG101$^+$	NRRL 3504 derivative carrying pNOVG101	This work
S. roseochromogenes pCLOG101$^+$	NRRL 3504 derivative carrying pCLOG101	This work
S. roseochromogenes pNOVG102$^+$	NRRL 3504 derivative carrying pNOVG102	This work
S. roseochromogenes pCLOG102$^+$	NRRL 3504 derivative carrying pCLOG102	This work
S. roseochromogenes pNOVG103$^+$	NRRL 3504 derivative carrying pNOVG103	This work
S. roseochromogenes pCLOG103$^+$	NRRL 3504 derivative carrying pCLOG103	This work
S. roseochromogenes pNOVG104$^+$	NRRL 3504 derivative carrying pNOVG104	This work
S. roseochromogenes pCLOG104$^+$	NRRL 3504 derivative carrying pCLOG104	This work
S. roseochromogenes pNOVG105$^+$	NRRL 3504 derivative carrying pNOVG105	This work
S. roseochromogenes pCLOG105$^+$	NRRL 3504 derivative carrying pCLOG105	This work
S. roseochromogenes cloH-I::pKOClo	NRRL 3504 mutant with the insertional inactivation of *cloH-I-J-K* operon	This work

Table 2 Oligonucleotide primers, used in this work

Name	Sequence (5'-3')*	Purpose
cloG_XbaI_up	AAATCTAGACCGAATCATTATGTCCTAC	*cloG* cloning into
cloG_EcoRI_rp	AAAGAATTCAGACCGTATTTCTCCGTC	the expression vectors
novG_XbaI_up	AAATCTAGACCAGACCATTAAGTCCTATG	*novG* cloning into the
novG_EcoRI_rp	AAAGAATTCAGTTGAACGTCAGGCGGTG	expression vectors
nov-clo-inKO_F	TTTTCTAGATGATCAGCGTGCTTGCCGTG	Cloning of the 2,300 bp
nov-clo-inKO_R	TTTGAATTCGTCGCCGCCGATCATCAGCC	internal fragment of *cloH-I* operon
aac(3)IV_F	ATCGACTGATGTCATCAGCG	Diagnostic primers for
aac(3)IV_R	CGAGCTGAAGAAAGACAAT	*aac(3)IV* region amplification (911 bp)
gusA_ver_F	GGCGGCTACACGCCCTTCGA	Diagnostic primers for the
gusA_ver_R	TGATGGGCCGGGTGGGGTC	amplification of 1,000 bp *gusA* internal fragment

* restriction endonuclease recognition sites are underlined

*Eco*RI/*Xba*I-treated *novG* and *cloG* amplicons were cloned into *Eco*RI/*Xba*I-digested replicative vector pOOB83e, to generate pNOVG105 and pCLOG105, where both genes would be under the control of *ermEp.*

To generate *adpA* expression plasmid pMoRT4181, pGM4181 (carrying *adpA* allele from *Streptomyces albidoflavus* (=*albus*) J1074 under the control of *moeE5p*; see Yushchuk et al. 2018) was digested with *Bam*HI/*Xho*I restriction endonucleases. 3,555-bp fragment, carrying *moeE5p-adpA* was then ligated with 3,362 bp fragment of pRT801 (Gregory et al. 2003) vector, treated with the same restriction endonucleases. To generate a replicative vector carrying an additional copy of *bldA* (leucyl(UUA)-tRNA gene from *S. albidoflavus* J1074), pTOSbldA (Table 1) was digested with *Hind*III and *Xba*I. The 777-bp fragment, carrying *bldA* and its putative promoter region, was subcloned into pKC1139 recognition sites of the same restriction endonucleases. The resulting plasmid was labeled pKCBA.

The plasmid for one-step insertional inactivation of the *cloH-I-J-K* operon was generated as follows. 2,300-bp fragment of the *cloH-I* (covering last 1,550 bp of *cloH* and first 747 bp of *cloI*) was amplified using nov-clo-inko_F/R primer

pair (Table 2). The amplicon was digested with *Xba*I/*Eco*RI restriction endonucleases and cloned into *Xba*I/*Eco*RI-digested pKC1132 suicide vector, yielding pKOClo.

Preparation of *S. roseochromogenes* spore suspensions

To obtain spore suspensions, *S. roseochromogenes* lawns were grown on ISP3 agar for up to 240 h. Each lawn was flooded with 10 mL of 15% (v/v) glycerol and the spores were scraped off the surface of the lawn with a spatula. Obtained suspensions were vigorously vortexed and filtered through sterile cotton wool to eliminate vegetative mycelia and agar debris. Filtered suspensions were examined microscopically to evaluate the homogeneity and the cotton wool filtration step was repeated in case some mycelial fragments were still present in the suspension. Homogeneous spore suspensions were then concentrated by centrifuging for 15 min at 8,000 rcf. Finally, spore titers were estimated by plating serial tenfold dilutions of the spore suspension on TSA plates, incubating for 96 h at 30 °C, and counting the number of colonies. Spore suspensions with defined spore titers were stored at -80 °C in 15% (v/v) glycerol.

E. coli—S. roseochromogenes conjugation

A standard intergeneric mating protocol (Kieser et al. 2000) was used to transfer vector DNA from *E. coli* ET12567 (pUZ8002) to germinating spores of *S. roseochromogenes*. In brief, germination of 10^8 *S. roseochromogenes* spores was induced by incubating spores at 50 °C for 10 min. Then, spores were cooled down at room temperature and mixed with 10^9 cells of an overnight culture of donor *E. coli* ET12567 (pUB8002), which carried plasmids mentioned in Table 1. Mixed cell suspensions were plated on well-dried SFM or ISP3 plates, optionally supplemented with $MgCl_2$ to 10 mM. After 14–18 h of incubation at 30 °C, plates were overlaid with 1 mL of sterile distilled water containing 1.25 mg of apramycin sulfate and 750 µg of nalidixic acid sodium salt. Emerging transconjugants, selected for apramycin resistance, were analyzed after 120 h. Conjugation efficiency was calculated as a ratio of the total transconjugants number per one plate to the number of spores applied to conjugation. Transconjugants were verified by isolating total DNA with Kirby procedure (Kieser et al. 2000) and amplifying *aac(3)IV* gene or internal fragment of *gusA* gene.

Production and extraction of Clo

To initiate the fermentation, 10^7 spores of *S. roseochromogenes* strains were inoculated into 50 mL Erlenmeyer flasks with 20 mL of TSB and 6 glass beads (ø5 mm) supplemented with 50 µg/mL apramycin sulfate when appropriate. These pre-cultures were incubated for 48 h at 30 °C on an orbital shaker (220 rpm). Clo-producing cultures were then initiated by inoculating 2.8% (v/v) of pre-culture into a 500 mL Erlenmeyer flask containing 125 mL of GYM medium and 12 glass beads (ø5 mm). Producing cultures were incubated at 30 °C on an orbital shaker (160 rpm) and sampled every 24 h for the downstream determination of biomass accumulation and Clo production. Biomass accumulation was measured as follows. 5 mL of whole cultural broth were collected and the cells were spun down (15 min at 8,000 rcf), the pellet was washed with 15% (v/v) glycerol and left to desiccate for 48 h at 60 °C; finally, the weight of the pellet was determined.

To extract Clo, the pH of the samples, containing 4 mL of whole cultural broth (containing cells and medium, harvested at regular intervals from GYM Clo-producing *S. roseochromogenes* submerged cultures) was adjusted to 4.0 with concentrated HCl and the samples were vortexed for 30 s. Then, pH was restored to 8.0 by adding NaOH (10 N) and samples were mixed with the equal volume of ethyl acetate. Mixtures were left for 90 min at the orbital shaker (220 rpm) at room temperature, then fractionated by centrifuging 10 min at 8,000 rcf. The organic (upper) phase, containing Clo, was collected. Finally, 2 mL of the organic phase were evaporated *in vacuo* and the dry pellet was re-suspended in 200 µL of methanol.

Bioassays to determine the antibiotic activity of *S. roseochromogenes* strains

Bioassays aimed to detect the antimicrobial activity of *S. roseochromogenes* culture broth samples (well diffusion assay, WDA) or extracts (Kirby–Bauer disk diffusion assay, DDA) were carried out using a similar approach and involved *M. luteus* ATCC 4698 as a Clo-sensitive test culture. Basic plates for both types of bioassays were made out of two agar layers: the bottom layer was 20 mL of TSA (2% (w/v) agar) and the upper was 10 mL of TSA (0.7% (w/v) agar) where 200 µL of fresh *M. luteus* cell suspension ($OD_{600}=0.6$) was re-suspended. Then, in the case of DDA, ø5 mm Whatman paper disks soaked with 10 µL of methanol-dissolved ethyl acetate extracts were placed on the surfaces of bioassay plates. In the case of WDA, ø7 mm agar plugs were removed out of the upper agar layer and 10 µL of whole cultural broth were poured into the well. Plates with DDA and WDA were incubated for 24 h at 37 °C, and *M. luteus* growth inhibition zones were examined and documented.

LC–MS analysis of *S. roseochromogenes* extracts

Ethyl acetate extracts were obtained as described above, dried *in vacuo,* and re-dissolved in 200 µL of methanol

before analysis. 1 µL of methanol solution was separated on C18 Phenomenex columns (100 × 2.1 mm, 1.7 µm) using Dionex Ultimate 3000 HPLC–DAD system coupled to MaXis Impact HD LC-Q-TOF mass-spectrometer (Bruker Daltonics). The LC runtime was 22 min, at a flow rate of 0.6 mL/min. Solvent system: water + 0.1% formic acid (HFo; solvent A), acetonitrile + 0.1% HFo (solvent B); from 95% A to 0% A in 12 min, then 3 min at 0% A, then reversion to 45% A within the remaining 7 min. Ionization was performed in positive and negative modes. Analysis of LC–MS data was performed using the program Compass Data Analysis 4.2 (Bruker Daltonics). The quantitative analysis of Clo production was done by comparing areas of extracted Clo mass-peak (697.2 Da, $[M+H]^+$) present in NRRL 3504 ethyl acetate extracts to the areas of 697.2-Da mass peak produced by the known amounts of authentic Clo run through the machine. A reference calibration curve was created using the values of areas for 697.2-Da mass peaks obtained when 6 different amounts of Clo were injected and run under conditions mentioned above (see Table S1, Fig. S1). See ESM for the access to raw LC–MS data.

Scanning electron microscopy (SEM)

Before SEM, NRRL 3504 lawns were grown on ISP3 agar for 216 h. Then, thin slices of the lawn surfaces were cut, sprayed with thin layers of copper *in vacuo,* and imaged with JEOL JSM-T220a SEM (Jeol, Japan) using a 25 kV electron beam.

Qualitative glucuronidase assay

A qualitative glucuronidase assay was utilized to test whether recombinant strains of *S. roseochromogenes* exhibit *gusA*-mediated conversion of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) into 5,5'-dibromo-4,4'-dichloro-indigo. For this, 2 µL of X-Gluc (25 mg/mL DMSO solution) was added to the surfaces of the lawns; plates were left for 2 h at 30 °C and then examined for chromogenic conversion of X-Gluc.

Results

Conditions and rates of conjugative transfer of different vectors into *S. roseochromogenes* using spores as recipients

Integrative, actinophage-based vectors are widely used in *Streptomyces* genetics because of their efficiency as a tool for stable expression of the cloned genes (Kieser et al. 2000). Currently, vectors based on the *int-attP* module of actinophages φC31, VWB, and φBT1 are the most popular ones (Bierman et al. 1992; Van Mellaert et al. 1998; Gregory et al. 2003; Ostash et al. 2009). Replicative vectors are a powerful additional tool for gene expression and among them are widely used pSG5 replicon-based moderate copy number (20–50 per genome) plasmids (Bierman et al. 1992; Muth 2018). We, therefore, decided to test the utility of the aforementioned expression systems for Clo producer. Although φC31-based plasmids are known to be transferable into NRRL 3504 (Eustáquio et al. 2003), the other vectors were not tested. NRRL 3504 genome sequence showed that the latter possesses a typical *attB* site for φC31, as well as intact *attB* sites for φBT1 and VWB (Fig. S2a, b, and c, respectively, ESM).

We choose the intergeneric conjugation with *E. coli* ET 12567 (pUZ8002) as a method to deliver recombinant plasmids into cells of NRRL 3504. This method was previously proved efficient for NRRL 3504 (Eustáquio et al. 2003); however, in that case, the recipient was applied to the mating as vegetative mycelium. Such an approach has certain drawbacks, such as low efficiency and reproducibility, as well as a high labor burden. Thus, we decided to test the efficiency of intergeneric conjugation using spores of NRRL 3504 as a recipient material.

First, we tested the growth of NRRL 3504 on a range of common agar media, aiming to detect the optimal sporulation conditions (data not shown). We found out that ISP3 supports abundant sporulation in NRRL 3504 (Fig. S3), allowing to prepare concentrated spore suspensions (see Materials and Methods section).

To test if conjugation with spores works in NRRL 3804 and how efficient it is for different plasmids, we used a previously designed approach (Yushchuk et al. 2020), involving the derivatives of pSET152 (φC31-based integrative vector), pRT801 (φBT1-based integrative vector), pSOK804 (VWB-based integrative vector), and pKC1139 (pSG5-replicon replicative vector)—pSAGA, pRAGA1, pSoAGA2, and pKAGA1, respectively. These plasmids all carry a reporter cassette *aac(3)IVp-gusA*, which allows to instantly rule out false-positive colonies among transconjugants by adding X-Gluc to the overlay. Thus, we tested the transfer efficiency of four vectors mentioned above from *E. coli* ET12567 (pUZ8002) to *S. roseochromogenes.* The pSAGA vector (φC31-based) exhibited the highest transfer efficiency, followed by pRAGA1 (φBT1-based) and pSoAGA2 (VWB-based) (Table 3). The lowest transfer efficiency was shown for a replicative vector pKAGA1 (pSG5-replicon based, Table 3). All transconjugants were able to convert X-Gluc, proving the absence of spontaneous apramycin-resistant colonies (Fig. S4a). Furthermore, we tested three random transconjugants for each platform by PCR and were successfully able to amplify a 1000-bp internal fragment of *gusA* (Fig. S4b).

We also noticed that conjugative transfer efficiency was medium-dependent: pSAGA- and pRAGA1-carrying transconjugants occurred on ISP3 agar more frequently than on SFM, while for pSoAGA2 and pKAGA SFM turned out to be more favorable (Table 3). Finally, we observed that Mg^{2+} ions have a positive impact on the conjugative transfer efficiency in *S. roseochromogenes*, although to a different extent (Table 3). These results are in agreement with many other described cases, where Mg^{2+} ions improve conjugation efficiencies of different *Streptomyces* spp.; however, the explanation of this fact remains unknown (Galm et al. 2008; Kim et al. 2008). All platforms mentioned above did not affect the growth, morphology, or antimicrobial activity of NRRL 3504 (data not shown).

Insertional inactivation of *cloH-I-J-K* operon in *S. roseochromogenes*

We focused on the combination of well and disk diffusion assays (WDA and DDA, see Materials and Methods section) as surrogates for measuring Clo production. First, WDA would be used to screen the antimicrobial properties of different recombinants at different growth time points and replicates. Then, Clo was extracted (see Materials and Methods section) and tested in DDA. Finally, Clo would be measured quantitatively in these extracts by LC–MS.

We first cultivated NRRL 3504 in GYM medium for 120 h. WDA showed that antimicrobial activity appears in the samples of NRRL 3504 cultural broth starting from 96 h, and reaches the peak at 120 h (Fig. S5a). At the same time, biomass accumulation reaches a peak at 96 h (Fig. S6a). The results of the DDA (Fig. 2a) were in agreement. Finally, we tested the extracts of NRRL 3504 cultural broth samples (collected at 120 h) by LC–MS to detect the presence of Clo (observed *m/z* 697.2165, calculated Clo $[M+H]^+$ 697.2164 Da, 0.14 ppm difference, Fig. 3).

The total antimicrobial activity revealed by the bioassays could be a net result of the production of Clo as well other uncharacterized compounds. Indeed, streptomycetes are known to produce different antibiotics with similar antimicrobial properties and *S. roseochromogenes* genome of carries numerous BGCs for specialized metabolic pathways (Rückert et al. 2014). To rule out this possibility, we decided to construct a strain with blocked Clo production. Using the established conjugation protocol, *cloH-I-J-K* operon (central to Clo production, see Fig. 1b) of NRRL 3504 was insertionaly inactivated with plasmid pKOClo. The bioassays showed the complete cessation of antimicrobial activity of *cloH-I*::pKOClo mutant against *M. luteus* (Fig. S5b, Fig. 2b), while its growth rate (measured as dry biomass) was at the slightly lower level than NRRL 3504 (Fig. S6b). Accordingly, LC–MS analysis revealed the absence of Clo mass peak in the extracts of *cloH-I*::pKOClo strain (Fig. 3). Hence, antimicrobial activity of NRRL 3504 is indeed caused by accumulation of Clo; WDA and DDA are sensitive and reliable methods for the detection of Clo production.

Properties of recombinant *S. roseochromogenes* strains carrying extra copies of *adpA* and *bldA* genes

Global positive regulator AdpA is known to improve production of different antibiotics in *Streptomyces* spp. (Ohnishi et al. 2005; Rabyk et al. 2018; Yushchuk et al. 2018). Moreover, AdpA action is tightly intertwined with the expression of *bldA*—a gene for leu(TTA)-tRNA, involved in the translation of rare TTA codon (Chater and Chandra 2008; Makitrynskyy et al. 2013; Koshla et al. 2017). Within the G-C rich genomes of *Streptomyces* spp., TTA codon often occurs in genes for morphogenesis and antibiotic biosynthesis, playing a regulatory role (McCormick and Flärdh 2012). The presence of a large number of TTA codons (sixteen, which is four times as many as in *nov* BGC, see Fig. 1b) in *clo* genes and multiple presumed operators for pleiotropic regulator AdpA within several intergenic regions of *clo* BGC (Fig. S7b) prompted us to investigate the effects of additional copies of *bldA* and *adpA* genes on Clo production. For this purpose, we have used heterologous genes from *S. albidoflavus* J1074. This was justified, because AdpA from NRRL 3504 (coded by the *M878_RS76560* locus) shares 84% of amino acid sequence identity with

Table 3 Efficiencies of conjugal transfer of different plasmids from *E. coli* ET12567 (pUZ8002) to NRRL 3504 spores. Significance of the differences in obtained data was accessed via Welch's *t* test, please see ESM Table S2

Vector	Conjugative transfer efficiency			
	ISP3	ISP3 10 mM $MgCl_2$	SFM	SFM 10 mM $MgCl_2$
pSAGA	$(2.32 \pm 0.06) \times 10^{-5}$	$(1.95 \pm 0.05) \times 10^{-5}$	$(0.83 \pm 0.12) \times 10^{-5}$	$(1.01 \pm 0.1) \times 10^{-5}$
pSoAGA2	$(0.5 \pm 0.1) \times 10^{-6}$	$(2.6 \pm 0.19) \times 10^{-6}$	$(0.63 \pm 0.13) \times 10^{-6}$	$(9.7 \pm 0.1) \times 10^{-6}$
pRAGA1	$(5.3 \pm 0.21) \times 10^{-6}$	$(5.96 \pm 0.23) \times 10^{-6}$	$(2.7 \pm 0.09) \times 10^{-6}$	$(3.73 \pm 0.11) \times 10^{-6}$
pKAGA1	$(0.145 \pm 0.032) \times 10^{-7}$	$(0.285 \pm 0.047) \times 10^{-7}$	$(0.255 \pm 0.051) \times 10^{-7}$	$(1.6 \pm 0.22) \times 10^{-7}$

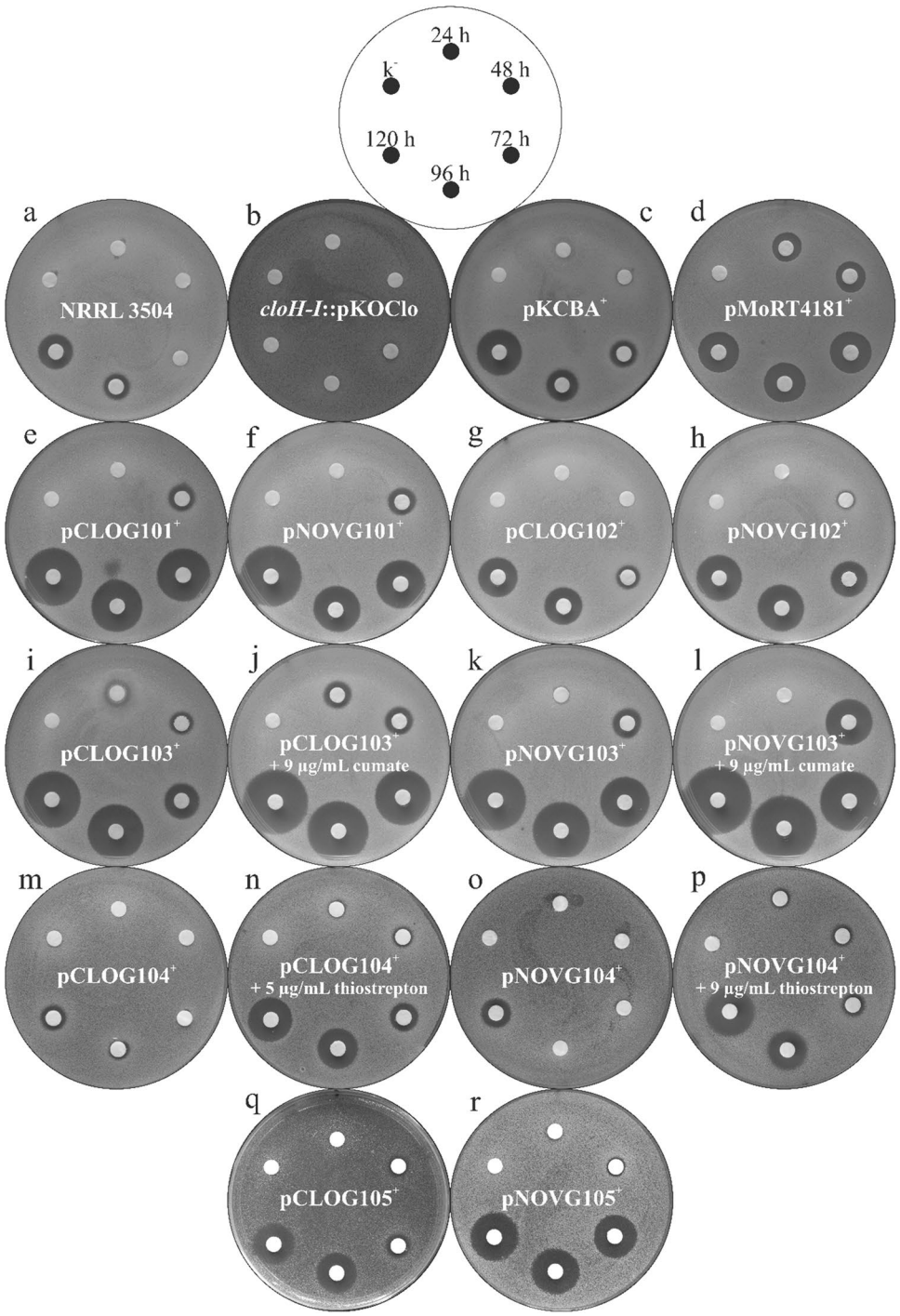

Fig. 2 Results of disk diffusion assays showing antimicrobial activity of ethyl acetate extracts obtained from the cultures of NRRL 3504 and recombinant strains. All strains were cultivated in GYM medium and samples for the extraction were collected at five time points from 24 to 120 h. k^- was always a disk soaked with 10 µL of solvent—methanol. Plates here show typical results of three replicates, for the numerical information on growth inhibition zones diameters and statistics, please see Table S4

its counterpart from J1074 (having identical DNA binding domains), while *bldA* genes of J1074 and NRRL 3504 (*M878_RS74880*) share 94% of nucleotide sequence identity. Thus, *bldA* under the control of native promotor was transferred into NRRL 3504 using pSG5-based low copy number replicative plasmid (pKCBA), while *adpA* gene under the control of *moeE5*-promotor was introduced on actinophage φBT1-based integrative plasmid pMoRT4181 (see also Table 1). Both $pKCBA^+$ and $pMoRT4181^+$ recombinants produced more Clo in comparison to the parent strain, as evident from the bioassays and MS measurements (Fig. 2c and d; Fig. S5c and d; Fig. 4). At the same time, biomass accumulation was comparable to the parent strain (Fig. S6c and d). Interestingly, in addition to Clo, $pMoRT4181^+$ accumulated two compounds whose masses fit known Clo intermediates: novclobiocins 101 (observed *m/z* 661.24, calculated $[M—H]^-$ 661.2391, 1.36 ppm difference) and 107 (observed *m/z* 554.2030,

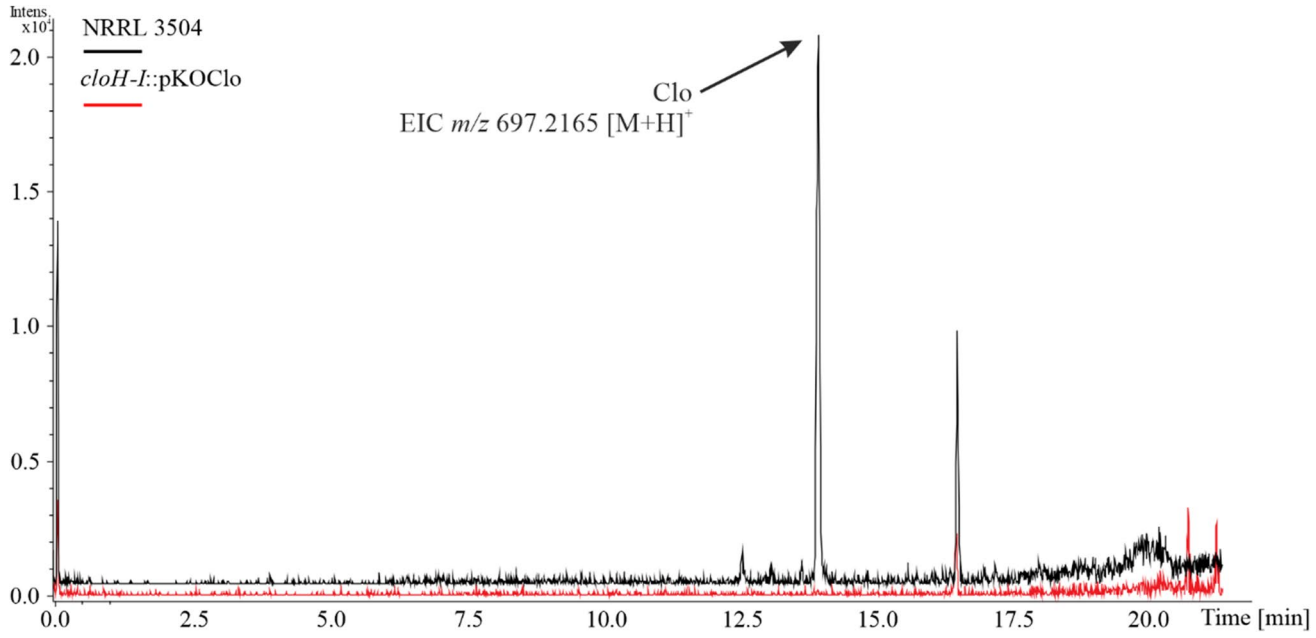

Fig. 3 Extracted ion chromatograms (EICs) of the masses corresponding to Clo (*m/z* 697.2165) in ethyl acetate extracts from cultivation broths of NRRL 3504 (black) and *cloH-I*::pKOClo mutant (red); desired mass is absent in the EIC corresponding to *cloH-I*::pKOClo extract

calculated $[M—H]^-$ 554.2021, 1.62 ppm difference; see also ESM Fig. S8; see Fig. 1a for chemical structures). We did not observe significant amounts of these intermediates in either NRRL 3504 or $pKCBA^+$ strain, or any other NRRL 3504 derivatives described below (data not shown).

Overexpression of cluster-situated regulatory genes *novG* and *cloG* using different expression platforms

The *nov* cluster-encoded transcriptional activator NovG is essential for the initiation and sustained production of Nov by *S. sphaeroides* (Eustáquio et al. 2005a, b). The same functional importance for Clo biosynthesis is suggested for CloG, the NovG ortholog (Heide 2009). Both NovG and CloG belong to the group of StrR-like transcriptional regulators often found to control the biosynthesis of antibiotics (van der Heul et al. 2018). Both proteins share 85% of amino acid sequence identity and so might be functionally interchangeable. Gene *cloG* contains three TTA codons within the reading frame, while only one TTA is present in *novG*. We speculated that the presence of three TTA codons might decrease the efficiency of *cloG* as a tool for Clo overproduction.

We cloned *novG* and *cloG* into replicative pKC1139-based vector and a series of actinophage-based vectors so that the expression of these genes would be under the control of different promoters (see Table 1). All *novG*/*cloG*-expressing plasmids were transferred into NRRL 3504 and the Clo production by the resulting recombinants and the parental strain was

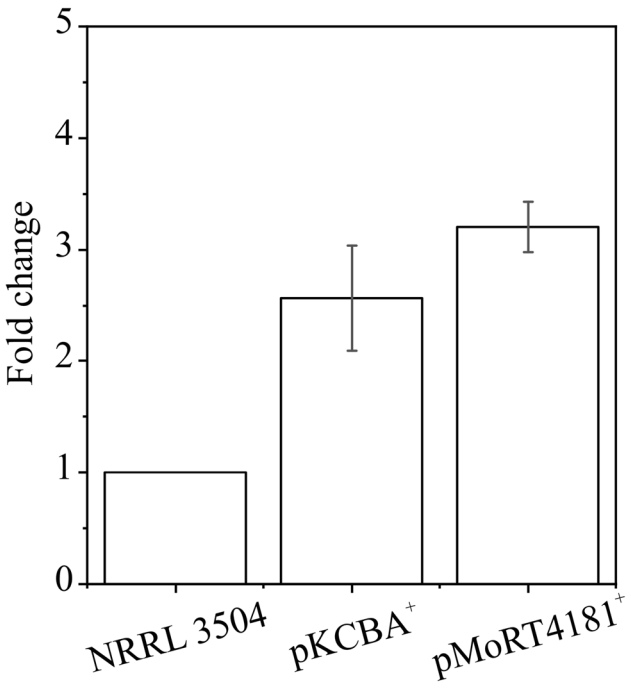

Fig. 4 Production of Clo in the recombinant strains carrying additional copies of *bldA* and *adpA* genes from *S. albidoflavus*, as judged from quantitative analysis of MS data. The diagram was derived from MS data as follows. Mass peaks of 697.2 Da, that corresponds to Clo cation ($[M+H]^+$), were extracted from the total ion chromatograms and ratios of the area of $pKCBA^+$ and $pMoRT4181^+$ strains to the wild type (NRRL 3504) were determined and normalized against the same amount of the biomass (dry weight from 5 mL). Results represent mean values (three repeats); error bars represent SD

first compared using bioassays. Almost all recombinant strains had better antimicrobial activity as compared to the parental strain (Fig. S5, Fig. 2) while showing no significant decrease in biomass accumulation (Fig. S6). Among them, pIJ6902-derived expression vectors (pCLOG104 and pNOVG104, where regulatory genes are under the control of thiostrepton-inducible promoters) exerted negative effect on antimicrobial activity of recombinants in the absence of inducer, but still increased antimicrobial activity when inducer was added (Fig. S5m-p, Fig. 2m-p). Notably, *novG*-overexpressing strains had more potent antimicrobial properties than *cloG*$^+$ strains (Fig. S5, Fig. 2). Then, we have estimated Clo production levels in cultural extracts (120 h time point) by means of LC–MS. Obtained data were again consistent with the WDA and DDA results, showing that *novG*$^+$ and *cloG*$^+$ strains specifically overproduced Clo (Fig. 5, Tables S3 and S4). The highest increase in Clo titers (up to 32-fold, Fig. 5, Fig. S9) was observed upon cumate-inducible expression of *novG* from *P21* promoter of vector pGCymRP21 (plasmid pNOVG103, see Table 1); at the same time, expression of *cloG* gave only 20-fold increase of Clo production under the same conditions (Fig. 5). Such productivity corresponded to 239 and 169 mg/L of Clo, respectively. Although overproducing Clo significantly, pNOVG103$^+$ and pCLOG103$^+$ strains accumulated biomass at the levels comparable with the parent strain (Fig. S6a and j).

Discussion

The natural producer of Clo, *S. roseochromogenes* NRRL 3504, is considered poorly amenable to genetic manipulation and genetic approaches to improve Clo titers in NRRL 3504 were not pursued. Here we report that intergeneric *E. coli—Streptomyces* conjugation, using spores of NRRL 3504 as a recipient, can be used to transfer integrative and replicative plasmids into *S. roseochromogenes* and to make gene knockouts in this strain. Overall, we have successfully transferred 12 integrative plasmids (φC31-, φBT1-, and VWB-based) and 4 replicative (pSG5-based) ones, demonstrating high conjugation efficiency. The utility of integrative vectors was demonstrated through the expression of pleiotropic and pathway-specific regulators of secondary metabolism, some of which led to significant enhancements in Clo production levels. This work therefore expands the genetic toolkit for *S. roseochromogenes* and facilitates the access to large quantities of Clo.

Additional plasmid-borne copies of *bldA* and regulatory gene *adpA* led to increased Clo titers. One of the possible explanations for the case of *bldA* might be the anomalously high frequency of rare TTA codon in *clo* genes (16 TTA codons across 28-gene BGC), which may limit the Clo production level. Likewise, the presence of putative AdpA operators within *clo* BGC motivated us to check the effects of *adpA* on NRRL 3504. Of note, *adpA* expression boosted the production of not only Clo but also novclobiocins 101 and 107. We tentatively suggest that this is caused by unbalanced activation of different *clo* operons by AdpA, although this idea requires further experimental verification. Alternatively, such effect might be due to the exhausted pool of Leu(TTA)-tRNA. Activation of some other biosynthetic pathways in NRRL 3504 by *adpA*-overexpression is also not excluded; this might explain diffused edges of *M. luteus* growth inhibition halos caused with pMoRT4181$^+$ culture broth samples (Fig. S5d). In fact, our recent experience in *Streptomyces cyanogenus* S136

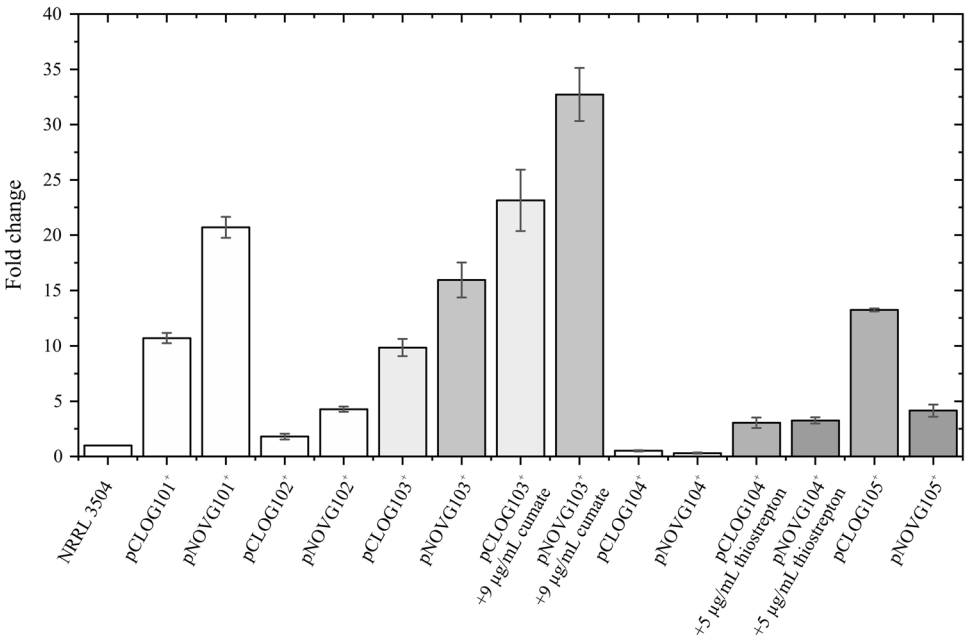

Fig. 5 Production of Clo in the recombinant strains, carrying additional copies of *novG* and *cloG* cluster-situated transcriptional regulatory genes under the control of different promoters. More than 30-fold increase in Clo production was achieved when *novG* was expressed from pGCymRP21 platform in presence of 9 μg/mL of cumate as an inducer. The diagram was built following the approach explained in the legend for Fig. 4; exact values of Clo peak areas for different measurements are given in Table S5

(Yushchuk et al. 2021), which used the same *adpA*-expression platform, makes this explanation very likely. The work to test these possibilities is underway in our laboratories. While manipulations of global regulatory genes positively influenced Clo production, introducing extra copies of either *cloG* or *novG*, which are pathway-specific regulators of biosynthesis of Clo and novobiocin, respectively, into NRRL 3504 led to even greater yields of Clo. The cumate-inducible expression of *novG*/*cloG* from a weak *P21* promoter yielded the best results. Both the bioassay and MS measurements agree with the estimate that under the described fermentation conditions the parental strain produced Clo at the level of *ca.* 7 mg/L (Table S5). Consequently, the aforementioned *novG*-expressing strain would yield around 200 mg/L of Clo, which is the highest titer for this natural product reported so far (Flinspach et al. 2010). Notably, we performed our Clo production experiments in the "generic" liquid medium—GYM—which lacks many properties of industrial production media. Nevertheless, Clo titers obtained here were already significantly higher than in *S. coelicolor* strains carrying *clo* gene cluster, which were fermented under highly optimized conditions. Therefore, we believe that recombinant strains overexpressing either *novG* or *cloG* might perform even better under more optimal production conditions.

Peculiarly, we noted that *novG* was more efficient than *cloG* in terms of increasing Clo production. Perhaps this is caused by the fact that *novG* is less saturated with TTA codons than *cloG* is, although we cannot exclude some other reasons. There is ample room for further improvements to Clo production level in NRRL 3504, either via fine-tuning the *cloG*/*novG* expression, or a combination of additional copies of pathway-specific and global regulators within one strain. The availability of three different actinophage-based integrative plasmids and a replicative one for manipulation of NRRL 3504 sets a reliable working ground for such endeavors.

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Author contribution BO and OY conceived and designed research, with conceptual input from OK, VF, and DK. SM, AS, and IO performed research and analyzed data. MM provided mass-spectrometry assistance and critical reagents. BO, OY, AS, and DK wrote the paper.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethical approval This article does not contain studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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