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ORIGINAL PAPER

Properties of *Streptomyces albus* J1074 mutant deficient in $tRNA^{Leu}_{UAA}$ gene *bldA*

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Abstract *Streptomyces albus* J1074 is one of the most popular and convenient hosts for heterologous expression of gene clusters directing the biosynthesis of various natural metabolic products, such as antibiotics. This fuels interest in elucidation of genetic mechanisms that may limit secondary metabolism in J1074. Here, we report the generation and initial study of J1074 mutant, deficient in gene *bldA* for $tRNA^{Leu}_{UAA}$, the only tRNA capable of decoding rare leucyl TTA codon in *Streptomyces*. The *bldA* deletion in J1074 resulted in a highly conditional Bld phenotype, with depleted formation of aerial hyphae on certain solid media. In addition, *bldA* mutant of J1074 was unable to produce endogenous antibacterial compounds and two heterologous antibiotics, moenomycin and aranciamycin, whose biosynthesis is directed by TTA-containing genes. We have employed a new TTA codon-specific β-galactosidase reporter system to provide genetic evidence that J1074 *bldA* mutant is impaired in translation of TTA. In addition, we have discussed the possible reasons for differences in the phenotypes of *bldA* mutants described here and in previous studies, providing knowledge to study *bldA*-based regulation of antibiotic biosynthesis.

Keywords *Streptomyces albus* · tRNA · Antibiotics · β-galactosidase

Introduction

The actinobacterium *Streptomyces albus* J1074 was isolated in 1980 as a *S. albus* G derivative deficient in the *Sal*GI restriction-modification system (Chater and Wilde 1980). Later, J1074 served as a model to study phage restriction phenomena in streptomycetes, and in 1990s it has gained prominence as a host for heterologous expression of antibiotic biosynthesis genes (Rodriguez et al. 1993, 2000). Fast, highly dispersed growth and genetic amenability make J1074 a strain of first choice for expression of heterologous gene clusters from actinobacteria (Baltz 2010). Previous studies (Lopatniuk et al. 2014; Iqbal et al. 2016) portray J1074 as an optimal starting point to develop an efficient platform for heterologous expression (metagenomic) purposes. It is expected that such a platform would greatly increase our ability to discover bioactive natural products encrypted in environmental DNA and silent gene clusters of actinobacteria. In this context, it is essential to elucidate genetic mechanisms that control secondary metabolism in J1074 to find limiting factors and the ways to circumvent them. It has to be mentioned that regulation of antibiotic production has been relatively well studied in few models of streptomycetes (Liu et al. 2013), although it is not yet understood to what extent this knowledge can be extrapolated onto new strains, such as J1074.

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Transcriptional control of gene expression is arguably the most important regulatory layer in bacteria, and antibiotic production is no exception. In addition, streptomycetes possess a highly unusual regulatory switch based on leucyl (TTA) codon and its cognate $tRNA^{Leu}_{UAA}$, encoded by gene *bldA*. TTA codons are located exclusively in genes for auxiliary functions such as morphogenesis and antibiotic production, and *bldA* deficiency is not lethal. There is a considerable temporal gap between occurrence of UUA-containing transcripts and their subsequent translation (Gramajo et al. 1993; Pope et al. 1996; Rebets et al. 2006). Failure to develop aerial mycelium and spores on top of substrate mycelium is a hallmark of all *bldA*-mutants (the "Bald" phenotype) studied to date. The latter is thought to be the result of blocked translation of TTA-containing gene for master regulator AdpA (Takano et al. 2003; Nguyen et al. 2003). The influence of *bldA* deficiency on antibiotic biosynthesis remains more controversial, as well as the exact chain of events linking *bldA* to delayed antibiotic production (Guthrie and Chater 1990; Gramajo et al. 1993; Chater and Chandra 2008; Makitrynskyy et al. 2013).

The effects of *bldA* deletion on J1074 have not been studied so far, and it remained unknown to what extent colony development and antibiotic production will be affected by the former. Here, we show that on certain (minimal) solid media *bldA*-deficient mutant J1074 produces aerial hyphae and spores less densely than the parental strain; in the same time, under many culture conditions parental and mutant strains are phenotypically indistinguishable. In line with many other reports, our *bldA* mutant's ability to express TTA codon-containing antibiotic biosynthesis gene clusters is strictly dependent on $tRNA^{Leu}_{UAA}$. Finally, we describe a novel reporter system that was used to study TTA codon-based regulatory mechanisms in *S. albus*.

Materials and methods

Microorganisms, vectors, and culture conditions

Streptomyces albus SAM2, a derivative of J1074 carrying single $attB^{\varphi C31}$ site (Bilyk and Luzhetskyy 2014), was used throughout the study. *Escherichia coli* DH5α was used for routine subcloning. *E. coli* ET12567 (pUB307) was used to perform intergeneric conjugation from *E. coli* to *S. albus*. *Escherichia coli* strains were grown under standard conditions (Sambrook and Russell 2001). *Bacillus cereus* ATCC19637 and *Debaryomyces hansenii* VKM Y-9 were used as antibiotic-sensitive test cultures (growth temperature −30 °C). RedET-mediated gene replacements (Datsenko and Wanner 2000) were carried out with the help of REDIRECT system (Gust et al. 2003). All vectors, cosmids, plasmids, and BAC DNAs are listed in Table 1. For intergeneric matings, *S. albus* strains were grown on soy-flour mannitol (SFM) agar (Kieser et al. 2000) at 30 °C. To reveal endogenous antibacterial and antifungal activities, *S. albus* strains were grown on SG2 agar (in g/L: glucose 20, yeast extract 5, soytone 10, pH 7.2 prior to autoclaving) and other solid media as detailed in Electronic Supplementary Materials (ESM). For submerged production of moenomycin and aranciamycin *S. albus* strains were grown in liquid medium SG1 (SG2 supplemented with 2 g/L $CaCO_3$) at 28 °C for 5 days. For phenotypic examinations, *S. albus* were grown on SFM and SMMS agar (Kieser et al. 2000), as well as other media as described in ESM. Wherever needed, strains were maintained in the presence of apramycin (25 μg/mL); chromogenic substrate X-Gal was added to the media to a final concentration of 50 μg/mL; cumate (50 mM) was used to induce *SCO3479* expression from plasmids pRV4 and pRV3.

Plasmids and BACs construction

Bacterial artificial chromosome (BAC) BAC_1N12 was used to prepare *bldA* (*XNR_1995*) knockout BAC. For this purpose, apramycin resistance cassette *aac(3)IV-oriT* flanked with P-GG and B-CC sites was amplified from plasmid patt-saac-oriT (Myronovskyi et al. 2014) with primers Am-bldAdelF and Am-bldAdelR (all primer sequences are given in Table s1, ESM). The resulting 1378-bp PCR product was used to replace *bldA* gene in BAC_1N12 using recombineering. The resulting BAC was named 1N12d_bldA. This and other constructs generated in course of the work were verified by sequencing. Plasmid pTOSbldA (complementation of the *bldA* deletion). The *bldA* gene together with putative promoter region was amplified with primers bldA-Hind-for and bldA-Xba-rev yielding a product 675 bp in length. The PCR product was digested with enzymes *Hin*dIII–*Xba*I and cloned into respective sites of vector pTOS to give pTOSbldA. Plasmid pTESsco3479 (expression of β-galactosidase gene *SCO3479* under promoter *ermEp**). Gene *SCO3479* was amplified with primers Sco3479upXbaI and Sco3479rpMfeIBgl from the chromosome of *S. coelicolor*. The PCR product, 3016 bp in length, was digested with restriction endonucleases *Xba*I and *Bgl*II, and then ligated to *Xba*I–*Bgl*II sites of vector pTES. Plasmids pOOB109 and pOOB110. The 3.3-kb fragment of *S. coelicolor* M145 genome that encompassed the entire coding sequence of *SCO3479* plus 0.3-kb putative promoter region was amplified with primers Sco3479upBglII and 3479rpMfeIBglII. The resulting amplicon was treated with restriction endonuclease *Bgl*II and cloned into *Bam*HI + *Bgl*II-digested vector pTES. Two clones were selected, referred to as pOOB109 and pOOB110, which contained *SCO3479* in two orientations within vector pTES. In pOOB109, *SCO3479* is under control of its own

Table 1 Plasmids, cosmids, and BACs used in this work

Name	Description	Source
BAC_1N12	Cm^r; pSMART-based bacterial artificial chromosome (BAC) carrying a segment of *S. albus* genome with gene *bldA*	This work
1N12_ bldA-I	BAC 1N12 derivative, replacement of *bldA* with IMES-flanked cassette *aac(3)IV-oriT*	This work
moeno38	SuperCos1-based, carries entire *moe* cluster 1 except *moeR5moeS5*, Km^rAp^r	Ostash et al. (2013)
moeno38-6	moeno38, replacement of *neo* with $int^{\varphi C31}$-$oriT^{RK2}$-*hyg* cassette (Hy^r), Δ*moeH5* (Km^r)	Ostash et al. (2013)
pOJ436ara	pOJ436-based cosmid, carries entire aranciamycin biosynthesis gene (*ara*) cluster	Luzhetskyy et al. (2007)
pTES	Am^r; phiC31-based *Streptomyces* integrative vector; expression of cloned gene from *ermEp**	Herrmann et al. (2012)
pTOS	Am^r; VWB-based *Streptomyces* integrative vector	Herrmann et al. (2012)
pIJ773	Source of FRT-flanked *aac(3)IV-oriT* cassette	Gust et al. (2003)
patt-saac-oriT	Source of IMES-flanked *aac(3)IV-oriT* cassette	Myronovskyi et al. (2014)
pALFLP	pSG5-based plasmid expressing recombinase Flp; Hy^r	Fedoryshyn et al. (2008)
pUWLint31	pIJ101-based plasmid expressing Int-phiC31; Hy^r	Myronovskyi et al. (2014)
pUWLDre	pIJ101-based, expresses SSR Dre to evict pTOS vector sequences	Herrmann et al. (2012)
pGCymRP21	Am^rSp^r; phiC31-based *Streptomyces* integrative vector; expression of a cloned gene from cumate-inducible promoter *cmt*-P21	Horbal et al. (2014)
pTESsco3479	pTES carrying *SCO3479* under control of *ermEp**	This work
pOOB109	pTES carrying *SCO3479* under control of tandem promoter—truncated *ermEp* + full-length *SCO3479p*	This work
pOOB110	pTES carrying *SCO3479* under control of full-length *SCO3479p*	This work
pOOB114	pTES carrying "dead" version of *SCO3479* (stop codon in 19th position) under control of *ermEp**	This work
pRV3	pGCymRP21 carrying mutant (TTA codon in 19th position) version of *SCO3479* under control of cmt-P21	This work
pRV4	pGCymRP21 carrying native version of SCO3479 under control of *cmt*-P21	This work
pTOSbldA	pTOS derivative carrying *bldA* gene along with upstream 0.6-kb region	This work

promoter as well as truncated ermEp*; and in pOOB110, *SCO3479* is under control of its own promoter. Plasmid pOOB114. 3017-bp fragment containing entire coding sequence of *SCO3479* plus 29-bp putative promoter was amplified from plasmid pOOB109 with primers lacZ_XbaI_TGA2 and 3479rpMfeIBglII. In this way, leucyl codon CTG in 19th position of *SCO3479* was replaced with stop codon TGA. The resulting amplicon was treated with XbaI and MfeI and cloned into XbaI–EcoRI sites of pTES to give pOOB114. Plasmid pRV3. 3017-bp fragment containing entire coding sequence of *SCO3479* plus 29-bp putative promoter was amplified from plasmid pOOB109 with primers lacZ_XbaI_TTA1 and Sco3479_6Hisrp. In this way, leucyl codon CTG in 8th position of *SCO3479* was replaced with synonymous codon TTA and six histidine codons CAC were appended upstream of stop codon. The resulting amplicon was treated with *Xba*I and *Mfe*I and cloned into *Spe*I–*Eco*RI sites of pGCymRP21 to give pRV3. In the latter, *SCO3479* replaced *gusA* gene. Plasmid pRV4. 3017-bp fragment containing entire coding sequence of *SCO3479* plus 29-bp putative promoter was amplified from plasmid pOOB109 with primers Sco3479upXbaI and Sco3479_6Hisrp. In this way, native sequence of *SCO3479* was appended upstream of hexahistidine-stop codon sequence $[(CAC)_6$-TGA]. The resulting amplicon was treated with *Xba*I and *Mfe*I and cloned into *Spe*I–*Eco*RI sites of pGCymRP21 to give pRV4.

Generation and verification of the *S. albus* recombinant strains

All constructs were transferred into *S. albus* conjugally, as described elsewhere; gene replacements in *S. albus* were generated as described in Myronovskyi et al. (2014). Apramycin resistance marker flanked with B-GG and P-GG sites was evicted from the genome of *bldA* mutant with recombinase Int-phiC31 (plasmid pUWLint31). pTOS vector sequences from *bldA* complementation strain were evicted with recombinase Dre (plasmid pUWLDre). PCR was employed to confirm the presence of the plasmids, cosmids, and expected gene replacements in the chromosomes of streptomycetes.

Determination of antibiotic production

Moenomycin production was analyzed in *S. albus* strains carrying cosmid moeno38-6. The latter leads to accumulation of two moenomycins in *S. albus*—nosokomycin

A_2 and its precursor lacking terminal galacturonic residue (Ostash et al. 2013; Lopatniuk et al. 2014). Strains were grown in SG1. Quantity of moenomycins was determined by antibiotic disc (Ø 5 mm, Whatman) diffusion assay against spores (10^7 per plate) of moenomycin-sensitive *Bacillus cereus* ATCC19637 as described in Makitrynskyy et al. (2010). Productivity index (PI) was calculated according to the equation: PI = (Ø growth inhibition zone, mm/Ø disc, mm) − 1. PI = 0 is the lowest possible value and corresponds to antibiotic production below detection limit of the bioassay (~2% of production level of the control strain). The PIs were referred back to equal amounts of dry biomass (10 mg) in different strains.

For aranciamycin production, control and pOJ436ara-carrying strains were grown for 24 h in preculture medium (tryptic soy broth, TSB; Himedia) at 30 °C and 1 mL of the preculture was used to inoculate 50 mL (in 300-mL flasks with glass beads) of main medium SG1. After 5 days of growth, biomass was separated from supernatant via centrifugation. Spent medium (10–20 mL) was extracted with equal volume of ethylacetate. Ethylacetate extract was dried in vacuo, then reconstituted in methanol, and subjected to TLC (immobile phase: silicagel plates F60, Merck; mobile phase: chloroform:methanol = 95:5) and disc diffusion assay against *B. cereus* ATCC19637 spores (10^7 per plate) on modified minimal agar (g/L: KH_2PO_4 3, K_2HPO_4 7, sodium citrate × $4H_2O$ 0.5, $MgSO_4$ × $7H_2O$ 0.1, $(NH_4)_2SO_4$ 1, glucose 2, bacto peptone 0.3, agar 16) (Anagnostopoulos and Spizizen 1961).

Aranciamycin production and native antibiotic activity of *S. albus* strains were also monitored using agar plug antibiotic diffusion assay. Briefly, strains were grown on SG1 agar for 5 days. Then, agar plugs (Ø 5 mm) were cut off the lawn and stacked on top of TSB agar plates with test culture *D. hansenii* spread immediately prior to the experiments or *B. cereus* spores as it is described above. Halos of growth inhibition around the plugs were measured after 18 h of incubation.

Scanning electron microscopy (SEM)

Small pieces of 3-day-old lawns were cut off the OM or SFM agar plate samples, vacuum-dried, and directly analyzed on a Jeol JSM-T220A scanning microscope.

Results

Generation and growth characteristics of *S. albus* strains carrying *bldA* knockout

For *S. albus* J1074 *bldA* gene knockout, we prepared BAC 1N12_bldA-I, where *bldA* was replaced with *aac(3)IV-oriT* cassette flanked with B-GG and P-GG sites for recombinase PhiC31. With this BAC, we successfully generated respective *S. albus* mutant (ΔbldA-I) and then its markerless derivative (upon expression of PhiC31 from plasmid pUWLInt31). The markerless *bldA* mutant was referred to as OK3. OK3 genotype was confirmed by PCR (see Fig. S1, Electronic Supplementary Materials, ESM). We also introduced *S. albus* SAM2 *bldA* gene on vector pTOS back into OK3, and, after pTOS sequences eviction, generated markerless complemented strain OK3 + bldA.

In submerged culture, OK3 and the parent (SAM2) strains exhibited similar dynamics of growth and biomass accumulation (Fig. S2). The strains were also incubated on a number of solid media and analyzed after 3 and 5 days. On most media, OK3 was macroscopically and microscopically indistinguishable from the parent (Figs. S3, S4). Nevertheless, we have found that certain minimal media did result in visible changes in lawn morphology of OK3 (see Fig. S4). The most pronounced morphological differences were seen on SFM and SMMS (Figs. 1 and S5). On these media, OK3 produced sparse aerial hyphae and spore chains without significant delay as compared to the parental strain.

Antibiotic production by OK3 strain

Genomic potential of *S. albus* J1074 for secondary metabolism is significant (Zaburannyi et al. 2014), yet, to date, its expression required genetic interventions (Olano et al. 2014; Brana et al. 2014). Compounds of paulomycin family are the only known antibacterially active metabolites to be accumulated by J1074, although their production pattern was unstable (Olano et al. 2014; Gonzalez et al. 2016) We tested over a dozen of the most common agar media used for *Streptomyces* cultivation, and found those that reproducibly elicit the production of antibiotic compounds by J1074 (Figs. S6, S7). Particularly, *S. albus* accumulated antibacterial and (to a lesser extent) antifungal compounds when growing on SG2 agar. Using SG2, we compared antibiotic activity of SAM2, *bldA* mutant OK3 and OK3 + bldA. The results are summarized in Fig. 2. OK3 did not accumulate detectable amounts of antibacterial compounds and introduction of *bldA* restored antibiotic production to the mutant. The antifungal activity of OK3 was not impaired and even slightly increased as compared to SAM2. Using HPLC–MS (as detailed in ESM), we confirmed that production of candicidin-like compounds by OK3 is increased as compared to parent strain, while paulomycins were not found in OK3 (Fig. S8).

Next, we compared the ability of SAM2 and OK3 to heterologously produce antibiotics whose biosynthesis depend on TTA codon-containing genes. These were moenomycin and aranciamycin biosynthesis gene clusters, carried on

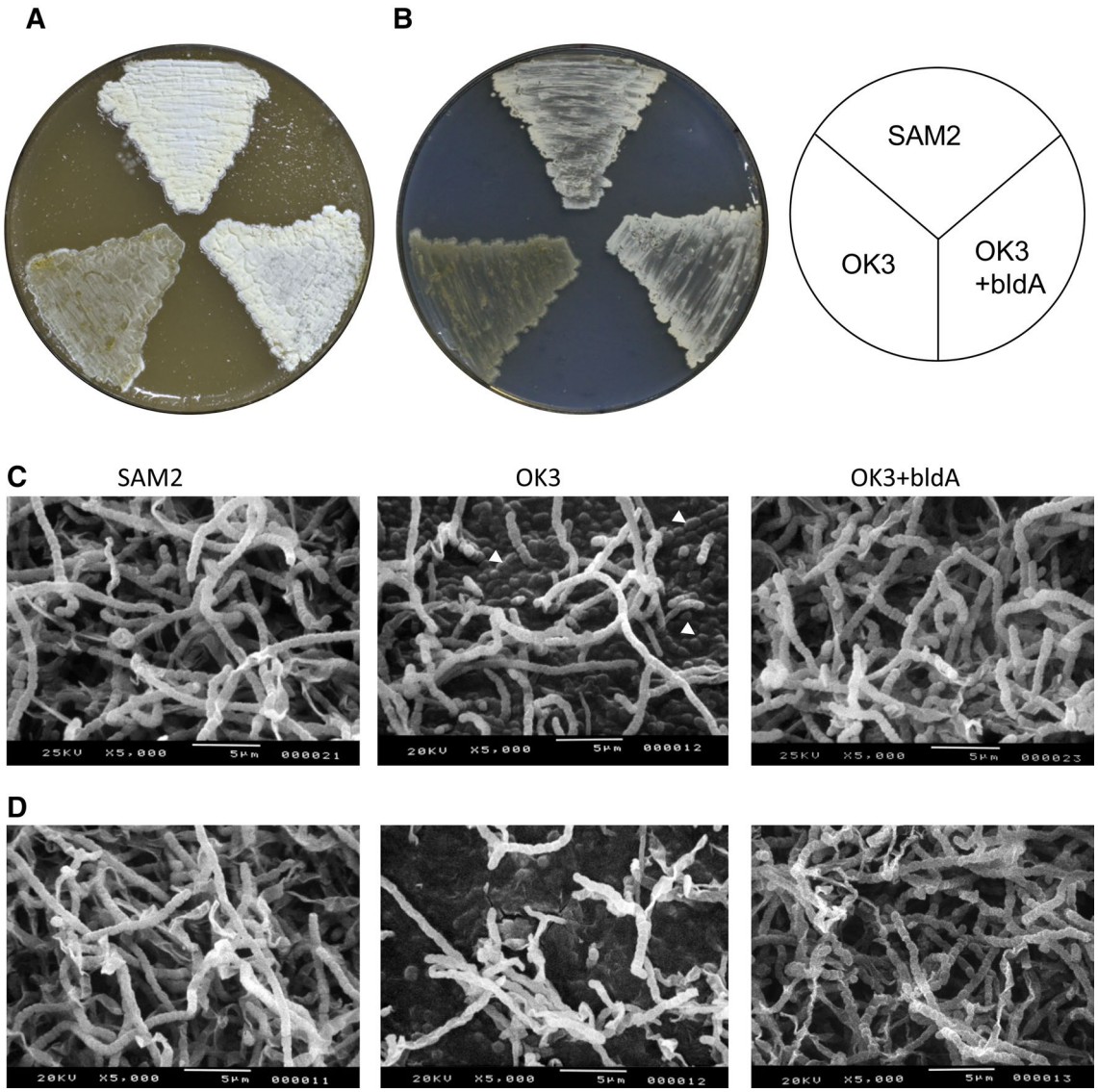

Fig. 1 *Streptomyces albus* J1074 *bldA* mutant phenotype. Parental (SAM2), mutant and complemented strains were grown for 3 days on SFM (**a**) and SMMS (**b**). The same lawns were used to obtain scanning electron microscopic views of colonial surfaces of SFM (**c**) and SMMS-grown cultures (**d**). The substrate mycelium of OK3 strain on SFM (**c**) appeared to be fragmented into spore-like chains (*white triangles*)

cosmids moeno38-6 (Lopatniuk et al. 2014) and pOJ436ara (Luzhetskyy et al. 2007), respectively. As can be judged from bioactivity and TLC-based assays, moeno38-6^{+} and pOJ436ara^{+} transconjugants of the parental strain abundantly produced respective antibiotics, while OK3 strain did not (Figs. 3, S9–S10).

The β-galactosidase reporter system to detect and elucidate *bldA*-based regulation

The above-described results imply that, in line with what is known for the other *Streptomyces* (Chater and Chandra 2008; Liu et al. 2013), failure to translate codon UUA in transcripts of secondary metabolism genes is the primary reason for the inability of OK3 to produce endogenous and heterologous antibiotics. Yet, with two exceptions (Wang et al. 2009; Makitrinskyy et al. 2013), direct evidence for involvement of *bldA* in regulation at the level of UUA translation is absent for all studied cases. In *S. albus* J1074, the *bldA* mutation and the observed phenotype (lack of antibacterial activity of the strain) are bridged by a complicated cascade of regulatory and structural genes. Codon-swap reporters based on β-galactosidase and luciferase genes are used in enterobacteria and yeast to peer into regulatory events at the level of translation of a certain codon (Urbonavicius et al. 2003; Lamichhane et al. 2013). We set out

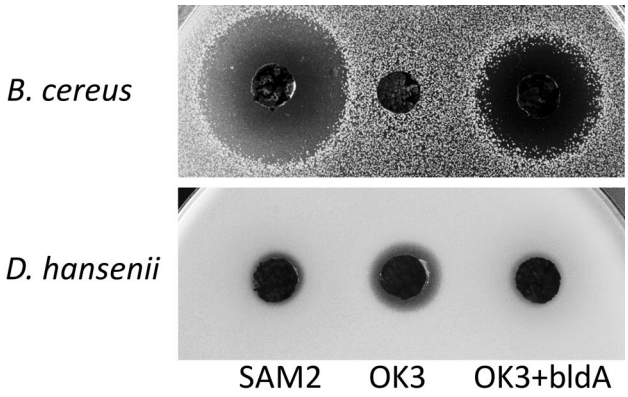

Fig. 2 The *bldA* knockout in *S. albus* abolished the production of antibacterial compound(s), but not antifungal one(s). For the bioassay, agar plugs were cut off 5-day lawns of *S. albus* strains grown on SG2 agar, and stacked on top of plates with test cultures. Note the small halos of *D. hansenii* growth inhibition around SAM2 and OK3 + *bldA* plugs, and increased halo around OK3 plug

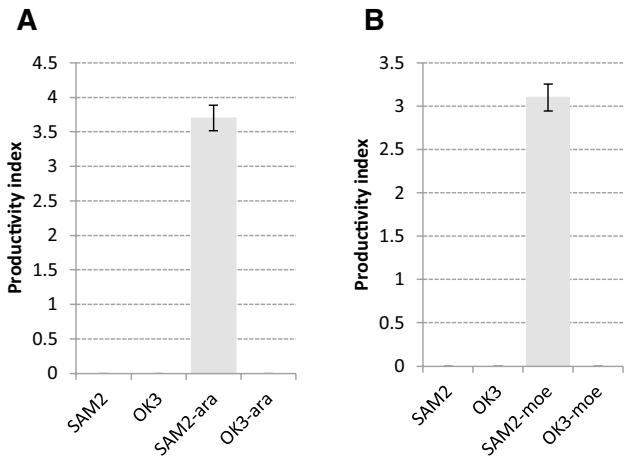

Fig. 3 Aranciamycin (**a**) and moenomycin (**b**) production is blocked in *bldA* mutant of *S. albus*. Strains were grown in SG1 medium, and productivity indices were calculated from disc diffusion assays as described in "Materials and methods". SAM2, OK3—strains carrying empty vector pSET152; SAM2/OK3-ara, SAM2/OK3-moe—*S. albus* strains carrying either cosmid pOJ436ara or cosmid moeno38-6, respectively

to develop an analogous reporter system for *Streptomyces albus* J1074, which would enable straightforward phenotypic detection of translation of UUA. Here, we took advantage of the fact that J1074 is one of the rare streptomycetes having no detectable intra- or extracellular enzymatic activity towards lactose analog, X-Gal (King and Chater 1986). Indeed, our analysis confirmed that none of the seven annotated *S. coelicolor* β-galactosidases had counterparts within J1074. Using reciprocal best BLAST hit strategy (Kuzniar et al. 2008), *S. coelicolor* M145 protein Sco3479 (referred hereafter to as $LacZ_{sc}$) was identified as an ortholog of *E. coli* LacZ. Several $lacZ_{sc}$-carrying plasmids were prepared (Fig. 4a) and expression of functional β-galactosidase from all of them (except for pOOB114 which carries in-frame stop codon in position 19 of $lacZ_{sc}$) was confirmed. Particularly, gene $lacZ_{sc}$ conferred *lacZ*-deficient *E. coli* to the ability to hydrolyze X-Gal; in liquid and solid media, different $lacZ^{+}_{sc}$ *S. albus* strains produced blue color of different intensity (Fig. S11). The latter most likely reflects different strength of transcription of $lacZ_{sc}$.

We introduced TTA, CTC, and stop codon-containing $lacZ_{sc}$ reporter plasmids (pRV3, pRV4, pOOB114, respectively) individually into OK3 strain. As expected, the plasmids did not cause the production of blue color (X-Gal hydrolysis) in the absence of inducer (cumate). In the presence of 50 mM cumate only the strain carrying CTC-specific reporter (pRV4) turned blue (Fig. 4). The ability of OK3-pRV3 to hydrolyze X-Gal was restored upon introduction of *bldA* (Fig. S12). All available data suggest that *bldA* deletion directly and specifically impaired the translation of TTA codon in $lacZ_{sc}$ reporter gene.

Discussion

In this work, we have carried out a set of experiments aimed to study the effects of *bldA* deletion on *S. albus* J1074 and to develop a simple reporter system for further investigation of *bldA*-mediated regulation in J1074.

Perhaps, the most striking property of Bld phenotype (displayed by *bldA* mutants of streptomycetes, vide supra) is its conditionality. That is, the degree of morphological and antibiotic synthesis defects strongly depend on growth conditions and level of transcription TTA codon-containing (TTA^{+}) genes (Guthrie and Chater 1990; Leskiw et al. 1993; Gramajo et al. 1993; Pope et al. 1996; Xu et al. 2008). The conditionality of Bld phenotype of *S. albus* OK3 has extreme manifestation. Indeed, we were able to identify media, where OK3 exhibits impaired morphological development, but under no conditions did we observe complete cessation of aerial mycelium and spore formation. To the best of our knowledge, there is no experimentally validated information on gene networks that govern morphogenesis in *S. albus* J1074. However, as far as we can judge from in silico analysis of J1074 genome (Zaburannyy et al. 2014), the *bld*- and *whi*-cascades are essentially the same in J1074 and other model streptomycetes. Gene *XNR4181* encoding ortholog of *S. coelicolor* AdpA (also known as BldH), a master regulator of colonial differentiation and (often) antibiotic biosynthesis (Chater and Chandra 2008), contains TTA codon, and thus should be under *bldA* control. Hence, either UUA within *adpA* mRNA is mistranslated efficiently enough to allow colonial development, or other (as-yet-unknown) genes compensate/bypass the loss of AdpA. In

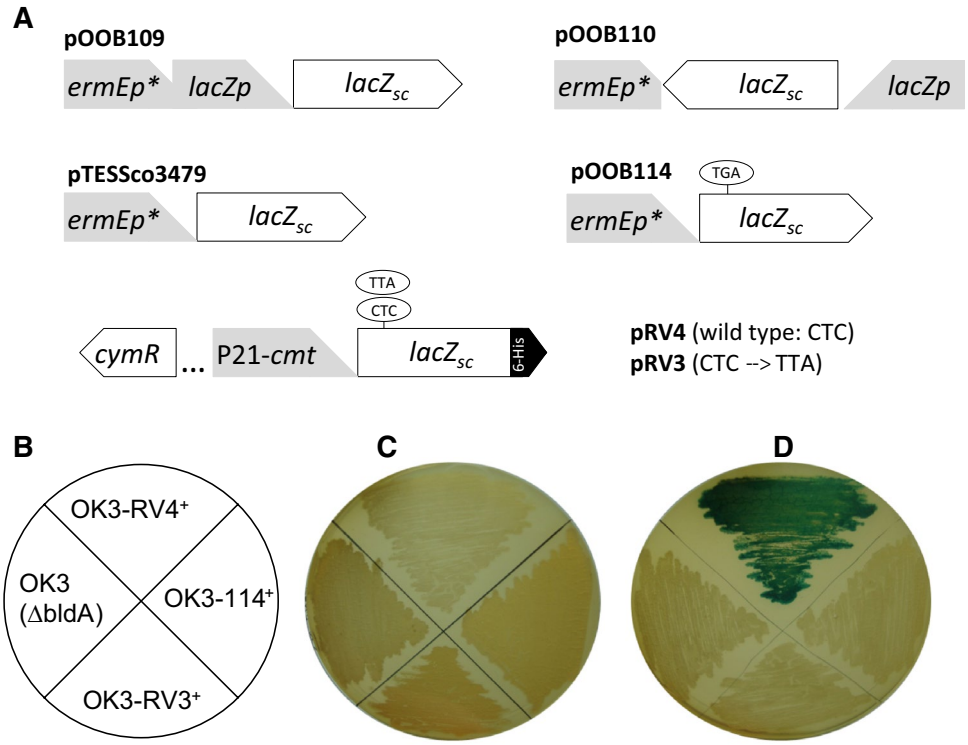

Fig. 4 Codon-specific β-galactosidase reporter for *S. albus* visualizes arrested TTA codon translation in *bldA* mutant OK3. All plasmids (**a**) carrying *S. coelicolor* M145 β-galactosidase gene *SCO3479* ($lacZ_{sc}$) contain actinophage ϕC31 *int-attP* integration module and apramycin resistance marker *aac(3)IV*. They differ in the type of promoter (*shaded gray*) that drives $lacZ_{sc}$ transcription, and presence of distinct codons (shown as *ovals*) in positions 8 and 19 of $lacZ_{sc}$ ORF (if not shown, then CTC codon is present). In pOOB109 and pOOB110, *ermEp* is rendered nonfunctional through truncation of its last 30 nts. In pRV3 and pRV4 $lacZ_{sc}$, transcription is cumate-inducible (Horbal et al. 2014). Four OK3 derivatives (**b**) were streaked onto TSA X-Gal-containing plates in the absence (**c**) and in presence (**d**) of cumate. CTC^+ reporter is expressed in OK3, while TTA^+ is not. The latter is expressed in wild type (SAM2) strain (see ESM, Fig. S11)

Streptomyces, there are several reports on mistranslation of various UUA^+ mRNAs, including *adpA*, in the absence of *bldA*-encoded $tRNA^{Leu}_{UAA}$ (Leskiw et al. 1991; Makitrinskyy et al. 2013). Although existence of a subset of TTA^+ genes capable of translation in *bldA* mutants appears to be paradoxical, an explanation for this was offered (Trepanier et al. 2002). Briefly, propensity for mistranslation is attributed to the nature of the first nucleotide downstream of the TTA codon. If either A or G follow TTA, then the ribosome is able to mistranslate the codon; otherwise (C/T downstream of TTA) translation is aborted. Thus, in the absence of $tRNA^{Leu}_{UAA}$, only mRNA containing the quadruplets TTAC/T will not be translated, and so regulatory effect will be attained. There is TTAC quadruplet in case of *S. albus* J1074 *adpA*; thus, codon context hypothesis does not seem to apply. The position of TTA within the coding sequence could be important. In most streptomycete genes, it is located close to the start codon (Zaburannyy et al. 2009), while in *adpA* gene family, it is positioned roughly in the middle of the ORF. Also, enhancement of UUA translation in *adpA* mRNA in the presence of *S*-adenosylmethionine was reported (Xu et al. 2008). These facts underscore the importance of environmental (rather than genetic) factors in translation of *adpA* mRNA, and they might play a crucial role in shaping the morphology of OK3 strain.

The *bldA* knockout abolished production of two heterologous antibiotics, moenomycin and aranciamycin, the biosynthesis of which is controlled by TTA-containing genes. Moreover, OK3 no longer produced endogenous antibacterial compounds. According to all available data, glycosylated antibiotics, paulomycins, are the principal source of antibacterial activity exhibited by *S. albus* J1074 (Majer and Chater 1987; Olano et al. 2014). We also believe that paulomycins accounted for most (if not all) of the antibacterial activity of SAM2, given the characteristic reddish coloration of *S. albus* lawns on SG2 agar, and the absence of that color in OK3, although detailed analysis was not pursued. Cessation of their production by OK3 strain is most likely due to presence of TTA codon within regulatory gene *SSGH_05342* (*plm30*) encoding LuxR family regulator essential for paulomycin production (Gonzalez et al. 2016). At the same time, level of antifungal activity of OK3 is not affected as compared to SAM2, and even appears to be elevated. *S. albus*

genome carries several gene clusters for biosynthesis of compounds that might target eukaryotic cells (Zaburannyy et al. 2014). One of these clusters (around gene *XNR_RS29020*), directing the production of candicidin-like compound, is free of TTA codons, and so could be expressed in *bldA*-minus background.

Our work has once again highlighted all the challenges associated with interpretation of complex, conditional, and composite phenotypes of *bldA* mutants. For example, since TTA codons are embedded into genes for pleiotropic and pathway-specific transcriptional factors, any observable defect of *bldA* mutant would be the net result of deregulated transcription and translation. It is thus difficult to account for and disentangle the relative contribution of each genetic and environmental factor to a final *bldA*-minus phenotype. This calls for development of a simple reporter system where effects of *bldA* knockout on translation can be reliably detected and studied in the most direct way. Several such systems (mostly based on antibiotic resistance genes) were employed in the past, and they gathered valuable information about *bldA* function (Leskiw et al. 1991; Pope et al. 1996). Nevertheless, available reporter genes are not without shortcomings, such as unnatural (for *Streptomyces*) codon usage biases; poor control of transcription; and absence, in most cases, of TTA-free version of the reporter gene. We developed, for *S. albus* J1074, a specialized codon reporter system based on *S. coelicolor* M145 β-galactosidase gene *SCO3479* ($lacZ_{sc}$). It allows tight transcriptional control over *SCO3479* expression, ease of phenotypic detection (in presence of chromogenic substrates), and His-tagged protein tracking. The simplicity of our reporter system lends itself to a rigorous elucidation of the regulatory role of TTA codons at the level of translation, as our initial experiments (vide supra) suggest.

A number of recent studies suggest that it is not the delayed expression of *bldA* gene but formation of mature (translationally competent) tRNA holds the key to its regulatory function (Leskiw et al. 1991; Xu et al. 2008; Pettersson and Kirsebom 2011). Yet, besides initial in silico predictions (Rokytskyy et al. 2016) nothing is known about the genetics of tRNA maturation in *Streptomyces*. Likewise, there is a growing body of data on the crucial role of environmental factors in tRNA biology (Laxman et al. 2013; Moukadiri et al. 2014; Sakai et al. 2016), although this area remains unexplored in case of *bldA* gene. We envision that the above-described reporter system will facilitate the screening of growth conditions and genes that modulate UUA translation efficiency, both in wild type and *bldA*-minus *S. albus* backgrounds.

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