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A GENETIC ASSAY SYSTEM TO STUDY MISTRANSLATION OF LEUCYL CODON UUA IN *STREPTOMYCES*

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Among all codons, TTA is the rarest one in GC-rich *Streptomyces* genomes. This codon is located exclusively in dispensable genes, involved in not essential processes, like morphological differentiation or secondary metabolism. Delayed (for reasons yet not fully known) translation of TTA codons limits antibiotic production and spore formation to a certain period of streptomycete life cycle, most often to stationary phase of culture development. During protein synthesis, UUA codon is decoded by the only $tRNA^{Leu}_{UAA}$, also known as *bldA* gene product. Together, TTA codon and its cognate tRNA form specific regulatory switch, coordinated functionality of which is needed for normal life cycle processing and biosynthesis of number of antibiotics. Intriguingly, some TTA-containing genes are still expressed (phenotypically) in *bldA* null mutants, even in absence of $tRNA^{Leu}_{UAA}$. Mistranslation of UUA codons is usually suggested in such cases, although evidence for the former is scant. Here we took advantage of recently generated *bldA* mutant of *Streptomyces albus* J1074 and β-galactosidase reporter to develop a genetic system for observation and study mistranslation. We describe pilot experiments that demonstrate function of the developed assay system.

Keywords: *Streptomyces*, *bldA*, mistranslation, reporter gene

Deficiency in *Streptomyces coelicolor* gene *bldA* for $tRNA^{Leu}_{UAA}$ leads to significant phenotypic changes, such as conditional arrest of sporulation and blocked production of some antibiotics [5, 9, 11, 13]. It has to be noted that TTA is the rarest codon in GC-rich actinobacterial genomes [20] which is absent in essential genes. In the model strain *S. coelicolor* A(3)2, whose genome harbors 142 TTA-containing (TTA^+) genes, *bldA* deficiency (the so called "bald" phenotype) causes major-to-infinite delay of the onset of secondary metabolism presumably because of inability to translate the UUA-containing mRNAs [19]. These effects are mediated by a few TTA^+ genes encoding pleiotropic regulators and regulators dedicated to antibiotic production, most notably those of *adpA* and *ram* genes [17]. Functions of the majority of TTA^+ genes in *S. coelicolor* and other species are not understood.

While *bldA* regulation is a well-documented phenomenon [2, 4, 14], there is evidence that certain TTA^+ genes escape translational arrest in *bldA* mutants and express at significant levels [10, 18]. Mistranslation of UUA is usually invoked as the most plausible explanation of such observation. Our recent *in silico* study provided some support in favor of mistranslation hypothesis [15]. However, there is no model system for *Streptomyces* which would provide simple experimental framework to observe and probe mistranslation. We decided to fill this gap, taking advantage of recently developed in our laboratory *bldA* knockout strain *S. albus* OK3 and a *sco3479* (*lacZ*) gene-based β-galactosidase reporter system [8]. Here we provide genetic evidence that *sco3479* expression is blocked at the level of translation and describe the conditions where presumable mistranslation of *sco3479* is reproducibly observed. Our work sets the working ground to understand the extent, molecular mechanisms and functional consequences of protein mistranslation in *Streptomyces*.

Materials and Methods

Strains and plasmids used and constructed in this work are listed in Table 1. Tryptic soy broth (Merck) and soy-mannitol agars [7] were used to grow *Streptomyces* and plate matings. *E. coli* strains were grown in liquid or agar LB medium supplemented with appropriate antibiotic (if needed) at either 30 or 37 °C, respectively. For visual and microscopic lawn examination, strains were grown on soy-flour and TSB media. Genomic DNA from *Streptomyces* and plasmid DNA from *E. coli* were isolated using standard protocols [16]. Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas).

The β-glucuronidase activity (GusA) was measured in cell-free extracts from 48-h TSB cultures of *S. albus* (beginning of stationary phase) as described in [10] and referred back to the same amount of dry biomass. The β-galactosidase activity (LacZ) of *S. albus* strains was measured both in liquid and solid media, in presence of inducer (cumate) and chromogenic substrate 5-Br-4-Cl-3-indolyl-β-D-galactopyranoside (X-Gal) under conditions specified in the main text for each experiment separately. Following classes of compounds were used in disc diffusion assay as potential *sco3479* mistranslation inducers: antibiotics (penicillin, cefalotin, imipenem, teicoplanin, kanamycin, gentamycin, streptomycin, neomycin, kasugamycin, erythromycin, lincomycin, thiostrepton, chloramphenicol, ciprofloxacin, rifampicin); sulfate salts of metals (cobalt, nickel, copper, chromium); organic dyes and enzyme inhibitors (Congo Red, ethidium bromide), Coomassie blue, reserpine, crystal violet, malachite green, bromophenol blue); hydrogen peroxide. Antibiotics were used at concentrations 30 μg/disc, metals and dyes – 3 mM per disc. Discs were soaked into 15 μl of aqueous solution containing hydrogen peroxide in concentrations ranging from 0.01 to 1 %. Assay plates were incubated at 30 °C for 20 days and were inspected daily to reveal the occurence of blue-coloured colonies.

Table 1

Plasmids and bacterial strains used in this work

Strain	Relevant characteristics	Source/ Reference
pGUS	Promoter probe vector, contains promoterless *gusA*; derived from pSET152	[12]
pGCymRP21	Derived from pGUS, contains P21 promoter, *cmt* operator and *cymR* gene	[6]
padpAscript	Derived from pGUS, contains *adpAghp-gusA* fusion	[10]
padpAtransl	Derived from pGUS, *adpAgh-gusA* fusion with HL4 linker	[10]
padpAcontrol	Derived from pGUS, promoterless *adpAgh-gusA* fusion with HL4 linker	[10]
pRV3	Derived from pGCymRP21, cloned TTA-containing *sco3479* (*lacZ* from *S. coelicolor*)	[8]
E. coli DH10B	Routine cloning host; F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ$^-$ *rps*L *nup*G.	Invitrogen
E. coli ET12567	Strain for conjugative transfer of coresident plasmids; *dam13*::Tn9 (Cm^r) *dcm6 hsdM hsdR zjj*202::Tn10 (Tet^r) *recF143 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136* (Str^r) *hisG4 tsx78 mtl1 glnV44* .	[7]
pUZ8002	pUZ8002 - Km^r	
S. *albus* SAM2	Derived from *S. albus* J1074 by deletion of *φ*C31 *pseB4.*	[1]
S. *albus* OK3	Derived from *S. albus* SAM2 by deletion of *bldA*	[8]
S. *albus* OK3-RV3	Derived from *S. albus* OK3 with integrated pRV3	[8]

Results and Discussion

Expression of *adpA* in *S. albus* OK3 is blocked at the level of translation. We recently have generated and described *bldA* knockout mutant *S. albus* OK3 [8]. Its phenotype, both macro- and microscopic, is reminiscent of the other described *bldA* mutants of *Streptomyces*. Nevertheless, it remained unproven that secondary metabolic and morphogenetic defects of OK3 are indeed a consequence of derailed translation of UUA mRNAs in the mutant. We decided

to reveal the step affected by *bldA* mutation using reporter system. For that purpose plasmids padpAscript, padpAtransl and padpAcontrol were individually transferred into *S. albus* SAM2 and OK3 strains (Table 1). The first plasmid carries fusion of *S. ghanaensis* ATCC14672 *adpA* gene promoter (*adpAp*) to β-glucuronidase gene *gusA*. The plasmid allows monitoring of the transcription from *adpAp* and it is expected to be the same in both SAM2 and OK3, if the *bldA* mutation has nothing to do with transcription. The second plasmid carries fusion of *adpAp-adpA* to *gusA* via helical linker, and it thus reports on translation of *adpA* gene. The latter carries codon TTA in 225th position, so it requires $tRNA^{Leu}_{UAA}$ (*bldA*) to decode this codon in *S. albus*, as it does in *S. ghanaensis* [10]. We anticipated that level of GusA activity from padpAtransl should be lower in OK3 than in SAM2 provided that our conjecture about *bldA* function is correct [8]. The last plasmid is a control one for translational fusions. All three plasmids integrate in a one copy into a single $attB^{\phi C31}$ site within *S. albus* genome. Indeed, analysis of GusA activity in the generated transconjugants (Fig. 1) confirmed our initial idea: there was no significant difference in transcription from *adpAp* between SAM2 and OK3 strains, while we detected almost no GusA activity in case of translational fusion expressed in OK3 strain. These data provide circumstantial genetic evidence that *bldA* mutation specifically affects translation step of *adpA* expression; no effects of *bldA* mutation on transcription (at least that of *adpA*) were revealed.

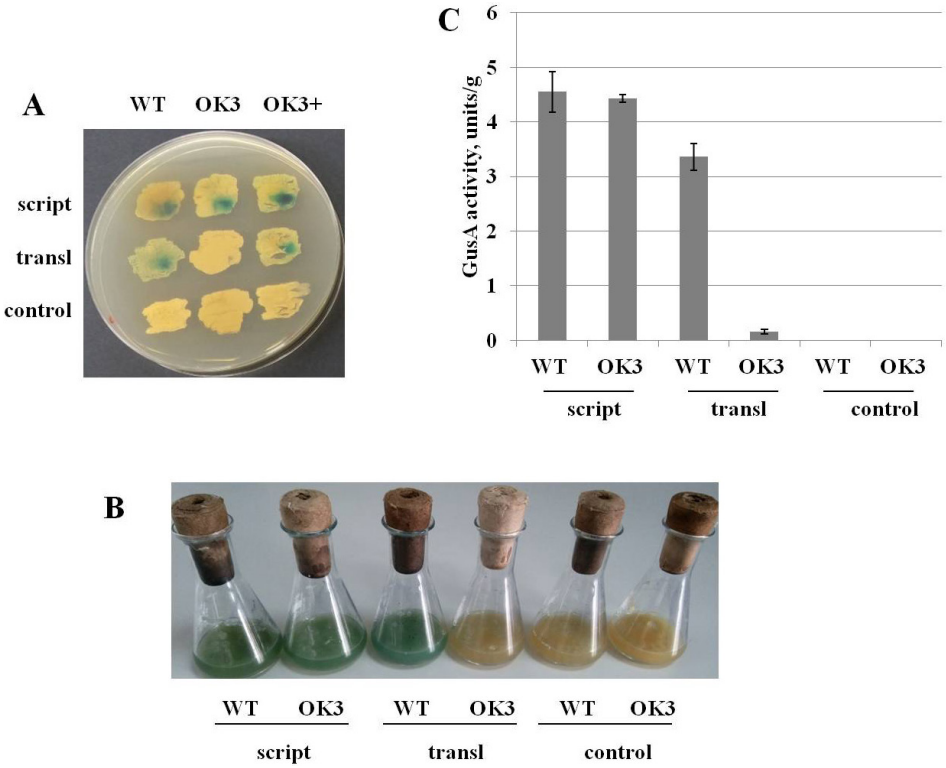

Fig. 1. Glucuronidase activity of *S. albus* strains, containing *gusA*-based reporter plasmids. WT – *S. albus* SAM2; OK3 – *S. albus* OK3; OK3+ – complemented *S. albus* OK3 with *bldA* gene; script – integrated padpAscript; transl – integrated padpAtransl; control – integrated padpAcontrol. Strains were grown on TSA medium **(A)** or in TSB medium **(B)** for 48 hrs, 30 °C. X-Gluc solution was added to right lower edge of lawns **(A)** or to the culture **(B)** and incubated for 2 hrs. Formation of blue compound indicates glucuronidase activity. **C** – measurement of glucuronidase activity in *S. albus* strains (48 hrs in TSB medium, 30 °C). GusA activity normalized against dried weight in 1 ml

Searching the format of assay system to detect and study mistranslation of UUA in *Streptomyces*. Mistranslation of UUA is often invoked as an explanation of leaky phenotypes of *bldA* mutations. However, the reports on presumable mistranslation in *Streptomyces* are scattered through different strains studied over the last 25 years. There were no attempts to develop straightforward experimental system which would allow facile detection of mistranslation and its downstream analysis (e.g., quantification, isolation of a mistranslated protein etc). In our previous work we developed TTA-specific LacZ-based reporter system for *S. albus* SAM2, and we suggested that it could be optimal starting point for *Streptomyces*-specific UUA mistranslation assay. Simplicity of visual screening, regulated cumate-dependent transcription of lacZ and availability of hexahistidine tag are the key advantages of the described reporter plasmid pRV3 [8]. We therefore set out to find conditions of *lacZ* mistranslation in a *bldA*-null background. A number of solid media, cumate concentrations and incubation times were tested; we finally revealed that tryptic soy agar supplemented with 50 μM cumate are optimal to reveal LacZ expression. Under these conditions, after 14 days of incubation at 30 °C and one more day at 4 °C, around 100 light blue-colored colonies became visible on the lawn of OK3-RV3 strain (Fig. 2). Given that 10^7 cfu of OK3-RV3 were plated onto TSA, the frequency of blue colored colonies would be equal to 10^{-5}; this roughly agrees with the mistranslation rate estimated for *E. coli* [3]. No blue colonies were observed when inducer was omitted from assay plates. The latter implies that occurrence of blue colonies strictly depends on activation of transcription of *lacZ* as a (expected) prerequisite of mistranslation. To conclude, our studies defined conditions where mistranslation can be reproducibly observed.

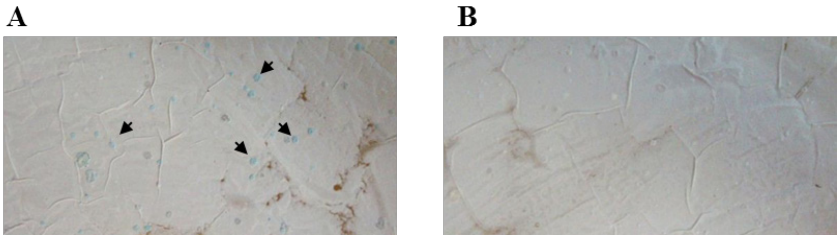

Fig. 2. Fragments of lawns of *S. albus* OK3-RV3 grown on TSA supplemented with X-Gal (100 mcg/mL) with (**A**) or without (**B**) of cumate. Arrows indicate blue colonies

As a pilot test of the developed system, we assayed a number of compounds as potential inducers of LacZ mistranslation. The compounds (fully listed in Materials and Methods) include commonly used antibiotics, metals, organic dyes and stressors, such as hydrogen peroxide. The disc diffusion assay revealed only one compound, chloramphenicol, that significantly enhanced the expression of *lacZ* from pRV3 plasmid (Fig. 3). Somewhat surprisingly, known inducers of

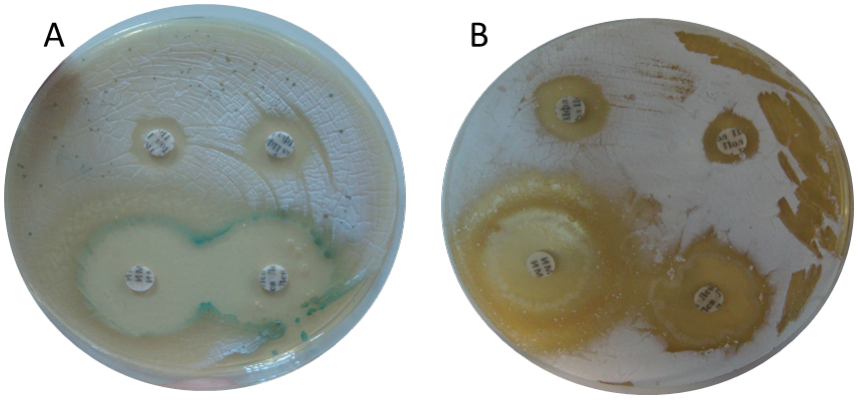

Fig. 3. Chloramphenicol induces lacZ expression in OK3-RV3 strain in presence of lacZ transcription inducer cumate (**A**). In the absence of cumate (**B**) no blue color was visible

mistranslation, such as streptomycin and kasugamycin, had no effects on development of blue colour by OK3-RV3 strain on assay plates. Nevertheless, as the chloramphenicol is well-known translation elongation inhibitor. Hence, our results are in line with the possibility that other compounds that target translation can also be inducers of UUA mistranslation.

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ГЕНЕТИЧНА ТЕСТЕРНА СИСТЕМА ДЛЯ ВИВЧЕННЯ МІСТРАНСЛЯЦІЇ ЛЕЙЦИНОВОГО КОДОНУ UUA В *STREPTOMYCES*

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Серед усіх кодонів TTA є найрідкіснішим для ГЦ-багатих геномів стрептоміцетів. Цей кодон характерний для генів, що не є важливими для виживання, зокрема, задіяних у морфологічній диференціації чи вторинному метаболізмі. Затримана в часі (з досі не з'ясованих причин) – порівняно з іншими кодонами – трансляція TTA кодону обмежує антибіотичну продукцію та формування спор до певного періоду життєвого циклу стрептоміцетів, найчастіше до стаціонарної фази розвитку культури. Під час синтезу білка кодон TTA декодується лише однією тРНК$^{Leu}_{UAA}$, також відомою як продукт гена *bldA*. Разом кодон TTA і тРНК, що його декодує, формують специфічний регуляторний «перемикач», скоординоване функціонування якого є необхідним для нормального перебігу життєвого циклу та біосинтезу низки антибіотиків. Цікаво, що деякі TTA-вмісні гени експресуються (фенотипово) у делеційних *bldA*-мутантів. Зазвичай у таких випадках припускають містрансляцію UUA кодонів, хоча доказів цього не вистачає. У цій роботі ми використали нещодавно сконструйованого *bldA*-мутанта *Streptomyces albus* J1074 та репортерний ген β-галактозидази для створення генетичної системи вивчення містрансляції. Ми описуємо перші експерименти, що демонструють функціонування розробленої аналітичної системи.

Ключові слова: *Streptomyces, bldA,* містрансляція, репортерний ген