Combining Transposon Mutagenesis and Reporter Genes to Identify Novel Regulators of the Topa Promoter in Streptomyces

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Abstract

Background

Identifying the regulatory factors that control transcriptional activity is a major challenge of gene expression studies. Here, we describe the application of a novel approach for *in vivo* identification of regulatory proteins that may directly or indirectly control the transcription of a promoter of interest.

Results

A method based on the combination of Tn5 minitransposon-driven random mutagenesis and *lux* reporter genes was applied for the first time for the *Streptomyces* genus. As a proof of concept, we studied the *topA* supercoiling-sensitive promoter, whose activity is dependent on unknown regulatory factors. We found that the *sco4804* gene product positively influences *topA* transcription in *S. coelicolor*, demonstrating SCO4804 as a novel player in the control of chromosome topology in these bacteria.

Conclusions

Our approach allows the identification of novel *Streptomyces* regulators that may be critical for the regulation of gene expression in these antibiotic-producing bacteria.

Background

Prokaryotic gene expression is a process that is adjusted to the growth phase and to the changes in environmental conditions. As bacterial gene expression is predominantly regulated at the transcriptional level, bacterial genomes encode numerous proteins that control transcription initiation. Among them, the key players are DNA-binding proteins such as sigma factors, which determine promoter recognition by RNA polymerase (RNAP), as well as other transcription factors (TFs), acting as repressors or activators, which may affect the binding of RNAP to a promoter (Lloyd et al, 2001; Browning and Busby, 2016). However, non-DNA binding proteins such as anti-sigma factors, proteases and other proteins can also control the accessibility of direct regulators to the DNA, thus acting indirectly and playing a critical role in transcriptional regulation. Therefore, the identification of all the components of a regulatory system is a challenging task.

Most often, studies on the regulation of gene expression have been limited to searching for promoters bound and controlled by certain regulatory proteins (Bush et al., 2019; Huang et al., 2019). To date, several powerful methods for the determination of the DNA-binding sites of known TFs and the genes regulated by them have been developed, such as SELEX, ChIP-chip, ChIP-seq and RNA-seq (Bouvet 2001; Meng et al., 2005; Wu et al., 2006; Johannes et al., 2010; Hrdlickova et al., 2017). All these tools aim to identify all the putative targets of a certain regulatory protein (Baptist et al, 2013). However, on the other hand, there are only limited strategies to identify TFs of a given promoter of interest (Wang et al, 2015). Currently available techniques to identify TFs that bind specific regions include a modified bacterial one-

hybrid reporter system (Guo et al., 2009) and *in vitro* DNA capture strategies (Park et al., 2005; Park et al., 2009; Burda et al., 2014; Truong-Bolduc and Hooper, 2018). To search for gene expression regulators *in vivo*, the combination of random transposon mutagenesis with reporter genes (predominantly *lacZ* or antibiotic resistance cassettes) was successfully developed. This strategy has been applied in a number of bacterial species (*Pseudomonas chlororaphis, Proteus mirabilis, Staphylococcus aureus* and *Vibrio cholerae*) (Luo et al., 2018; Szostek and Rather 2013; Burda et al. 2014, McDonough et al., 2014). Such approaches may be highly beneficial for the identification of global regulatory factors and the dissection of complex regulatory networks, such as those controlling secondary metabolite synthesis in *Streptomyces.*

Streptomyces are soil-dwelling bacteria that undergo morphological differentiation, which encompasses vegetative growth and sporulation (Flärdh and Buttner, 2009). They are used as producers of numerous biologically active secondary metabolites, such as antibiotics (approximately 60% of the world's natural antibiotics are *Streptomyces*-obtained), immunosuppressants and cytostatics (Chater, 2006). The pathways for the synthesis of secondary metabolites are encoded by gene clusters that are activated only at specific growth phases or physiological conditions (Gehrke et al., 2019; Szafran et al., 2020). Thus, the production of secondary metabolites is tightly controlled by complex regulatory systems, many of which remain uncharacterized. *In silico* predictions revealed that the genome of any *Streptomyces* species may encode up to 1100 transcriptional regulators (Romero-Rodriguez et al. 2015), a large fraction of which fall into one of the two main clades: pathway-specific regulators (PSRs) and pleiotropic/global regulators (McLean et al., 2019; Xia et al., 2020). PSRs are regulatory proteins (such as ActII-orf4, RedD and CdaR or TetR, LacI, MerR, and LuxR family regulators (Wilson et al., 2001; Kuscer et al., 2007; Chater 2016; Wei et al., 2018; McLean et al., 2019; Xia et al., 2020, Nett et al., 2009)) that are usually situated in secondary metabolite biosynthetic gene clusters and directly control the expression of their nearby genes, while pleiotropic regulators (e.g, AdpA (Yamazaki et al., 2004; Wolański et al., 2011), AfsR (Hong et al., 1991; Floriano and Bibb, 1996), BldD (den Hengst et al., 2010), and DasR (Hillerich and Westpheling, 2006)) are scattered throughout the chromosome and positioned distantly from the genes they regulate. While the identification of PSRs is relatively straightforward, the identification of global regulators that control a particular gene of interest may be challenging. A deep understanding of all aspects of *Streptomyces* gene expression, particularly transcription, is crucial to better exploit these bacteria as producers of widely used compounds.

Notably, in *Streptomyces*, similar to other studied bacteria (*Streptococcus pneumoniae, Haemophilus influenzae, Escherichia coli, Salmonella enterica*), DNA supercoiling also plays a role in global gene regulation by directly affecting the transcriptional activity of a number of promoters (Gmuender et al., 2001; Rui and Tse-Dinh, 2003; Peter et al., 2004; Dorman and Corcoran, 2009; Ferrándiz et al., 2014; Webber et al., 2013; Szafran et al., 2019). In *Streptomyces*, chromosome supercoiling is a global regulatory factor that controls the transcription of 3–7% of genes (Szafran et al., 2019). Proper DNA supercoiling in the cell is controlled by a set of enzymes called topoisomerases. The opposing activities of topoisomerase I (TopA), which removes negative supercoils, and gyrase, which can introduce negative supercoils, maintain topological homeostasis in bacterial cells (Champoux, 2001). Inhibition of

topoisomerase activity or alteration of their level leads to changes in chromosome topology and affects DNA transactions, including replication and transcription. One of the most important mechanisms that maintains the balance of topoisomerases activity is transcriptional control of their cellular level (Menzel and Gellert, 1983; Tse-Dinh, 1985). In contrast to many model bacterial species, TopA is the only type I topoisomerase in *S. coelicolor;* thus, it is essential and must be precisely regulated to maintain the proper level of chromosomal supercoiling (Szafran et al., 2016). TopA depletion in *Streptomyces* causes severe growth retardation, including increased DNA supercoiling and altered gene expression, including the expression of secondary metabolite genes (Szafran et al., 2013; Donczew et al., 2016). As in other bacteria, in *S. coelicolor*, the TopA level is predominantly regulated by the transcriptional control of the *topA* gene (Tse-Dinh, 1985; Ferrandiz et al., 2014; Ahmed et al., 2016; Szafran et al., 2016). In *S. coelicolor*, transcription of *topA* is driven from at least two promoters, with equal contributions of both promoters during both vegetative growth and spore production. The *p1* promoter was shown to be supercoiling sensitive, which corroborates the shortened distance between motifs − 10 and − 35 (Szafran et al., 2016). On the other hand, the comparison of the *p2* promoter to other known promoter sequences did not identify the known recognition site for sigma factors or other transcriptional regulators. Moreover, apart from transcriptional regulation, no other mechanism regulating TopA activity has been described in *Streptomyces*.

Here, to identify regulators of the *topA* promoter in *S. coelicolor*, we used an approach based on Tn5 minitransposon (mini-Tn5)-driven random transposon mutagenesis combined with the *lux* reporter system. As a proof of concept, we demonstrated that disruption of the *sco4804* gene lowers the TopA level, while its overexpression results in enhanced *topA* promoter activity. We infer that SCO4804 is an indirect and pleiotropic regulator, which influences *topA* and gyrase gene transcription. Thus, our approach allowed us to identify a new component of the chromosome supercoiling-related regulatory network in *S. coelicolor*.

Results

Application of transposon mutagenesis combined with the lux reporter gene system identifies potential regulators of topA promoter activity

Our previous studies have shown that *S. coelicolor topA* promoter activity is highly dependent on chromosome supercoiling, but neither negative nor positive protein regulators of the *topA* promoter have been identified (Szafran et al., 2016). Previously, to detect *topA* activity changes, we used a *lux* reporter plasmid (pFLUX*ptopA*) in which the *topA* promoter controls the transcription of the *luxCDABE* reporter genes (Szafran et al., 2016). The *luxCDABE* operon encompasses the *luxAB* genes that encode luciferase (a heterodimer of LuxA and LuxB) and the *luxCDE* genes that encode enzymes necessary for luciferase substrate (tetradecanal) biosynthesis (Craney et al., 2006). Here, to search for unknown regulators of *topA* promoter activity, we combined *lux* reporter genes and random transposon mutagenesis.

The *S. coelicolor* WT-lux strain (pFLUX*ptopA* in the wild-type background) was subjected to random transposon mutagenesis (Fig. 1, stage 1), performed using a transposon plasmid (pHL734) containing the mini-Tn-5 transposon (Xu et al., 2017). pHL734 harbours a codon-optimized, highly efficient Tn5 transposase (under the control of the *ermE* promoter), which inserts mini-Tn-5 transposons randomly along the chromosome. The conjugation was repeated 4 times to deliver a WT-lux-tn library consisting of at least 8300 single colonies (Fig. 1, stage 2). Since transposition with mini-Tn5 occurs once per genome (as pHL734 cannot replicate in *Streptomyces*), each of the 8300 obtained colonies was assumed to carry a single transposon insertion in the genome. The luminescence of all obtained single colonies was measured during their growth on plates (Fig. 1, stage 3). Next, clones with altered luminescence intensity compared to the paternal WT-lux strain were selected (23 colonies with decreased luminescence and 18 colonies with increased luminescence) and re-streaked on fresh MS plates, and their altered luminescence in comparison to the paternal strain was verified (Fig. 1, stage 4). Subsequently, the clones were cultured in liquid medium, and the luminescence of selected clones was measured again (Fig. 1, stage 5). After the second round of selection, we obtained 12 colonies with significantly lowered or abolished luminescence signals and 2 colonies exhibiting elevated luminescence intensity. The presence of intact *lux* genes in clones with diminished fluorescence was confirmed by PCR (Fig. S1). Since modification of *topA* promoter activity detected by changes in *lux* gene activity was also expected to affect the TopA protein level (although earlier we observed that high activity of the *topA* promoter may not lead to high protein level; Szafran et al., 2016), we next aimed to estimate the TopA level in 14 transposant clones. To this end, the selected clones were cultivated in liquid, and the TopA level in the cell lysate was detected using Western blotting with anti-TopA antibodies and compared to the wild-type strain (Fig. 1, stage 6). Lower TopA protein levels (in comparison to those in the wild type strain) were observed in 2 clones, but we did not observe a significant increase in TopA protein levels in any of the mutants with elevated luminescence levels. Mutants with altered TopA protein levels were used for further analysis.

In summary, among the transposon mutants, we detected clones with both increased and decreased reporter gene activity. This indicates that the application of transposon libraries in combination with *lux* reporter genes may be used to identify both positive and negative transcriptional regulators.

Transposon mutation leads to modified transcription of the topA gene but does not affect chromosome supercoiling

One of the WT-lux strain transposants, named lux-tn66, emitted very weak luminescence when cultured on solid and in liquid media (Fig. 2A and 2B). Western blot analyses showed that the level of TopA protein in lux-tn66 was approximately 50% of the protein level in the wild-type strain (Fig. 2C, left), which was also verified by the measurement of *topA* transcript levels (Fig. 2C, right). RT-qPCR showed that the *topA* transcript level was approximately 70% of the *topA* transcript level in the wild-type strain. The observed discrepancy between the very low luminescence (indicating a very low level of *topA* promoter controlling reporter gene activity) and moderate lowering of the *topA* transcript level and protein level suggests the

complex regulation of the TopA protein level. However, the modified level of the *topA* transcript and its protein product confirmed the analysis of the reporter gene activity.

Based on our earlier studies, which showed that lowering the TopA protein level slows the growth of *S. coelicolor* in liquid and solid medium (Szafran et al. 2013), we measured the rate of growth of the lux-tn66 strain. The growth rate analysis in liquid culture showed a slight retardation of transposon strain growth compared to the WT-lux paternal strain, but the growth rate of the transposon strain was still significantly faster than that of the TopA-depleted strain (in which the TopA protein level was approximately 20-fold lower than the wild-type TopA level (Szafran et al., 2013)) (Fig. 2D).

Since the severe TopA depletion increases chromosome supercoiling (Szafran et al., 2013), we checked whether decreased TopA protein levels in the transposon lux-tn66 strain caused any changes in global DNA supercoiling. To this end, we determined the level of global DNA supercoiling using modified strains containing the reporter plasmids pWHM3Hyg or pWHM3Spec. We compared the supercoiling of plasmids isolated from modified lux-tn66 (lux-tn66-RP), the wild-type strain derivative (MS10) and the TopA-depleted strain derivative (MS11), which was used as a positive control. DNA supercoiling in transposon mutant lux-tn66-RP was found to be unaffected by decreased TopA protein level (Fig. 2E). Since chromosome supercoiling is maintained by concerted action of gyrase and TopA, we expected that unaltered DNA supercoiling despite lowered TopA protein level in the lux-tn66 strain may result from changes in gyrase protein level. To test this hypothesis, we determined the activity of the *gyrB* gene encoding one of the gyrase subunits. RT-qPCR analysis of the *gyrB* transcript level in the lux-tn66 transposon mutant showed significantly lower transcription level than wild-type *gyrB* transcription (Fig. 2F). This observation suggests that the lowered gyrase protein level compensated for the undesirable supercoiling changes triggered by the lowered TopA protein level in the lux-tn66 transposon mutant.

Based on our experiments, we infer that the modified level of *topA* gene transcription may either be linked to inactivation of the direct regulator of *topA* gene expression or to the indirect regulation of *topA* involving changes in the gyrase protein level in the mutant strain.

SCO4804 is a potential candidate for a topA promoter activator/regulator

Analysis of the transposon insertion site in the lux-tn66 strain (performed as described by Xu et al., 2017) showed that transposition occurred within the *sco4804* gene, 85 bp downstream of its predicted start codon (Fig. 3A). The *sco4804* gene encodes a hypothetical protein, SCO4804, composed of 815 amino acids (predicted molar mass 86.04 kDa), that is rich in glycine and proline residues, and that is conserved in *Streptomyces* species. Structural prediction was performed using Robetta software (Raman et al., 2009) and indicated the presence of putative alpha-helical structures in the central region of the protein and unstructured regions at both the C- and N-termini. Another analysis performed using PredictProtein (Yachdav et al., 2014), also showed three possible DNA-binding regions within the SCO4804 protein structure, which indicates that this protein may act as a transcriptional regulator (Fig. S2). Comparative analysis in the HOGENOM database (Penel et al., 2009) showed only a few homologues in other bacterial

families (such as Alphaproteobacteria, Bacteroidetes and Cyanobacteria); however, no annotated role was provided for the protein in any species.

Positioning of the *sco4804* gene (103 bp and 171 bp of non-coding regions upstream and downstream of the *sco4804* gene, respectively) suggests that it may not form an operon with adjacent genes; however, its genomic location is conserved within the *Streptomyces* genus. RNA-seq experiments performed previously using *S. coelicolor* wild-type and TopA-depleted strains (Szafran et al., 2019) showed significant, 8-fold induction of the *sco4804* gene, as well as adjacent genes, under TopA-depleted conditions. This result was confirmed by RT-qPCR experiments using the WT-lux strain (MG03) and TopA-depleted lux strain (MG04) (Fig. 3B). This strongly suggests that *sco4804* transcription is supercoiling-dependent, corroborating its potential function in controlling topoisomerase levels.

SCO4804 overproduction increases topA **promoter activity**

Since *sco4804* transcription was shown to be induced by TopA protein depletion and disruption of the *sco4804* gene lowered TopA protein level, we predicted that SCO4804 acts as a positive regulator of *topA* transcription. To confirm this hypothesis, we constructed a strain overexpressing the *sco4804* gene in the WT-lux strain background.

In the obtained strain (MG66), *sco4804* (as a second gene copy in the integrative vector pIJ6902) was controlled by a thiostrepton-inducible *tipA* promoter. Overexpression of *sco4804* in MG66 cells was confirmed by RT-qPCR, revealing significantly elevated *sco4804* transcript levels in comparison to the WT-lux strain background (Fig. 4A). Having confirmed the induction of SCO4804 in the MG66 strain, we set out to analyse the influence of SCO4804 on growth and *topA* promoter activity, as well as DNA supercoiling. Induction of SCO4804 led to slight retardation of growth compared to the control WT strain (with pIJ6902 empty vector) (Fig. 4B). The *topA* promoter activity, measured using the *lux* reporter genes, was significantly increased by *sco4804* induction either in 24-hour liquid or 48-hour plate cultures of the MG66 strain cultured in the presence of inducer (10 µg/ml thiostrepton) (Fig. 4C and D). This result indicated that overexpression of *sco4804* caused significant activation of the *topA* promoter.

Next, to confirm that *sco4804* is a positive regulator of the *topA* promoter, we performed RT-qPCR analysis of *topA* transcript level in the MG66 strain with induced *sco4804* overexpression. The results showed that increased *topA* transcript level was observed immediately after induction of *sco4804* (30 minutes after the addition of thiostrepton to the medium), but after 60 minutes of incubation in the presence of inducer, *topA* transcript level decreased to the wild-type level (Fig. 4E). The discrepancy between long-term elevated *lux* activity after *sco4804* induction and *topA* transcript elevation only during a very short period of time after induction suggests other post-transcriptional regulation modifications of *topA* transcript level, reinforcing our previous experiments (Szafran et al., 2019). Interestingly, the level of *gyrB* transcript exhibited similar changes, with an increase 30 minutes after SCO4804 induction and a decrease 60 minutes after induction (Fig. 4F). This indicates that the balance between gyrase and TopA activity was established and that the native supercoiling level could be restored. Indeed, the analysis of

the reporter plasmid supercoiling showed no changes in the MG66 strain upon SCO4804 induction (Fig. S3).

To check whether SCO4804 is a direct regulator of *topA* promoter activity, we tested its binding to the *topA* promoter *in vitro*. To this end, we purified the 6His-SCO4804 protein using the *E. coli* BL21 (DE3) groEL-groES strain (Goloubinoff et al., 1989) (Supplementary info, Fig. S4A) and performed an electrophoretic mobility shift assay (EMSA) using a 458 bp DNA fragment encompassing the *topA* promoter and 632 bp promoter of the *sco4697* gene, as well as 654 bp of a part of the *sco3928* gene as the negative controls. While 6His-SCO4804 bound all tested DNA fragments at a concentration of 1 μM, it was non-specific towards the *topA* promoter (Fig. S4B). Moreover, the addition of poly(dIdC) competitor DNA eliminated all non-specific interactions. A further pull-down assay and topoisomerase activity tests in the presence of 6His-SCO4804 excluded the possibility of a direct interaction between SCO4804 and TopA (Figs. S5 and S6). These experiments suggest that SCO4804 influences *topA* promoter activity in an indirect manner.

Discussion

Our approach combining *lux* reporter genes and a random transposon library allowed us to perform high-throughput screening for potential regulatory proteins that control TopA protein level in *S. coelicolor*. Genome-wide transposition, as a powerful genetic tool, is widely used for systematic genetic studies of different bacterial species, including the construction of random insertion *Streptomyces* mutants with IS6100, Tn4560, IS493, Tn5, and Himar1 transposons (McHenney and Baltz 1996; Volff and Altenbuchner 1997; Weaden and Dyson 1998; Widenbrant and Kao 2007; Bilyk et al. 2013). The Tn5 minitransposon together with the codon-optimized Tn5 transposase displays high efficiency, less codon bias and lower host specificity than other transposases (Xu et al., 2017). In *Streptomyces*, random mutagenesis has previously been used to find repressors or activators of genes of interest, the products of which are easy to monitor within the cell, such as antibiotic or pigment production. This technique has been successfully applied for the identification of actinorhodin and landomycin E negative regulators (Chen et al., 2012; Horbal et al., 2013). Moreover, transposon mutagenesis combined with a reporter system based on an antibiotic resistance cassette was previously applied to search for repressors for daptomycin production in *S. roseosporus* (Luo et al., 2018a). However, this approach, based on antibiotic resistance genes, limited the screening to negative transcriptional regulators. The advantage of our approach, in comparison to the abovementioned approaches, is its suitability for high-throughput searches of both negative and positive regulators in *Streptomyces* transposon libraries. An approach similar to our approach and based on the combination of random mutant library construction and the *lux* reporter gene has been successfully applied for the identification of regulatory proteins of *lecA* in *Pseudomonas aeruginosa* (Diggle et al., 2002) and the *acs* gene in *E. coli* (Baptist et al., 2013). As our screen combines a mutant library with luciferase reporter constructs, the changes in gene of interest expression can be readily monitored in both liquid and solid cultures over time in different environmental conditions, which also enables the identification of regulators active only in particular environmental conditions. However, it must be considered that this method is limited to non-essential regulators. The other disadvantage of

using *lux* reporter genes is the formation of artefacts due to metabolic influences on luciferase activity, but this can be overcome by using a second reporter system, for example, based on *gfp* expression (Baptist et al, 2013). Nevertheless, because of the compatibility of all genetic elements, we believe that the strategy tested here for the identification of regulators may be widely used in *Streptomyces*.

By applying our screening approach, we expected to find any proteins that influence TopA protein level with either transcriptional or translational/post-translational modes of action. It was shown earlier that the *topA* promoter was activated by increased chromosome supercoiling and was inhibited due to chromosome relaxation after novobiocin treatment (Szafran et al., 2016). In addition to supercoiling sensitivity, no other factor controlling promoter activity has been identified to date; thus, the identification of either a *topA* activator or repressor was of interest. We found that SCO4804 acts as a *topA* transcriptional activator, since its elimination decreased *topA* promoter activity and protein level, while induction of SCO4804 resulted in higher activity of the *topA* promoter. Notably, our previous studies showed that elevated *topA* transcript level does not correspond with elevated TopA protein level, as well as or with significant changes in DNA supercoiling levels (Szafran et al., 2016). However, the fact that the *topA* transcript level increased and subsequently diminished shortly after SCO4804 induction suggests that other mechanisms of maintaining TopA protein level are also activated. We previously suggested that the *topA* transcript and TopA protein levels are controlled by multiple regulatory strategies that act concertedly to preserve constant supercoiling level in *Streptomyces* (Szafran et al., 2016; Szafran et al., 2020). Additionally, we observed that SCO4804 induction influences not only TopA but also gyrase gene expression, indicating that the newly identified protein may be a component of the chromosome supercoiling maintenance system. The fact that transcription of *sco4804* is activated in response to increased negative supercoiling corroborates its potential function as the regulator of topoisomerase activity.

Conclusions

To summarize, our screening approach is optimized for *Streptomyces* and allows the identification of both positive and negative regulators that control the expression of genes of interest by either direct or indirect mechanisms. As proven by our concept, the protein SCO4804 was found to be a component of a complex regulatory network involved in *S. coelicolor* chromosome supercoiling maintenance. Since the production of secondary metabolites is regulated by chromosomal topology, understanding complex transcriptional regulation in *Streptomyces* is crucial for the industrial application of these bacteria.

Methods

Bacterial strains, plasmids, and growth conditions

Basic DNA manipulation procedures were performed according to standard protocols (Sambrook and Russell, 2001). Unless otherwise stated, all enzymes and isolation kits were obtained from Thermo Fisher Scientific (Waltham, MA) and NEB (Ipswitch, MA). Bacterial media and antibiotics were purchased from

Difco Laboratories (Detroit, MI) and Carl Roth (Karlsruhe, Germany), respectively. The *S. coelicolor* growth conditions and antibiotic concentrations, as well as the conjugation procedure, followed the general protocols described by Kieser et al, 2000. For induction, thiostrepton at concentrations of 0.5-10 µg/ml was added. Growth curves of the *S. coelicolor* strains were determined using the Bioscreen C device (Oy Growth Curves Ab Ltd., Helsinki, Finland). Cultures were grown in triplicate in 79 medium (Prauser and Falta, 1968) (300 µl/well), inoculated with 0.01 U/ml spores (1 U is defined as the volume of spore stock solution diluted up to 1 ml with OD_{600nm} = 1). The *S. coelicolor* and *E. coli* strains used in this study are shown in Table 1. The plasmids used in this study are shown in Table S1 (Supplementary materials).

Table 1: Strains used in this study.

Name	Genotype	Source or reference
S. coelicolor		
M145 (WT)	SCP1- SCP2-	Bentley et al., 2002
PS04 (TopA↓)	M145 Δ*topA*::scar *attB* ΦC31::pIJ6902*topA*	Szafran et al., 2013
MG03 (WT-lux)	M145 attBΦBT1::pFLUXH*ptopA*	Szafran et al., 2016
MG04 (TopA↓-lux)	M145 Δ*topA*::scar attBΦC31::pIJ6902*topA* attBΦBT1::pFLUXH*ptopA*	Szafran et al., 2016
MG02	M145 attBΦBT1::pFLUXH_*permE*	Szafran et al., 2016
MG01	M145 attBΦBT1::pFLUXH	Szafran et al., 2016
lux-tn66	MG01 *tn5*::*sco4804*	This study
lux-tn66RP	lux-tn66 pWHM3Spec	This study
MS10	M145 pWHM3Hyg	Szafran et al., 2016
MS11	M145 Δ*topA*::scar attBΦC31::pIJ6902*topA* pWHM3Hyg	Szafran et al., 2013
MG66	MG01 attBΦC31::pIJ6902_*sco4804*	This study
MG66_RP	MG66 pWHM3Hyg	This study
M145_pIJ6902	M145 attBΦC31::pIJ6902	This study
E. coli		
DH5α	F− endA1 glnV44 thi-1 *recA1relA1 gyrA96deoRnupG* purB20 φ80dlacZΔM15 Δ(*lacZ*YA-argF)U169, *hsdR*17(rK−mK+), λ−	Lab stock
ET12567 pUZ8002	*dam*-13::Tn*9dcm cat tet hsd zjj-201*::Tn*10 tra neo* RP4	Kleser et al., 2000
BW25113/pIJ790	K12 derivative; Δ*araBAD* Δ*rhaBAD* λ-Red (*gam bet exo*) *cat araC rep101*(Ts)	Gust et al., 2001
BL21 (DE3) groEL-groES strain	F− *ompTgal dcm lon hsdSB*(rB−mB−) λ(DE3 [*lacI lacUV*5-T7p07 ind1 sam7 nin5]) [*malB*+]K-12(λS) pGroESL	(Goloubinoff et al., 1989)

Transposon mutagenesis

To perform random transposon mutagenesis in *S. coelicolor* MG03, we used the pHL734 vector. Mutagenesis was performed according to a procedure described earlier (Xu et al., 2017). Briefly, *E. coli* ET12567 pUZ8002 harbouring the pHL734 plasmid was grown to OD_{600nm} = 0.5 and conjugated with 5 U of *S. coelicolor* MG03 (WT-lux) spores. The conjugated cell mixture was diluted (10^{-4} - 10^{-6}) and plated on MS agar supplemented with 10 mM $MgCl_2$ and 60 mM $CaCl_2$ to obtain single colonies. After 17 hours of growth at 30°C, the plates were overlaid with 20 µl per plate of each antibiotic below: nalidixic acid (25 mg/ml), apramycin (50 mg/ml) and hygromycin B (50 mg/ml). The obtained colonies were tested for luminescence intensity (see below), re-streaked on fresh MS agar plates supplemented with apramycin and hygromycin and used to establish liquid cultures. The positions of mini-Tn5 insertions in the *S. coelicolor* MG03 chromosome were identified using the rescue plasmid method. First, chromosomal DNA was isolated from a 24-hour culture in 79 medium of *S. coelicolor* transposon strains. Subsequently, 2 µg of chromosomal DNA was digested with the ApaI restriction enzyme (at 37°C overnight, 50 µl total reaction volume), and then DNA was purified using a CleanUp kit (A&A Biotechnology, Gdynia, Poland) and eluted with 15 µl of ultrapure water. Then, 100-200 ng of the ApaI-digested DNA was re-ligated (at 4°C overnight, 20 µl total volume of the reaction) using 1 µl of T4 DNA ligase (NEB) to allow formation of a mini–*E. coli* replicative plasmid. Electrocompetent *E. coli* DH5α cells were transformed with the ligation products (using half of the reaction volume) and selected on apramycin LB agar plates. Plasmid DNA was isolated from single *E. coli* colonies using a Plasmid Screening Kit (Syngen Biotech, Wrocław, Poland) according to the manufacturer's instructions. The isolated plasmids were digested with the ApaI restriction enzyme and analysed using gel electrophoresis, and plasmids exhibiting different digestion patterns were picked for subsequent DNA sequencing. Sequencing (Sigma-Aldrich, Saint Louis, MO) using UpS oligonucleotides identified the sites of mini-Tn5 insertion.

Strain construction

For inducible overexpression of the *sco4804* gene, the pIJ6902_*sco4804* plasmid was constructed. A DNA fragment encompassing the *sco4804* gene with flanking EcoRI and NdeI restriction sites was synthesized and cloned into the pUC57 mini plasmid, yielding pUCmini_4804 (GenScript Biotech Corporation, New Jersey, US). The pIJ6902_*sco4804* plasmid was obtained by restriction cloning of the *sco4804* insert into the pIJ6902 plasmid using NdeI and EcoRI sites. The construct was then conjugated from *E. coli* ET12567 pUZ8002 into *S. coelicolor* MG03 (WT-lux strain), apramycin-resistant exconjugants were selected, and the plasmid presence in the obtained strain MG66 was confirmed by PCR using M13pUCr and sco4804_rv oligonucleotides.

To analyse DNA supercoiling in the *S. coelicolor* transposon mutant lux-tn66 strain, we modified the pWHM3Hyg reporter plasmid (Szafran et al., 2013) by substituting the hygromycin resistance cassette with the spectinomycin resistance gene using the Redirect system (Gust et al., 2004), oligonucleotides spect_fwd_2 and spect_rv and plasmid pIJ778 as a template, yielding pWHM3Spec. Next, we introduced the pWHM3Spec plasmid into the lux-tn66 transposon mutant strain via conjugation with *E. coli* ET12567 pUZ8002 (Kieser et al., 2000). The MG66_RP strain, which was also used for analysis of DNA

supercoiling, was obtained by conjugation of the pIJ6902_*sco4804* plasmid into the MS10 strain (WT harbouring the pWHM3Hyg reporter plasmid).

As a control for the growth analysis of the MG66 strain induced with thiostrepton, we also constructed the M145_pIJ6902 strain, in which the empty plasmid pIJ6902 was introduced via conjugation into the M145 *S. coelicolor* strain.

Reporter gene activity assays

To measure luciferase activity in liquid culture, strains containing the *luxCDAEB* operon (under the control of the *topA* promoter or under the control of the *erm* promoter) in the pFLUXH ΦBT1 integrating vector were grown in liquid 79 medium for 24 hours at 30°C (in three biological replicates for each strain). Subsequently, the mycelium was collected by centrifugation, wet weight was determined, and mycelium was resuspended in 300 µl of 79 medium. Measurement of the luciferase activity was performed in triplicate directly from the mycelium suspension for each biological sample in a 100 µl volume in 96-well microplates (Perkin Elmer, Waltham, MA) using the Infinite PRO Multimode Plate Reader (Tecan, Männedorf, Switzerland). The luminescence intensity was normalized against wet weight (units/100 mg of mycelium). Luminescence visualization on solid medium was performed on MS agar plates after 48 hours of growth at 30°C, and luminescence detection was performed using a ChemiDocXRS+ device (Bio-Rad, Hercules, CA).

RNA isolation and RT-qPCR

For gene expression analysis, RNA was isolated from *S. coelicolor* cultures grown for 18 hours (unless otherwise stated) in liquid 79 medium. Before harvesting, a 1/10 culture volume of 95% EtOH saturated with phenol was added at a 5% final concentration to stabilize cellular RNA (Romero et al, 2014), and then mycelium was harvested by centrifugation and frozen in liquid nitrogen. Next, total RNA was isolated using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The RNA solution was transferred to a Total RNA Mini column (A&A Biotechnology) and processed according to the manufacturer's instructions. The RNA samples were digested with Turbo DNase I (Invitrogen, Carlsbad, CA) to remove traces of chromosomal DNA and then purified and concentrated using Clean-Up RNA Concentration (A&A Biotechnology). Five hundred micrograms of RNA was used for cDNA synthesis with the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA samples were diluted 5 times and used as templates for quantitative PCR (qPCR, each reaction performed in triplicate) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The level of the *topA* transcript was quantified using *hrdB* as a reference gene (ΔΔCT method) (StepOnePlus Real-Time PCR system; Applied Biosystems, Foster City, CA). Isolated RNA was tested for DNA contamination by qPCR with oligonucleotides complementary to the *S. coelicolorhrdB* gene. The difference > 5 Ct after 30 PCR cycles between the RNA sample and the corresponding cDNA sample as a template showed that the RNA samples were DNA-free.

DNA supercoiling assay

Global DNA supercoiling in *S. coelicolor* strains was quantified using the pWHM3Hyg reporter plasmid (Szafran et al., 2013) or its modified version, pWHM3Spec (Table S1). The plasmids were isolated using alkaline lysis and column purification based on a modified version of the manufacturer's (Plasmid Screening Kit, Syngen) procedure. After 48 hours of growth in liquid 79 medium supplemented with hygromycin or spectinomycin, *S. coelicolor* mycelium was collected by centrifugation, resuspended in PZ buffer containing 25 mg/ml lysozyme and incubated at 30°C for 5 min. The subsequent steps followed the manufacturer's protocol. The isolated reporter plasmids were resolved in 0.8% agarose in Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.3) in the presence of 4.6 μM chloroquine at a voltage of 20 V. To visualize topoisomers, the gel was stained with ethidium bromide for 30 min at room temperature. The experiment was repeated twice. The topoisomer distribution was analysed using ImageJ software.

TopA level quantification using Western blotting

For TopA level quantification, *S. coelicolor* 5 ml liquid cultures in 79 medium were cultivated for 24 hours. Next, the cell pellet was collected by centrifugation, resuspended in phosphate-buffered saline (PBS), sonicated and centrifuged. The cell lysates (5 μg of total protein) were separated by 10% SDS-PAGE according to standard procedures (Laemmli, 1970). After electrophoresis, the resolved proteins were stained overnight with PageBlue Protein Staining Solution (Thermo Fisher Scientific) or transferred to a nitrocellulose membrane and blocked with 2% milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4–7.6). The blots were subsequently incubated with rabbit polyclonal TopA antiserum (1:10,000 in TBST; 1-hour incubation; Szafran et al., 2013) and visualized using alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:5000) (Sigma-Aldrich). The band intensities were analysed using ImageJ software, comparing the TopA band intensity of particular mutants to the wild-type reference.

Structure prediction and homologue analysis

SCO4804 protein structure prediction was performed using the Robetta Web Server and "TrRefineRosetta" modelling method (Raman et al., 2009; Song, et al., 2013), as well as using PredictProtein (Yachdav et al., 2014). Homologue searching was performed using HOGENOM (Penel et al., 2009).

Abbreviations

WT- wild type; RNAP- RNA polymerase, TF- transcription factor; TAE- Tris-acetate-EDTA buffer; TBE-Tris-borate-EDTA buffer , MS- mannitol soya flour agar; PBS- phosphate-buffered saline; TBST- Tris-buffered saline with 0.1% Tween 20 detergent.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

MGJ performed all experiments described in this paper and was a major contributor in writing the manuscript. MS contributed to the conception of the work, data interpretation and revision of the manuscript; DJ made substantial contributions to interpretation of data and writing the manuscript.

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Figures

Fig. 1

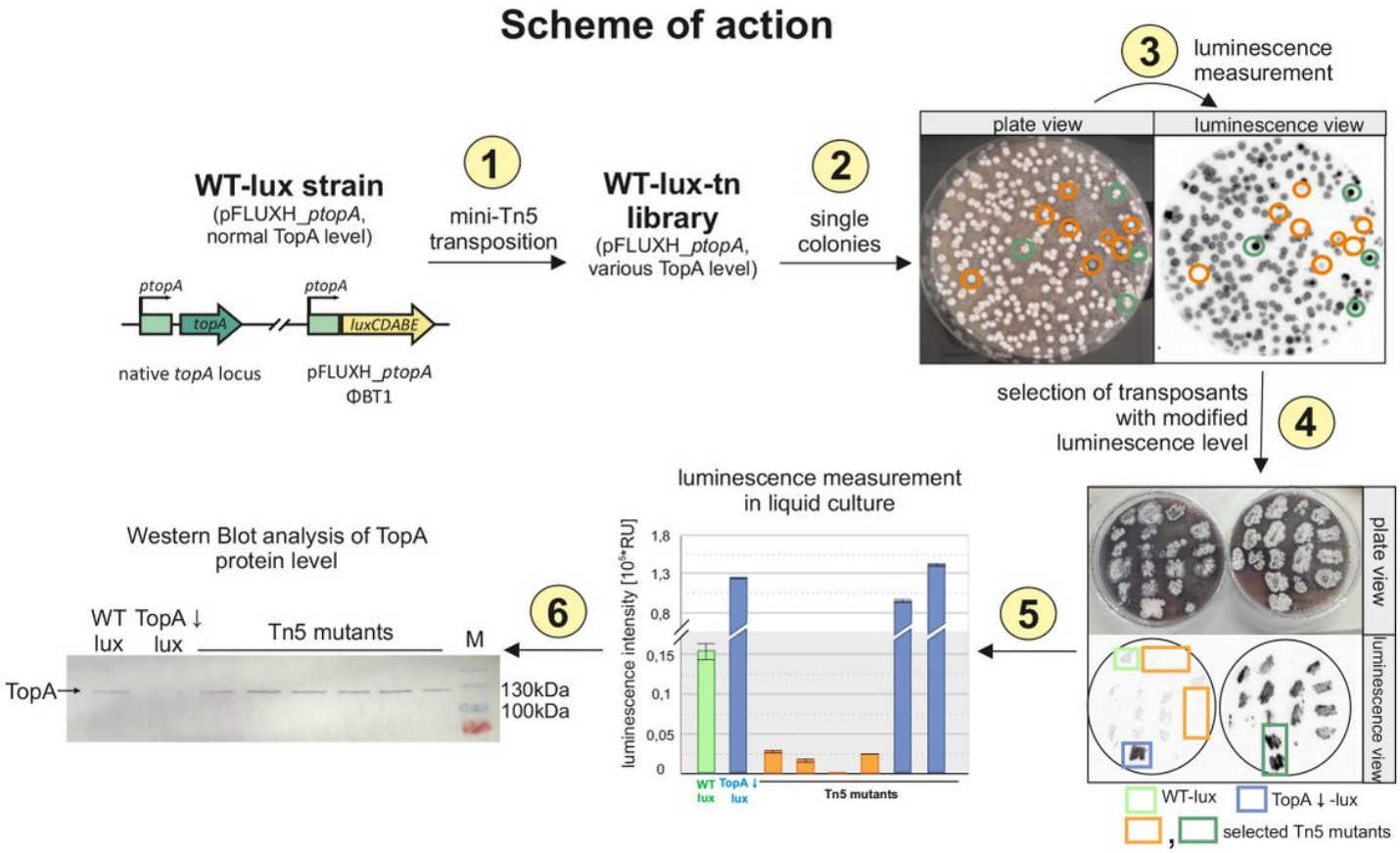

Figure 1

Scheme of random Tn5 transposon mutagenesis in the S. coelicolor WT-lux reporter strain. 1. Random transposon mutagenesis of the WT-lux strain (MG03) with a mini-Tn5 transposon. 2. The WT-lux-tn mutant library consisted of approximately 8300 single colonies obtained on MS agar plates. 3. Measurement of the luminescence of WT-lux-tn library single colonies. 4. Selection of colonies with altered light emission compared to the WT-lux paternal strain and TopA-depleted lux strain (MG04, high activity of topA promoter). 5. The luminescence of selected colonies from the WT-lux-tn library measured in liquid culture compared with the WT-lux strain and TopA-depleted lux strain (high activity of the topA promoter). RU - relative luminescence units. 6. Western blot analysis of TopA protein level in cell lysates of selected colonies from the WT-lux-tn library with anti-TopA polyclonal antibodies. M - molecular mass marker.

Fig. 2

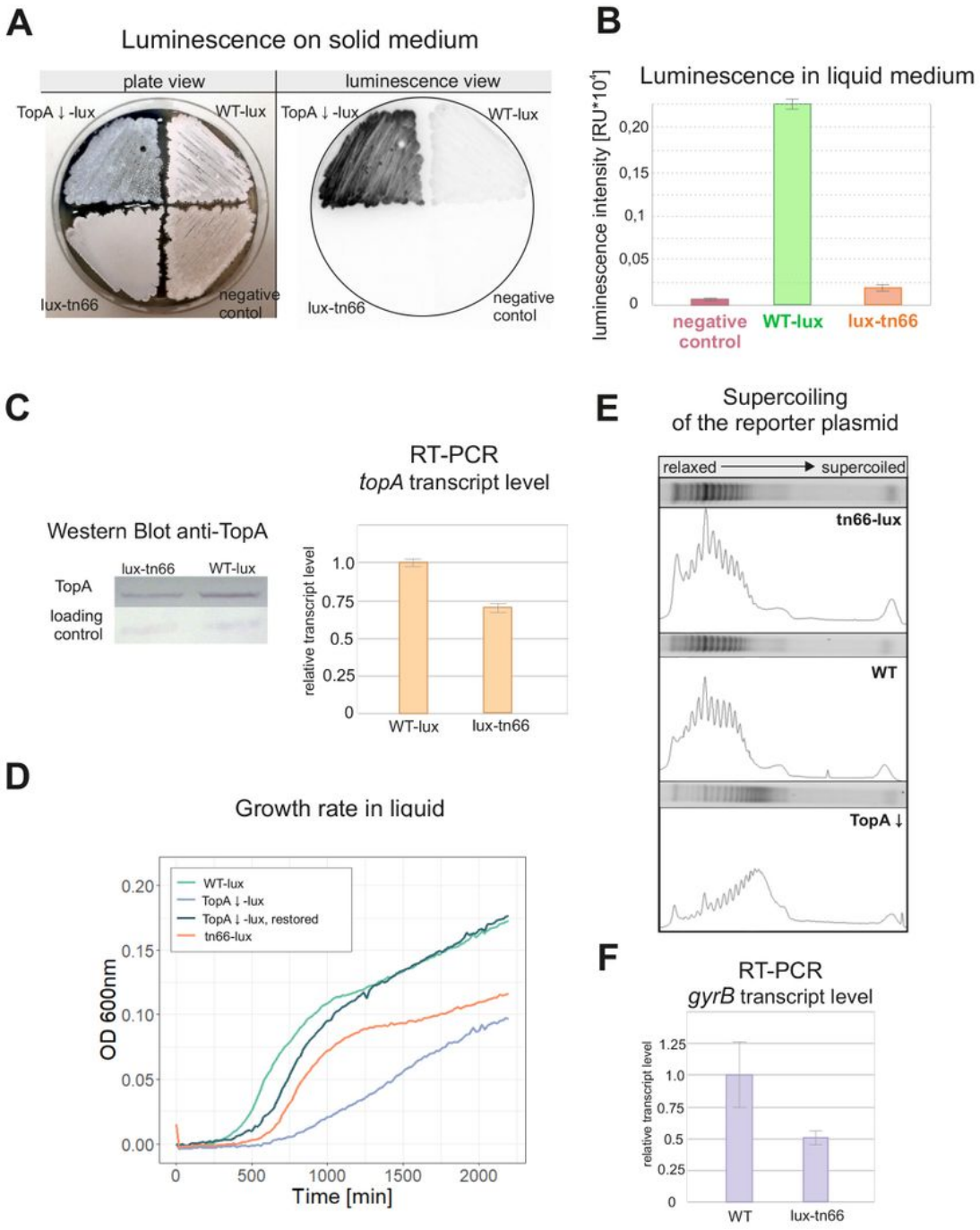

Figure 2

Phenotype of the lux-tn66 transposon strain. A. Growth and luminescence of the lux-tn66 transposon strain on solid MS medium in comparison to the WT-lux strain (MG03), TopA-depleted lux strain (MG04) and the negative control - wild type strain with empty pFLUXH vector (MG01). Left panel: plate view (after 5 days of growth), right panel: luminescence intensity (after 48 hours). B. Luminescence of mutant reporter strains after 24 hours of growth in liquid 79 medium compared to the WT-lux (MG03) and the

negative control—wild-type strain with empty pFLUXH vector (MG01). C. Western blot analysis of TopA protein level (top panel) and the relative transcription of the native topA gene in the mutant lux-tn66 strain determined using RT-qPCR analysis performed on 24-hour 79 medium cultures, compared to the WT-lux strain. D. The growth curves of the lux-tn66 strain (79 medium, Bioscreen C, measurements every 20 minutes) compared to the WT-lux (MG03) and TopA-depleted lux strain (MG04), as well as to MG04 with restored TopA protein level (after induction with 0.5 µg/ml thiostrepton). E. Supercoiling density of the reporter plasmids pWHM3Hyg or pWHM3Spec isolated from the transposon mutant lux-tn66 derivative (lux-tn66_RP) strain, the wild-type strain derivative (MS10) and the TopA-depleted (MS11) strain (representative image of two independent experiments). The figure shows topoisomers detected in agarose gel as well as band intensity measurements performed using ImageJ software. F. The level of gyrB transcript in lux-tn66 strain determined using RT-qPCR analysis performed on 24-hour 79 medium cultures, compared to the WT-lux strain (MG03).

Fig. 3

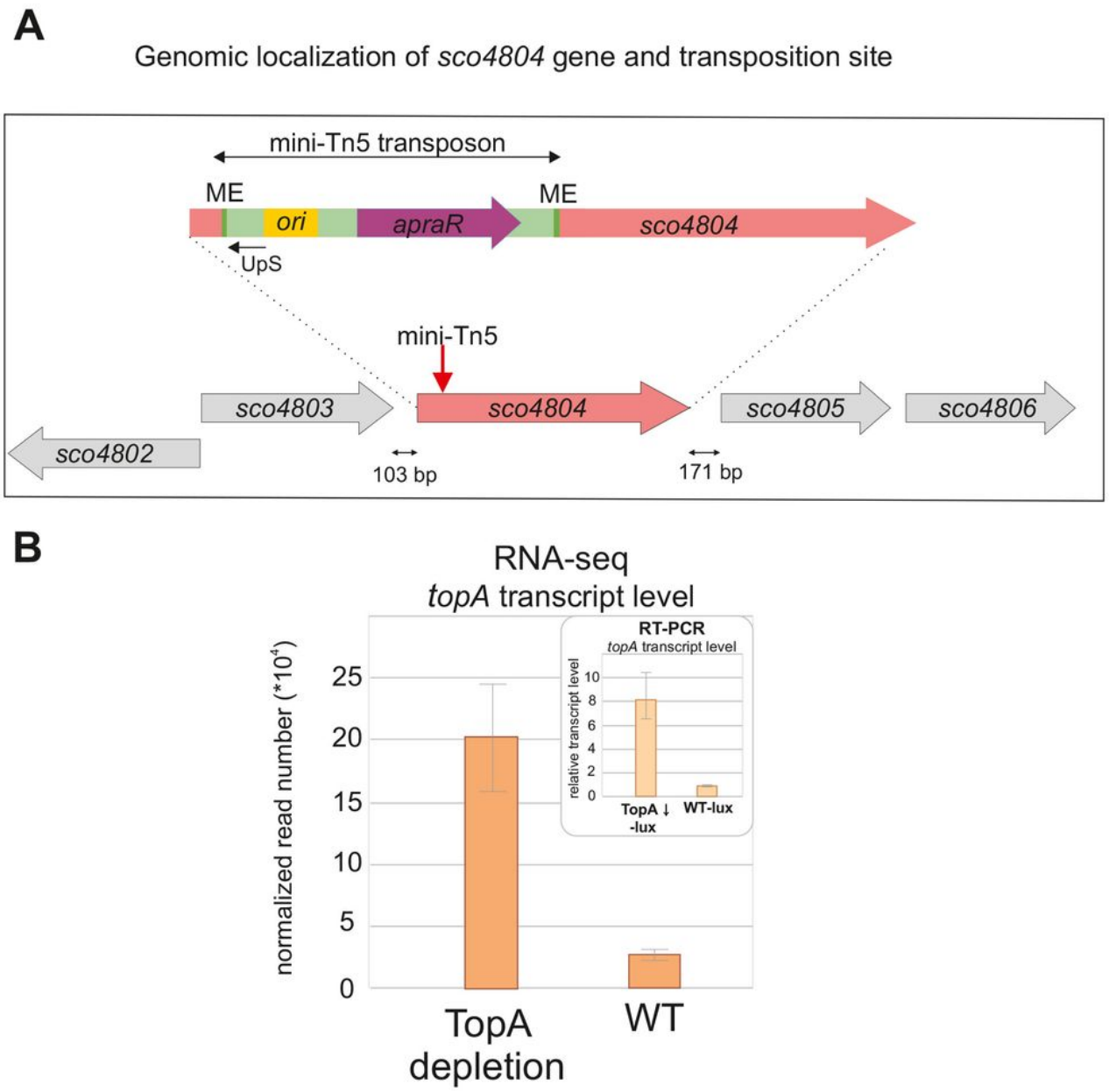

Figure 3

Genomic localization and supercoiling-dependent transcription of sco4804. A. Transposition site in lux-tn66 strain. ME (dark green) - the mosaic end sequence; ori (yellow) - origin of replication from pUC vector for DNA replication in E. coli; apraR (dark violet) - apramycin resistance gene; UpS - primer used for recognition of mini-Tn5 insertion site. The red arrow shows the identified site of the mini-Tn5 insertion. The black arrows at the bottom of the scheme show the distance between neighbouring genes. B. RNA-

Seq-based analysis of the expression level of sco4804 in the TopA-depleted (PS04) and control wild-type (M145) strains performed for 18-hour YEME/TSB cultures, normalized by the upper quartile (Szafran et al., 2019). The error bars correspond to standard deviations calculated for four independent biological replicates. Inset: the relative transcription of sco4804 in the TopA-depleted lux (MG04) and WT-lux reporter (MG03) strains calculated using RT-qPCR analysis performed for 24-hour cultures in 79 medium.

Fig. 4

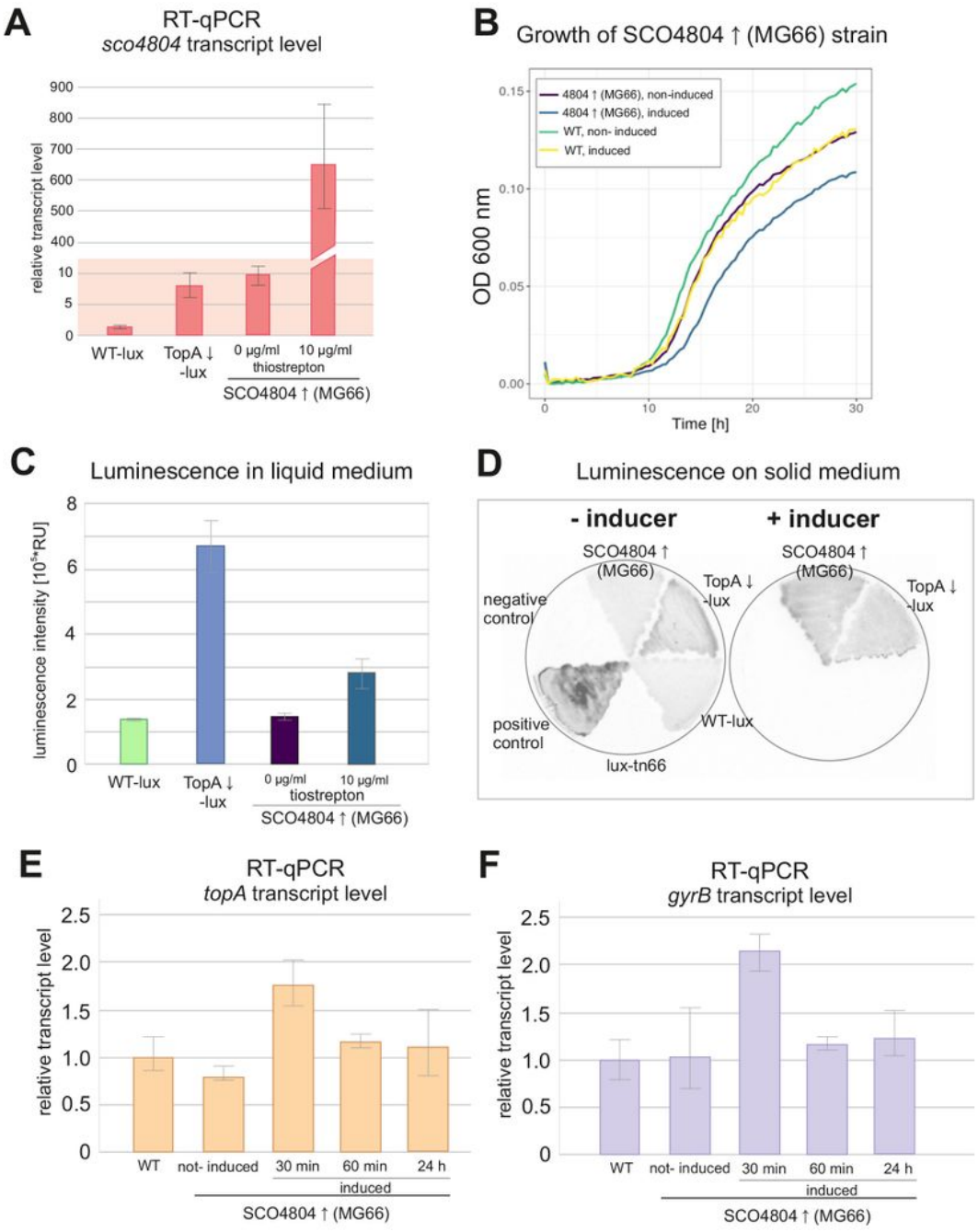

Figure 4

Phenotype of the SCO4804 overproducing strain. A. RT-qPCR analysis of the sco4804 transcript level in the MG66 strain (non-induced and induced with 10 µg/ml thiostrepton) compared to the WT-lux (MG03) and TopA-depleted lux (MG04) strains performed for 24-hour cultures grown in 79 medium. B. The growth curves of the non-induced MG66 strain and MG66 induced with 10 µg/ml thiostrepton (79 medium, Bioscreen C, measurements every 20 minutes) compared to the WT-lux strain (MG03). C. Measurement of luminescence of the MG66 strain in the absence or in the presence of the sco4804 inducer (0 and 10 g/ml of thiostrepton) indicating the changes of topA promoter activity, performed in liquid 79 medium after 24 hours of growth and compared to the WT-lux (MG03) and TopA- depleted lux (MGO4) strains. D. Luminescence of MG66 indicating the activity of the topA promoter controlling the lux reporter genes after 48 hours of growth on solid MS agar plates, without and with the inducer (10 µg/ml thiostrepton), as compared to the WT-lux (MG03) and TopA- depleted lux (MGO4) strain, negative control (the wild-type strain with empty pFLUXH vector (MG01)) and positive control (the wild type strain with pFLUXH_permE (MG02)). E. RT-qPCR analysis of the relative transcription of the topA gene in the SCO4804 overproducing strain (MG66) cultured in 79 medium for 24 hours and induced with 10 µg/ml thiostrepton for 30 minutes, 60 minutes or cultured for 24 hours in the presence of the inducer. The data were compared to the non-induced control and WT-lux strain (MG03) grown for 24 hours in 79 medium. F. RT-qPCR analysis of the relative transcription of the gyrB gene in the SCO4804-overproducing strain (MG66) induced after 24 hours of growth with 10 µg/ml thiostrepton for 30 minutes and 60 minutes and/or cultured for 24 hours in the presence of the inducer. The data were compared to the non-induced control and WT-lux strain (MG03) grown for 24 hours in 79 medium.

Supplementary Files

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