The pathway specific regulatory genes, *tei15** and *tei16**, are the master switches of teicoplanin production in *Actinoplanes teichomyceticus*

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Liliya Horbal, Anton Kobylyanskyy, Andrew W. Truman, Nestor Zaburranyi, Bohdan Ostash, Andriy Luzhetskyy, Flavia Marinelli, Victor Fedorenko*

L. Horbal, N. Zaburranyi, B. Ostash, V. Fedorenko*

Department of Genetics and Biotechnology, Ivan Franko National University of Lviv, Ukraine

v_fedorenko@franko.lviv.ua

A. Kobylyanskyy, F. Marinelli

Department of Biotechnology and Life Sciences, University of Insubria, Varese and "The Protein Factory" Research Center Politecnico of Milano, ICRM CNR Milano and University of Insubria, Varese Italy

A. Luzhetskyy

Helmholtz-Institute for Pharmaceutical Research Saarland, Saarbrucken, Germany

A. W. Truman

Department of Molecular Microbiology, John Innes Centre, Colney Lane, Norwich, UK

*Corresponding author: Victor Fedorenko

Abstract Pathogenic antibiotic-resistant bacteria are an unprecedented threat to healthcare worldwide. The range of antibiotics active against these bacteria is narrow; it includes teicoplanin, a "last resort" drug, which is produced by the filamentous actinomycete *Actinoplanes teichomyceticus*. In this report, we determine the functions of *tei15** and *tei16**, pathway specific regulatory genes that code for StrR- and LuxR-type transcriptional factors, respectively. The products of these genes are master switches of teicoplanin biosynthesis, since their inactivation completely abolished antibiotic production. We show that Tei15* positively regulates the

transcription of at least 17 genes in the cluster, whereas the targets of Tei16* still remain unknown. Integration of *tei15** or *tei16** under the control of the aminoglycoside resistance gene *aac(3)IV* promoter into *attBφC31* site of the *A. teichomyceticus* chromosome increased teicoplanin productivity to nearly 1 g/L in TM1 industrial medium. The expression of these genes from the moderate copy number episomal vector pKC1139 led to 3-4 g/L teicoplanin, while under the same conditions wild type produced approximately 100 mg/L. This shows that a significant increase in teicoplanin production can be achieved by a single step of genetic manipulation of the wild type strain by increasing the expression of the *tei* regulatory genes. This confirms that natural product yields can be increased using rational engineering once suitable genetic tools have been developed. We propose that this new technology for teicoplanin overproduction might now be transferred to industrial mutants of *A. teichomyceticus*.

Keywords: Actinoplanes, Teicoplanin, Pathway-specific regulators, Strain improvement, Overproducer.

Introduction

There has been a recent alarming rise of diseases caused by bacteria resistant to all β-lactams, and to most macrolides, aminoglycosides, and tetracyclines. This has prompted the use of glycopeptide antibiotics, such as vancomycin and teicoplanin, as the only drugs that effectively treat certain life-threatening infections. However, vancomycin possesses a number of negative features including low lipophilicity, which means that it cannot penetrate through some tissues (Craig 2003), and comparatively high nephrotoxicity (Wood 1996; Svetitsky et al. 2009). In comparison to vancomycin, teicoplanin is two- to fourfold more active against both methicillin-susceptible and methicillin-resistant isolates of *Staphylococcus aureus*, and much less toxic (Chow et al. 1993; Van Bambeke et al. 2004). For these reasons teicoplanin can even be used for the treatment of serious infections in children. Therefore, there is a strong interest in the development of industrial overproducers of teicoplanin and its derivatives.

Teicoplanin is a mixture of closely related glycopeptide compounds, and is produced by *Actinoplanes teichomyceticus* (Somma et al. 1984). Its biosynthesis starts with the non-ribosomal synthesis of a linear heptapeptide, which is oxidatively cross-linked, halogenated, glycosylated and acylated (Kahne et al. 2005). The low yield of teicoplanin seriously limits the commercial production of this compound. Several strategies have been applied in attempts to increase the production of teicoplanin, including empirical mutagenesis and selection (Jung et al. 2009), optimization of fermentation conditions (Taurino et al. 2011; Beltrametti et al. 2007) and total chemical synthesis (Boger et al. 2001). In an industrial environment, recursive mutagenesis and selection (classical strain improvement, CSI) still represents the most successful approach for a rapid increase in the production yield of antibiotic-producing microbes. However, this is a time-consuming and cumbersome procedure, which introduces unwanted mutations that can hamper further improvements (Medema et al. 2011). Targeted manipulations by recombinant DNA technology can provide an alternative strategy for improving titers that complements the empirical methods used in industry, and successful cases have been recently reported (Chen et al. 2010). Nevertheless, the application of such molecular approaches has often been hindered by a poor knowledge of the genes to be targeted, as well as by the lack of molecular tools to manipulate the producing microorganisms. This has limited the engineering of *A. teichomyceticus*, which belongs to a group of filamentous non-streptomycetes actinomycetes that are challenging to genetically manipulate (Marcone et al. 2010). The earliest report on conjugal DNA recombination in *A. teichomyceticus* was published in 2008 (Ha et al. 2008), whereas the teicoplanin biosynthesis gene cluster was sequenced and fully annotated almost ten years ago. The cluster (named *tei* by Li et al. 2004, and *tcp* by Sosio et al. 2004) spans approximately 89 kb and includes 49 open reading frames (ORFs) that are predicted to participate in teicoplanin biosynthesis. This includes genes putatively responsible for the assembly of the antibiotic, resistance, export, and regulation of its synthesis (Fig. 1).

There are five putative regulatory genes in the cluster. The products of the *tei2* and *tei3* genes encode a response regulator and a kinase, respectively, which show high level of homology to the

VanR/VanS system of *S. coelicolor* (Hutchings et al. 2006) and are most likely involved in the regulation of resistance gene expression (Beltrametti et al. 2007). The *tei31** gene codes for putative SARP family transcriptional regulator. The products of two other genes, *tei15** (named *tcp28* according to Sosio et al. 2004) and *tei16** (named as *tcp29* according to Sosio et al. 2004), have been assigned to the families of StrR-type and LuxR-type transcriptional regulators, respectively (Li et al. 2004; Sosio et al. 2004). In most cases, regulators of these families are pathway-specific, located in the clusters and involved in the positive control of antibiotic production (van Wezel and McDowall 2011). Recently, we demonstrated that it is possible to moderately increase teicoplanin production through manipulation of the cluster-situated regulatory genes *tei15** and *tei16** (Horbal et al. 2012; Horbal et al. 2013). However, the exact function and mechanism of action of the Tei15* and Tei16* proteins remained obscure, the influence of increased expression of the regulatory *tei* genes on teicoplanin production was not studied in optimized fermentation conditions, and no strains were studied that used constructs with *tei15** or *tei16** genes based on integrative vectors.

In this work, we performed gene deletion in *A. teichomyceticus* by the in-frame substitution of the chromosomal genes with the apramycin resistance cassette. The efficiency of this method in *A. teichomyceticus* is very high, since 97% of transconjugants were selected as resistant mutants. Using this genetic tool, we inactivated the *tei15** and *tei16** genes and investigated their role in teicoplanin production. We demonstrated that Tei15* and Tei16* are the key switches for antibiotic biosynthesis in the *tei* cluster. These findings, along with our previous studies (Horbal et al. 2013), allowed us to extend the choice of genetic engineering strategies for *A. teichomyceticus*. A variety of teicoplanin overproducing strains were generated by using integrative or replicative vectors carrying the heterologous promoter *aac(3)IV* and either the *tei15** or *tei16** regulatory genes. Teicoplanin production was compared to wild type in an optimized fermentation medium.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium and antibiotics were added to cultures when required to maintain plasmids at the following concentrations per millilitre: ampicillin 65 µg; kanamycin 50 µg; chloramphenicol 25 µg; apramycin 50 µg, hygromycin 120 µg. Medium components and antibiotics were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

A. teichomyceticus NRRL-B1672 (ATCC 31121) and its recombinant strains were maintained at -80°C in 15% v/v glycerol at a biomass concentration of approximately 0.08 g/mL dry weight. Working cell banks (WCB) were prepared as previously described (Taurino et al. 2011). For conjugation, *A. teichomyceticus* strains were grown on oatmeal (Kieser et al. 2000) or MS-medium (Kieser et al. 2000) for vigorous sporulation. Selection of the transconjugants was performed on the same media supplemented with appropriate antibiotics when required. For DNA isolation, transconjugants were grown in 25 mL liquid seed medium (g/L: glucose 30, yeast extract 5, peptone 5, K_2HPO_4 4, KH_2PO_4 2, $MgSO_4 \times 7H_2O$ 0.5, pH 7.2).

Recombinant DNA techniques

Isolation of genomic DNA from *A. tecihomyceticus* and plasmid DNA from *E. coli* was carried out using standard protocols (Kieser et al. 2000). Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers Thermo Scientific (Schwerte, Germany), Promega (Madison, USA).

Plasmid construction

tei15 inactivation.* The kanamycin resistance gene in cosmid 4B2 (Fig. 1) was replaced with the *hyg* cassette (pHYG1) by the use of λRed recombination process (Gust et al. 2002) and HygRVSKm Forw and HygRVSKmRev primers (Table 2). This generated cosmid 4B2hyg. The *tei15** gene was replaced with the *aac(3)IV* cassette (pIJ774) within cosmid 4B2hyg using λRed

recombination process. The tei15*delForw and tei15*delRev primers used for replacement are listed in Table 2, and were used to generate cosmid 4B2del15.

tei16 inactivation.* The *tei16** gene was replaced with the *aac(3)IV* cassette (pIJ774) within cosmid 4B2hyg by the use of λRed recombination process. The tei16*delForw and tei16*delRev primers used for replacement are listed in Table 2, and were used to generated cosmid 4B2del16.

Generation of the chromosomal mutants of *A. teichomyceticus* NRRL-B16726

Two gene disruption cosmids were conjugally transferred from *E. coli* into *A. teichomyceticus* NRRL-B16726. Exconjugants were selected for resistance to apramycin (10 μg/ml). For the generation of *A. teichomyceticus* Δ*tei15** and *A. teichomyceticus* Δ*tei16**, single-crossover apramycin and hygromycin resistant mutants were screened for loss of hygromycin resistance that is a result of a double-crossover event. Replacement of the *tei15** and *tei16** genes was confirmed by PCR using the primer pairs tei15*Forw and tei15*Rev, tei16*Forw and tei16*Rev, respectively (Table 2). The size of the PCR fragments was 1.35 kb when chromosomal DNAs of *A. teichomyceticus* Δ*tei15** and Δ*tei16** mutants were used, while the same primers for *tei15** and *tei16** genes produced amplicons of 1.11 and 2.6 kb, respectively, when genomic DNA of the wild-type strain was used as a template (Fig. S1).

Overexpression of the *tei15** and *tei16** genes under the control of the apramycin resistance gene promoter

A 0.35 kb fragment containing the apramycin gene resistance promoter (*aac(3)IVp*) from pIJ733 was amplified using aacPF and aacPR primers (Table 2), digested with *Bam*HI and *Eco*RV, and cloned into respective sites of the integrative shuttle vector pSET152 yielding pSET152A (Table 1).

A 1.12 kb DNA fragment containing the *tei15** gene was amplified from the 4B2 cosmid (Table 1) using tei15*F and tei15*R primers (Table 2), and cloned into pSET152A digested with *Eco*RV. This generated pSET152Atei15 (Table 1).

A 2.6 kb DNA fragment carrying *tei16** gene was amplified from the *A. teichomyceticus* chromosome using tei16*EF-1 and tei16*ER primers (Table 2). The amplified DNA fragment was cloned into *Eco*RV site of pKC1139A (Table 1) and pSET152A giving pKC1139Atcp29 and pSET152Atei16, respectively. In both plasmids, the *tei16** gene is under the control of the *aac3(IV)* promoter.

Complementation of the *tei15** and *tei16** mutants

The apramycin resistance gene in the plasmid pKC1139tcp28 (Table 1) was replaced with the *hyg* cassette (pHYG1) by the use of the λRed recombination process and of P1Am-Hyg-up and P2Am-Hyg-rp primers (Table 2), yielding pKCtcp28hyg.

Substitution of the apramycin resistance gene with hygromycin (pHYG1) in the plasmid pSET152Atei16 (Table 1) using the λRed recombination process and the primers P1Am-Hyg-up and P2Am-Hyg-rp (Table 2) generated plasmid pSETAtei16hyg.

Teicoplanin production and analysis

To start the fermentation process, one vial of the WCB was inoculated into 300-mL baffled flasks containing 50 mL of vegetative medium E25 (Beltrametti et al. 2004). Flask cultures were incubated for 72 hours on a rotary shaker at 220 rpm and 28°C and then used to inoculate (5% v/v) 500-mL baffled Erlenmeyer flasks containing 50 mL production medium TM1 (Taurino et al. 2011), or 3 L P-100 Applikon glass reactor (height 25 cm, diameter 13 cm) equipped with a AD1030 Biocontroller and AD1032 motor, containing 2 L of the same production medium (Beltrametti et al. 2004). Flasks were incubated at 28°C and 220 rpm for 144-168 hours.

Cultivations in fermenters were carried out for 144-168 hours at 30°C, with stirring at 500-900 rpm (corresponding to 1.17- 2.10 m/s of tip speed) and 2 L/min aeration rate. Dissolved oxygen (measured as $_{\%}DO_2$) was monitored using an Ingold polarographic oxygen electrode and eventually controlled by setting agitation speed in cascade with a set point of 20% of saturation with DO_2. The pH values of culture broths were monitored using a pH meter. Foam production was controlled by adding Hodag antifoam through an antifoam sensor. The samples of fermentation broth were collected at regular time intervals and analyzed. Teicoplanin was extracted by mixing 1 volume of broth with 1 volume of borate buffer [100 mM H_3BO_3 (Sigma-Aldrich), 100 mM NaOH (Sigma-Aldrich), pH 12]. Mixtures were kept shaking on a rotary shaker at 200 rpm and 36°C and centrifuged (16,000 *x g* for 15 min) afterwards. The supernatant was collected and filtered through a Durapore membrane filter (0.45 μm) (Millipore, Billerica, MA, USA). The glycopeptide production was estimated by HPLC performed on a 5-μm particle size Syncronis C18 (Superchrom, Thermo Scientific, Milano, Italy) column (4.6 × 250 mm) eluted at 1 mL/min flow rate with a 30-minute linear gradient from 15% to 65% of Phase B, followed by 10 minutes with 100% Phase B. Phase A was 32 mM $HCOONH_4$ (Sigma-Aldrich) pH 4.5:CH_3CN (Sigma-Aldrich) 90:10 (v/v) and Phase B was 32 mM $HCOONH_4$ pH 4.5:CH_3CN 30:70 (v/v) mixture. The chromatography was performed with a model 1100 HPLC system (Elite Lachrom VWR Hitachi LLC) and UV detection was done at 236 nm. As a standard, pure samples of teicoplanin (Targocid, Sanofi-Aventis) were used. Three HPLC analyses were repeated on the same sample and data were calculated as mean values of three replicated analyses.

Teicoplanin concentration was measured as total T-A_2 complex as the sum of five factors (T-$A_{2\text{-}1}$, T-$A_{2\text{-}2}$, T-$A_{2\text{-}3}$, T-$A_{2\text{-}4}$, T-$A_{2\text{-}5}$) calculated as previously reported (Taurino et al. 2011). To estimate growth, mycelium was collected by centrifugation (4000x *g* for 10 min). To measure biomass production, 10 mL culture was collected and centrifuged to determine packed mycelium volume (PMV). Glucose was analyzed using the Trinder assay (Sigma Diagnostics, St. Louis, MO, USA). Data was calculated as mean values from three replicated fermentations.

LCMS spectra were obtained using a Hewlett-Packard HPLC 1100 series instrument coupled to a Finnigan MAT LCQ ion trap mass spectrometer fitted with a positive mode ESI source. Cell culture (100 μL) was diluted with acetonitrile (100 μL) and centrifuged to pellet cell debris. Samples were injected onto a Phenomenex Luna C18(2) column (250 mm x 2.0 mm, 5 μm), eluting with a linear gradient of 5 to 60% acetonitrile (Fisher, HPLC grade) containing 0.1% trifluroacetic acid (TFA) in water (Rathburn, HPLC grade) + 0.1% TFA over 20 minutes with a flow-rate of 0.3 mL/min.

Overexpression of Tei15* and Tei16*

Codon-optimized copies of the *tei15** and *tei16** genes, named *tei15*s* and *tei16*s*, were synthesized by Shine Gene Company (Shanghai, China), digested with *Nde*I and *Xho*I, and cloned into respective sites of the vector pET28a, yielding pET28tei15 and pET28tei16 (Table 1), respectively.

E. coli Rosetta (pLysS) harboring the pET28tei15 plasmid, and *E. coli* BL21 (DE3) harboring the pET28tei16 plasmid, were grown overnight at 37ºC. LB (400 mL) containing 50 μg/mL of ampicillin was inoculated with 2 mL of the overnight culture and incubated at 37ºC until the OD_{600nm} reached 0.5-0.6. Tei15* and Tei16* expression was induced with 0.5 mM IPTG. After incubation for an additional 3 hours, the cells were harvested by centrifugation and washed with ice-cold column buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl). Cell lysis and purification of Tei15* (calculated mass 39.97 kDa) and Tei16* (calculated mass 89.55 kDa) with His-tag-binding resins were performed according to Novagen instructions. Both proteins (Fig. S3, S4) were eluted with column buffer containing 200 mM imidazole. The purest fractions were pooled, washed with storage buffer (50 mM potassium phosphate [pH 8.0], 300 mM NaCl, 10% v/v glycerol), concentrated using Amicon Ultra (Millipore). The protein yield was 0.8 mg/ml in the case of Tei15* and 0.45 mg/ml in the case of Tei16*. Aliquots of Tei15* and Tei16* fusion proteins in storage buffer were stored at – 80ºC, or used immediately in DNA-binding assays.

Electrophoretic mobility shift DNA-binding assays (EMSA)

DNA fragments containing putative promoters of *tei15** (300 bp), *tei16** (280 bp; 60bp), *teiA* (530 bp; 60 bp), *tei17** (219 bp; 70 bp), *tei2** (300 bp), *tei14** (360 bp), *tei27** (220 bp), *tei31** (370 bp) (Fig. 1) were used in EMSA. These promoter regions were amplified from the chromosomal DNA of *A. teichomyceticus* using primers listed in Table 2. Each EMSA contained 50 ng of a target DNA and 0.08 µg, 0.4 µg, 1.2 µg of the His-Tei15* or His-Tei16* protein in a total volume of 20 µL in a binding buffer (20 mM Tris HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 100 mM KCl, 1 mM MgCl2, 10% v/v glycerol). After incubation for 25 min at room temperature, protein-bound and free DNA were separated by electrophoresis at 4ºC on a 4.5% nondenaturing polyacrylamide gel in 0.5×TBE buffer. The gel was stained with ethidium bromide and analyzed using an UV-imaging system (Fluorochem 5330). A negative control assay was performed in the presence of the part of the *teiA* coding region, amplified with the use of primers teiAForw and teiARev (Table 2).

Results

Inactivation of the *tei15** and *tei16** genes in *A. teichomyceticus* prevents teicoplanin production

To elucidate the function of the *tei15** and *tei16** regulatory genes, we substituted the chromosomal copies of these genes with their respective mutant alleles that contain the apramycin resistance gene *aac3(IV)*. Among the 43 transconjugants obtained, one had the Am^rHyg^r phenotype, while the remaining ones were Am^rHyg^s, indicating that a second crossover had already occurred in these strains. Therefore, mutants *A. teichomyceticus* Δ*tei15** and Δ*tei16** were obtained and the inactivation of the genes was confirmed by PCR analysis (Fig. S1). Both strains showed the growth characteristics of the wild type strain. HPLC analysis showed that teicoplanin production is completely blocked in both Δ*tei15** and Δ*tei16** strains (Fig. 2, S2). No teicoplanin or

intermediates were detected in these mutants, whereas the wild type strain produced approximately 110 mg/L of the antibiotic. This indicated that the *tei15** and *tei16** genes are essential for the onset of teicoplanin production.

To verify that the abolition of teicoplanin biosynthesis was the result of the aforementioned gene inactivation and that there were no any polar effects, we carried out complementation analysis of the Δ*tei15** and Δ*tei16** mutants. This used two plasmids: pKC1139tcp28hyg and pSETPAtei16hyg (Table 1). The pKCtcp28hyg plasmid is based on the replicative shuttle vector pKC1139 (Muth et al. 1989) and contains the intact allele of the *tei15** gene under the control of its own promoter. The plasmid pSETPAtei16hyg is based on the integrative shuttle vector pSET152 and contains the *tei16** gene under the control of the apramycin resistance gene promoter (*aac(3)IVp*). Both plasmids were transferred into respective deletion mutants to generate two recombinant strains, *A. teichomyceticus* Δ*tei15** pKCtcp28hyg and Δ*tei16** pSETPAtei16hyg. The biosynthesis of antibiotic was restored in both the recombinant strains (Fig. 2).

Testing DNA binding activity of Tei15* and Tei16*

Close counterparts of Tei15*, functions of which were investigated recently, are Bbr (49% identity) from balhimycin biosynthesis gene cluster (Shawky et al. 2007) and Dbv4 (53% identity) from A40926 gene cluster (Alduina et al. 2007). These proteins are StrR-type regulators that contain DNA-binding domains and govern expression of the genes at the transcriptional level. Since Tei15* is highly similar to above mentioned proteins and contains helix-turn-helix DNA-binding motif (Li et al. 2004; Horbal et al. 2012), we predict that this one is also a transcriptional regulator.

To identify the promoter binding regions for Tei15* (a StrR-type regulator), we carried out EMSA analysis of the DNA binding activity of Tei15* against a set of intergenic regions (IGR) cloned from the *tei* cluster. This analysis used His-tagged Tei15* (Fig. S3) and eight DNA fragments containing putative promoters of: the regulatory genes *tei15**, *tei16** and *tei31**; the NRPS coding gene *teiA*; the deacetylase gene *tei2** (Truman et al. 2006); the chorismate mutase

gene *tei14**; the DpgA coding gene *tei17**; and the putative gene *tei27** (Li et al. 2004) (Fig. 1). Clearly visible shifted bands were obtained in the case of the promoter regions of regulatory genes *tei16** and *tei31**, the NRPS coding gene *teiA*, and the *tei17**, *tei2** and *tei27** genes (Fig. 3A). No DNA retardation was detected for the *tei15** promoter (Fig. 3A). We also observed no shifted bands with the putative promoter region of the *tei14** gene under the tested conditions (Fig. 3A). A set of control assays confirmed the specificity of Tei15* binding (Fig. S5).

The product of the *tei16** gene belongs to the LuxR family of regulators and contains DNA-binding domain at the C-terminus (Li et al. 2004; Horbal et al. 2012). To verify putative targets of this regulator, we carried out EMSA analysis using the above mentioned eight putative promoter regions and recombinant His-Tei16* protein. As shown on the Fig. 3B, no band shifts were obtained with any of the tested promoter regions.

In silico prediction of the Tei15* binding sites

Both homologs of the Tei15* protein, Bbr and Dbv4, bind to promoter regions that contain an inverted repeat: GTCCAa$(N)_{17}$TtGGAC (Shawky et al. 2007; Alduina et al. 2007). Therefore, we looked for this inverted repeat in the entire *tei* cluster and identified identical or similar palindromic sequences in twelve putative promoter regions. In particular, an identical motif was present in the promoters of the genes encoding LuxR-type regulator Tei16* and the NRPS TeiA (Fig. 4A). Motifs with only one base pair (bp) mismatch were identified in the putative promoters of the *tei2*, *tei2** and *tei5** genes (Fig. 4). The upstream region of the *tei31** gene contains a similar palindrome with a 2 bp mismatch, and the putative promoters of the *tei23** and *tei27** genes had similar motifs with 3 bp mismatches (Fig. 4). The promoters of the *tei8**, *tei17**, *tei28**, and *tei30** genes contain inverted repeats that differ by 4 bp to the consensus (Fig. 4). The right part of the inverted repeat is completely identical to the consensus sequence in promoters from all the genes, except *tei2* and *tei27**. Alternatively, the left part of the repeat is more variable (Fig. 4). This analysis demonstrates the importance of the EMSA analysis, which identified Tei15* binding sites (such as the *tei17** and

*tei27** promoter sites) which would have been missed if the gene cluster was just scanned for regions with perfect homology to the consensus sequence. This data does show some significant differences with Bbr regulation of balhimycin genes. Bbr clearly binds to the promoter region of the *bbr* gene, wherease Tei15* did not bind to the *tei15** promoter region (nor was a binding motif identified).

Increasing teicoplanin production by manipulation of the *tei15** and *tei16** genes

The data reported above, along with previous results (Horbal et al. 2012; Horbal et al. 2013), indicated that *tei15** and *tei16** are genes that are essential for teicoplanin biosynthesis. Therefore, we attempted to generate teicoplanin overproducers using *tei15** or *tei16** genes carried on replicative or integrative vectors. We first compared the teicoplanin productivity of a set of recombinant *A. teichomyceticus* strains carrying additional copies of the *tei15** or *tei16** genes cloned in pKC1139 under the control of either the native promoter or the heterologous promoter *aac(3)IVp* (Table 1), previously shown to be functional in this strain (Horbal et al. 2013). pKC1139 is a replicative vector maintained at the rate of 10 to 40 copies per chromosome in *Streptomyces* cells (Muth et al. 1989). The experiments were conducted at a flask level in the industrial medium TM1 (Taurino et al. 2011), which was previously optimized to sustain productivity in the wild type strain. None of the strains differed from the wild type in the rate of growth, pH profile and glucose consumption (data not shown). Morphology of the colonies when plated on soil extract agar medium (Kieser et al. 2000) was similar to the wild type. The results of HPLC analysis of teicoplanin production are presented in Fig. 5A. The recombinant strain with additional copies of the *tei16** gene under the control of native *tei16** promoter on pKC1139 vector (pKC1139tcp29) produced approximately the same amount of teicoplanin as the wild type and as the control strain transformed with the empty vector (pKC1139), i.e. 100 mg/L (Fig. 5A). Strain over-expressing *tei15** regulator under the control of native promoter on pKC1139 vector (pKC1139tcp28) showed more than a three–fold increased production level (375 mg/L) (Fig. 5A). The introduction of the

heterologous promoter *aac(3)IVp* upstream to both the *tei* regulatory genes in the replicative vector led to a substantial increase in antibiotic production, indicating that this promoter is much more effective then the native ones. Namely, *A. teichomyceticus* pKC1139Atcp28 produced nearly 4000 mg/L of teicoplanin, whereas *A. teichomyceticus* pKC1139Atcp29 produced 2900 mg/L (Fig. 5A, S6A). As with the wild type strain (Taurino et al. 2011), teicoplanin is produced as a complex of five compounds designated T-$A_{2\text{-}1}$-$A_{2\text{-}5}$ differing in the length and branching of the fatty acid moiety linked to the glucosamine residue on the heptapeptide scaffold (Fig. S7).

Recombinant strains based on replicative vector are often considered unsuitable for industrial scaling up as manufacturing guidelines prefer avoiding antibiotic addition to maintain selective pressure during production due to antibiotic cost and risk of chemical cross-contamination. Therefore, we constructed a second set of *A. teichomyceticus* recombinants by cloning *tei15** and *tei16** into the integrative vector pSET152 (based on the actinophage φC31 integration system), that has only one site of integration in the teicoplanin producer chromosome (Ha et al. 2008). Two recombinant strains (pSET152Atei15 and pSET152Atei16) were generated carrying one additional copy of *tei15** or *tei16** under the control of *aac(3)IVp* (Table 1). They were fermented at flask level in TM1 in parallel with the control strains carrying the empty vector pSET152 or its derivative pSET152A: none of them differ from the wild type in morphology, growth rate and time courses of pH and glucose consumption (data not shown). The results of HPLC analysis of teicoplanin production are presented in Figure 5B. *A. teichomyceticus* pSET152Atei15 and pSET152Atei16 produced more than 800 and 1000 mg/L teicoplanin, respectively, which represent an eight- to ten-fold improvement in comparison to the wild type and controls strains (Fig. S6B). As before, the teicoplanin complex composition in T-$A_{2\text{-}1}$-$A_{2\text{-}5}$ did not significantly change in comparison to the wild type (data not shown).

Scaling up teicoplanin production in a bioreactor

The productivity of the highest-producing strain *A. teichomyceticus* pKC1139Atcp28 was tested in parallel with the wild type strain in a 3 L fermenter, taking the advantage of the robust optimized process previously developed in our laboratories for wild type *A. teichomyceticus* (Taurino et al. 2011). This protocol was already optimized for the wild type, and involved maintaining dissolved oxygen (DO_2) at over 20% of saturation, and pH was naturally self-regulated during the fermentation.

The fermenter trials of the recombinant strain were conducted in the absence of apramycin, which was used indeed to maintain selection for the replicative plasmid pKC1139Atcp28 in vegetative and fermentation media at shake flask scale. This was done to adhere to common industrial guidelines that prefer to avoid antibiotic addition during production to reduce fermentation and purification costs. Fig. 6 shows the time courses of *A. teichomyceticus* pKC1139Atcp28 and of the wild type fermentations in TM1 at a 3-L fermenter scale. Kinetics of growth (measured by packed mycelium volume, PMV), of oxygen consumption and medium pH changes were highly similar between the recombinant and control strain (Fig. 6A, C). The oxygen consumption profile shows that the wild type grew slightly faster in the first 48 hours of fermentation, whereas the comparison of the growth curves indicate a 24-hours prolonged stationary phase (from 120 to 144 hours) in the recombinant strain. Glucose consumption was much faster in the wild type, where the sugar was completely depleted in 48 hours, whereas it lasted for 120 hours in the recombinant strain. The kinetics of antibiotic production was highly similar between the two strains and both reached a peak of teicoplanin production after 96 hours from inoculation. Indeed the maximum productivity achieved by the recombinant strain was 815 mg/L ($\sigma = \pm$ 20 mg/L), which is 3.5-fold higher than that observed for control strain in the same conditions (Fig. 6B, D). HPLC profile of the samples harvested after 96 hours of fermentation from the recombinant strain and the wild type showed that the mixture of teicoplanins produced was identical to the composition observed at flask scale (data not shown).

Discussion

Understanding the function of regulatory genes and those factors that influence their expression is fundamental for rationally engineering microbial producers of clinically valuable and industrially important antibiotics. This is the case for the "last resort" glycopeptide drug teicoplanin. The cost of the production process and the quality of the drug mostly depend on the generation and maintenance of teicoplanin-overproducing strains.

In this work, for the first time we performed gene deletion in *A. teichomyceticus* by in-frame substitution of the chromosomal copy of gene with the antibiotic resistance gene. This approach is more efficient than that earlier used by Truman *et al.* (Truman et al. 2008) that inactivated *tei2** by in-frame removal of part of the gene, since double crossover mutants possess resistance to antibiotic and consequently their selection is straightforward. Consequently, 97% of transconjugants that were selected were resistant mutants.

Using this tool we elucidated the function of two regulatory genes, *tei15** and *tei16**, in the *tei* cluster. These genes code for StrR- and LuxR-type regulators, respectively. Both Tei15* and Tei16* appear to be the key toggles of teicoplanin production in *A. teichomyceticus* since their disruption completely abolished antibiotic biosynthesis, while their over-expression led to dramatic overproduction. The binding of Tei15* to conserved motifs in DNA promoter regions confirmed the involvement of Tei15* in the regulation of teicoplanin production at transcriptional level.

The Tei15* protein binds to six out of the eight tested putative promoter regions, namely with the promoters of the regulatory genes *tei16** and *tei31**, the NRPS coding gene *teiA*, the DpgA coding gene *tei17**, the deacetylase coding gene *tei2** and the putative gene *tei27**. Based on the results of *in silico* analysis, we predict that these promoters orchestrate expression of three monocistronic and three polycistronic transcriptional units. Our analysis shows that the expression of the *teiA*, *teiB*, *teiC*, *teiD* and *tei1** genes is controlled from *teiAp* (Fig. 7). These genes are responsible for the assembly of the peptide core of teicoplanin. The transcription of another polycistronic unit (*tei17*-tei22**, Fig. 7) is governed from the *tei17*p* promoter (Fig. 7). These genes control the synthesis of the nonproteinogenic amino acid dihydroxyphenylglycine, a putative integral membrane transposter and GTP cyclohydrolase. Another Tei15*-controlled operon is the

one governed from the *tei2** promoter and is likely to consist of *tei2** and *tei3**, encoding the N-acetylglucosaminyl deacetylase and a mannosyltransferase, respectively. According to the EMSA results (Fig. 3), the aforementioned genes are under the control of the Tei15* protein. EMSA analysis also showed that expression of the two other regulatory genes, *tei16** and *tei31**, and *tei27**, a gene encoding an unknown protein, are also under the control of Tei15* (Fig. 7). However, the expression of *tei14** as well as of its own *tei15** gene does not seem to be controlled by Tei15*. Tei15* therefore does not seem to be an autoregulatory protein, which differs from its close homologue *bbr*, which was previously identified as a StrR-type regulator in the balhimycin biosynthetic gene cluster (Shawky et al. 2007). Taking these data into account, the expression of at least 17 genes in the *tei* cluster is directly governed by Tei15* (Fig. 7). To our knowledge, this is the first case that a StrR-type regulator orchestrates the expression of other regulators in either *Actinoplanes* or *Streptomyces* spp.

Operator sequences either identical or similar to those recognized by Bbr (Shawky et al. 2007) and Dbv4 (Alduina et al. 2007) (consensus sequence GTCCAa$(N)_{17}$TtGGAC) were identified within the putative promoters of twelve *tei* genes. Tei15*-specific binding to six of them was confirmed by EMSA analysis. However, the promoters of the *tei14** and *tei15** genes did not interact with Tei15*, and they do not possess operator sequences that are similar to the one described above. The Bbr-like operators in the *tei* cluster are 374 bp, 260 bp and 120 bp away from start codons of *teiA*, *tei16** and *tei17** genes, and we demonstrated using EMSA analysis that no shifted bands were observed when Tei15* binding was tested with small segments encompassing 60-70 bp upstream of start codons from the aforementioned genes (data not shown). Taken together, our findings strongly suggest that the identified repeats are Tei15* binding sites. Mismatches by 2 and 4 bases, respectively, in the inverted repeats of the promoters for the *tei27** and *tei17** genes do not appear to abolish Tei15* binding to them. Therefore, we predict that six other promoters that contain such operators, might also be under the control of the Tei15* protein (Fig. 7). We also predict that Tei15* regulates transcription of the *tei2* and *tei3* genes, as these genes form one transcriptional unit and the Bbr-like palindromic repeat (with one base mismatch) is present in the

tei2 promoter (Fig. 4B). These genes encode a response regulator and a kinase homologous to the VanR/S pair from *S. coelicolor* (Hutchings et al. 2006) and are most likely involved in the control of the expression of genes for teicoplanin resistance. Previous work indicates that the Tei3 kinase is not functional (Beltrametti et al. 2007), whereas the activity of the response regulator Tei2 has not yet been studied. To conclude, we hypothesise that by modulating the expression of the *tei2* gene, the StrR-type response regulator Tei15* also indirectly governs the expression of teicoplanin resistance genes (Fig. 7). Experimental verification of this assumption is underway in our laboratories.

The targets of Tei16* LuxR-type regulator in the *tei* cluster remain obscure, since it did not interact with any of the tested promoter regions in EMSA analysis. A possible explanation for this is that the protein regulates expression of some other genes in the cluster by binding promoter regions that we have not yet tested. Alternatively, the heterologously expressed protein could have poor EMSA activity, or the recombinant protein needs additional ligands for the activity. Further investigations are needed to better understand the role of Tei16* in controlling teicoplanin production.

We have also showed that optimization of the expression of the *tei15** and *tei16** regulatory genes via promoter substitution and vector selection led to high yields of teicoplanin. Valuable *A. teichomyceticus* strains producing grams per litre of teicoplanin are reported in the literature (Jung et al. 2009; Lee et al. 2003; Jung et al. 2008), but they were generated by time-consuming and labour-intensive CSI, based on recursive mutagenesis and phenotypic selection, that resulted in randomly modified genome backgrounds.

Herein, recombinant strains able to produce teicoplanin in the range of 1-4 grams per litre were generated by introducing the positive *tei15** and *tei16** regulatory genes into the wild type, using the integrative vector pSET152 or the autonomous high copy number plasmid pKC1139, and the heterologous promoter *aac(3)IVp*. The wild type produced around 100 mg/L in the same conditions, so this represents a remarkable increase of three orders of magnitude that favourably compares with the productivity of industrial mutants used to produce teicoplanin.

The improved productivity of the strains carrying the autonomous replicative vector in comparison with those containing the integrative vectors can be explained by the difference in the gene copy number per chromosome, which range from 10 to 40 for pKC1139 (Muth et al. 1989), to one for pSET152 (Ha et al. 2008). However, scaling up the best performing strain *A. teichomyceticus* pKC1139Atcp28, we directly experienced the instability that is typical of autonomous replicative vectors. In the absence of apramycin selection in the production phase, the replicative vector-based strain gave a reproducible teicoplanin productivity of 815 mg/L which is three times less than the one achieved at flask-level. Further trials to better adapt the fermentation process to the over-producing recombinant strains need to be conducted. These challenges notwithstanding, we believe that the use of stable integrative plasmids such as pSET152 derivatives, coupled with the search for more efficient and stronger promoters, can help to mimic the effects of the high copy number of autonomous replicative vectors. The use of such recombinant strains in industrial scaling up is favoured, since it is not limited by the need to perform antibiotic selection for plasmid maintenance. The data presented in this work indicate that the replacement of the native promoters of aforementioned regulatory genes with the heterologous *aac(3)IVp* markedly improve the level of productivity. These results support a more thorough investigation of a wider set of promoters for use in the teicoplanin producer, which may eventually lead to even better tools for strain improvement.

In conclusion, the recombinant strains hereby generated by rational genetic engineering have the potential to replace the heavily mutated industrial strains currently in use in industrial scaling up. Alternatively, we expect that the novel rational strategy based on recombinant DNA technology described in this study might be successfully transferred to achieve further improvements in industrial *A. teichomyceticus* strains.

Competing interests

The authors declare that they have no competing interests.

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Figures

Fig. 1 Genetic organization of the teicoplanin gene cluster.

Fig. 2 Teicoplanin production of *A. teichomyceticus* Δ*tei15** and Δ*tei16** mutants and their complemented strains in comparison to the wild type. T-A_2 production was analyzed by HPLC at 120 hours after inoculation in TM1 medium. Each value represents the average of three different experiments.

Fig. 3 Gel mobility shift assays with **A** His-Tei15* or **B** His-Tei16* and DNA fragments from *tei* cluster. In each assay 50 μg of DNA was used. *tei15*p, tei16*p, tei31*p* – the promoters of the regulatory genes *tei15**, *tei16** and *tei31**, respectively; *teiAp* – the promoter of the NRPS coding gene *teiA*; *tei2*p* – the promoter of the deacetylase coding gene *tei2**; *tei14*p* – the promoter of the chorismate mutase gene *tei14**; *tei17*p* – the promoter of the DpgA coding gene *tei17**; and *tei27*p* – the promoter of the *tei27** gene.

Fig. 4 Sequence alignment of the putative Tei15* binding sites with the consensus sequence of the Bbr –binding site. **A** DNA sequences of putative Tei15* binding sites in DNA regions identified by EMSA. **B** the Bbr-like palindromic structures within the promoters that were not tested by EMSA in this work. **C** Web logo analysis of the putative Tei15* binding sites. Weblogo was generated using WebLogo 3 (http://weblogo.threeplusone.com/). Arrows indicate inverted repeats and the double arrow denotes the spacer. The most highly conserved nucleotides are bold and marked with dark red color, nucleotides that differ from the consensus sequence are indicated in blue.

Fig. 5 Production of teicoplanin in TM1 medium by recombinant strains of *A. teichomyceticus*. T-A_2 production was analyzed by HPLC at 96, 120 and 144 hours after inoculation. **A** the wild type and recombinants carrying replicative vectors pKC1139, pKC1139tcp28, pKC1139tcp29, pKC1139Atcp28, and pKC1139Atcp29; **B** the wild type and recombinant strains carrying

Table 1 Bacterial strains and plasmids used in this work

integrative vectors pSET152, pSET152A, pSET152Atei15, pSET152Atei16. The *tei15** gene corresponds to *tcp28* and *tei16** - to *tcp29*.

Fig. 6 Growth and teicoplanin production of *A. teichomyceticus* pKC1139Atcp28 and of the wild type in a 3-L batch bioreactor in TM1. The pH value was naturally self-regulated (*i.e.*, it was not controlled by adding acid/base during the fermentation), whereas DO_2 was kept over 20% of saturation by adjusting agitation speed. In (**A** and **C**), time courses of pH (●), DO_2 (□), glucose (▲), and growth curve measured as PMV (■) of *A. teichomyceticus* pKC1139Atcp28 and of the wild type. In (**B** and **D**), production of T-A_2 by *A. teichomyceticus* pKC1139Atcp28 and by the wild type measured by HPLC analysis as mg/L.

Fig. 7 A model of teicoplanin production regulation by Tei15* and Tei16* transcriptional factors (for details see the text).

Bacterial strains and plasmids	Description	Source or reference
A. teichomyceticus	Producer of teicoplanin	NRRL-B16726
A. teichomyceticus Δ*tei15**	Derivative of *A. teichomyceticus* with inactivated *tei15** gene	This work
A. teichomyceticus Δ*tei16**	Derivative of *A. teichomyceticus* with inactivated *tei16** gene	This work
E. coli DH5α	Host for routine subcloning experiments	MBI Fermentas
E. coli ET12567 (pUZ8002)	(*dam*-13::Tn*9 dcm-6*), pUZ8002^{+} (*ΔoriT*). Used for conjugative transfer of DNA	Dr. A. Luzhetskyy, HIPS Institute, Germany
E. coli Rosetta pLysS	Host for the heterologous expression of His6-tagged Tei15*	Novagen
E. coli BL21 (DE3) pLysS	Host for the heterologous expression of His6-tagged Tei16*	Novagen
4B2	Supercos1 containing part of the teicoplanin gene cluster	Dr. A. Truman, John Innes Centre, England
4B2hyg	Supercos1 containing part of the teicoplanin gene cluster with hygromycin resistance gene instead of neomycin	This work
4B2del15	Derivative of 4B2hyg with the inactivated *tei15** gene	This work
4B2del16	Derivative of 4B2hyg with the inactivated *tei16** gene	This work
pHYG1	pLitmus38 containing hygromycin resistance cassette *hyg*	C. Olano Univ. de Oviedo, Spain
pIJ774	pUC19 containing *aac(3)IV-oriT* cassette	Dr. A. Luzhetskyy, HIPS Institute, Germany
pIJ773	pUC19 containing *aac(3)IV-oriT* cassette; source of the *aac(3)IV* promoter (*aac(3)IVp*)	Dr. A. Luzhetskyy, HIPS Institute, Germany
pET28a	Vector for His-tagged protein expression	Novagen
pET28tei15	Derivative of pET28a harboring codon-optimized copy of the *tei15** gene	This work
pET28tei16	Derivative of pET28a harboring codon-optimized copy of the *tei16**	This work

	gene	
pKC1139	*E. coli*/*Streptomyces* shuttle vector with temperature sensitive replicon pSG5, Am^r	Muth et al. 1989
pSET152	φC31-based *Streptomyces* integrative vector, Am^r	Kieser et al. 2000
pSET152A	pSET152 derivative containing *aac(3)IVp* from pIJ773	This work
pSET152Atei15	pSET152A derivative containing *tei15** regulatory gene under the control of *aac(3)IVp*	This work
pSET152Atei16	pSET152A derivative containing *tei16** regulatory gene under the control of *aac(3)IVp*	This work
pSETAtei16hyg	pSET152Atei16 derivative containing hygromycin resistance gene instead of apramycin	This work
pKC1139A	pKC1139 carrying *aac(3)IVp*	Horbal et al. 2012
pKC1139tcp28	pKC1139 derivative containing *tei28* (*tei15** according to Li et al., 2004) along with its upstream region with putative promoter	Horbal et al. 2012
pKCtcp28hyg	pKC1139tcp28 derivative containing hygromycin resistance gene instead of apramycin	This work
pKC1139Atcp28	pKC1139A derivative containing *tcp28* (corresponds to *tei15**) under the control of *aac(3)IVp*	Horbal et al. 2012
pKC1139tcp29	pKC1139 derivative containing *tcp29* (*tei16** according to Li et al., 2004) along with its upstream region with putative promoter	Horbal et al. 2012
pKC1139Atcp29	pKC1139A derivative containing *tcp29* (corresponds to *tei16**) under the control of *aac(3)IVp*	This work

Table 2 Primers used in this work

Primer	Nucleotide sequnce (5' – 3')	Utility	Gene name
HygRVSKm Forw HygRVSKm Rev	ATGGCGCAGGGGATCAAGATCTGATCAAGAGAC AGGATGCCCGTAGAGATTGGCGATCCC TCGCTTGGTCGGTCATTTCGAACCCCAGAGTCC CGCTCACAGGCGCCGGGGGCGGTGTC	replacement of the neomycin resistance gene	*hyg*
tei15*delForw tei15*delRev	GCCTCCAGCGCGCGCGTCACCAGCTTAGGAGCT GCATTGATTCCGGGGATCCGTCGACC GGGCCGGCGCCCGTACTGTCCGGGCGCGGCGCG CGGTCATGTAGGCTGGAGCTGCTTC	inactivation of the *tei15** gene	*tei15**
tei16*delForw tei16*delRev	TCGTGTGGTAGCGGGATTGCTCGGCCGGGGG GGCCCGTGATTCCGGGGATCCGTCGACC GGCTGCGGAATCGGCGACACGCCCGCCG CGGGATGATCATGTAGGCTGGAGCTGCTTC	inactivation of the *tei16** gene	*tei16**
tei15*Forw tei15*Rev	TCACCAGCTTAGGAGCTGCATTG TGGCAGAAGCGAGACGGTGGACGCC	gene inactivation confirmation	*tei15**
tei16*Forw tei16*Rev	GGTAGGGTTCGATCTCGTGTGG GATCGGCTGCGGAATCGGCGAC	gene inactivation confirmation	*tei16**
P1Am-Hyg-up P2Am-Hyg-rp	GTGCAATACGAATGGCGAAAAGCCGAGCTCA TCGGTCAGCCCGTAGAGATTGGCGATCCC TCATGAGCTCAGCCAATCGACTGGCGAGCGGC ATCGCATCAGGCGCCGGGGGCGGTGTC	replacement of the apramycin resistance gene	*hyg*
aacPF aacPR	TTGATATCGACATTGCACTCCAC TTGGATCCGTTGGATACACCAAG	cloning of the apramycin gene resistance promoter	*aac(3)IVp*
tei15*F tei15*R	TAGGAGGCTTAGGAGCTGCATTG GATATCTGGCAGAAGCGAGACGG	*tei15** over-expression	*tei15**
tei16*EF-1 tei16*ER	AGGAGGTAGGGTTCGATCTCGTGT GGATGATCAGTGGCTGTATCGCC	*tei16** over-expression	*tei16**
tei2*Forw tei2*Rev	TCGCAGATCAGCGACTGAGCGTC GAGGCATGGGGATCAGTTTGGTG	EMSA	*tei2*p*
tei14*Forw tei14*Rev	TGTCGCCGGGATCCGGACCGAC CATCACAACGACCATGACAGTC	EMSA	*tei14*p*
tei31*Forw tei31*Rev	TGCCGCTGTTGTTGACGGCGGAC GGTGGGTCCGCACATGCTCAC	EMSA	*tei31*p*
tei27*Forw	TTCCGGGGCCATCGTGATCTCC	EMSA	*tei27*p*

tei27*Rev	AATACGTGTCCGGACATGCGTG		
tei15*PForw tei15*PRev	GGCAGGCCGGACCGACGGTCT AGTGAACTGATCTCCATCTCC	EMSA	*tei15*p*
tei16*PForw tei16*PRev	GGAACCGGCGGTTGGACACGG CTGATGTTTACGCAGTGTCAC	EMSA	*tei16*p*
tei17*Forw tei17*Rev	TTTTTTCTAGATACAGCCACTGATCATCCC TTTGGTACCGTCATCGGTACATCCACCC	EMSA	*tei17*p*
teiAPForw teiARRev	TTTTTTCTAGAGATCGTCGGTTCCCGCGTG TTTGGTACCTGCGCTGCGGAATTCATCGTC	EMSA	*teiAp*
tei16*miniForw tei16*miniRev	GGTAGGGTTCGATCTCGTGTGGTAGCGGGA TTGCTCGGCCGGGGGGGCCCGTGACACTGC GCAGTGTCACGGGCCCCCCCGGCCGAGCAA TCCCGCTACCACACGAGATCGAACCCTACC	EMSA	*tei16*minip*
teiAminiForw teiAminiRev	CGCTCCGGGTCCGGACCGCCGGCCGGTGCG CCGGGTGGCTCTTGAAGGGGGACGATGAAT ATTCATCGTCCCCCTTCAAGAGCCACCCGGC GCACCGGCCGGCGGTCCGGACCCGGAGCG	EMSA	*teiAminip*
tei17*miniForw tei17*miniRev	GAGGTCAGCGATGTCCGCGGAAAAGGTCAA GGAACTGGTACGCAGTAAGCGCCGGATGTG GGGGTGGATG CATCCACCCCCACATCCGGCGCTTACTGCGT ACCAGTTCCTTGACCTTTTCCGCGGACATCG CTGACCTC	EMSA	*tei17*minip*
teiAForw teiARev	TGGCGTACGTGATGTACACC GCGGTCAGATGCGCCCTGGT	EMSA	*teiA*

Fig. 1

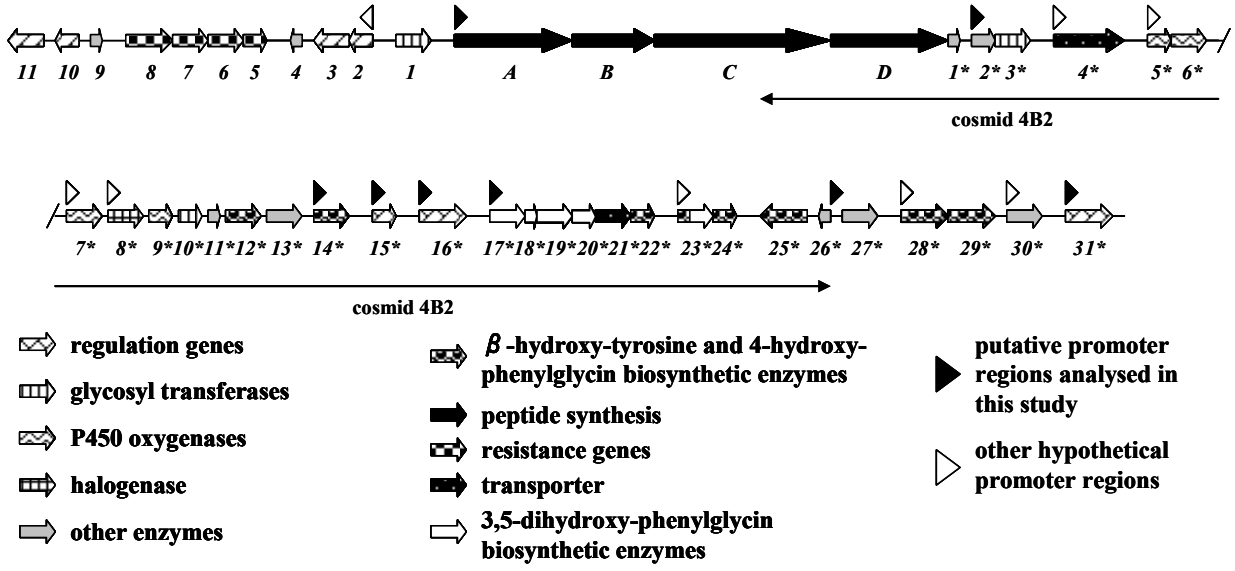

11 10 9 8 7 6 5 4 3 2 1 A B C D 1* 2* 3* 4* 5* 6*
cosmid 4B2
7* 8* 9*10*11*12* 13* 14* 15* 16* 17*18*19*20*21*22* 23*24* 25* 26* 27* 28* 29* 30* 31*
cosmid 4B2
regulation genes
glycosyl transferases
P450 oxygenases
halogenase
other enzymes
β-hydroxy-tyrosine and 4-hydroxy-phenylglycin biosynthetic enzymes
peptide synthesis
resistance genes
transporter
3,5-dihydroxy-phenylglycin biosynthetic enzymes
putative promoter regions analysed in this study
other hypothetical promoter regions

Fig. 2

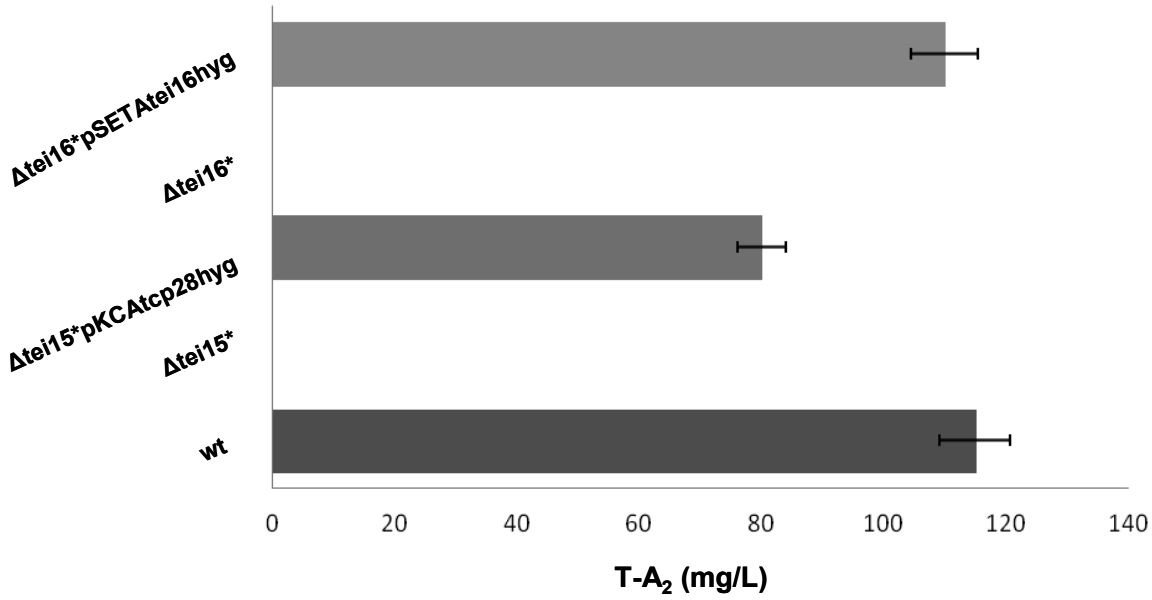
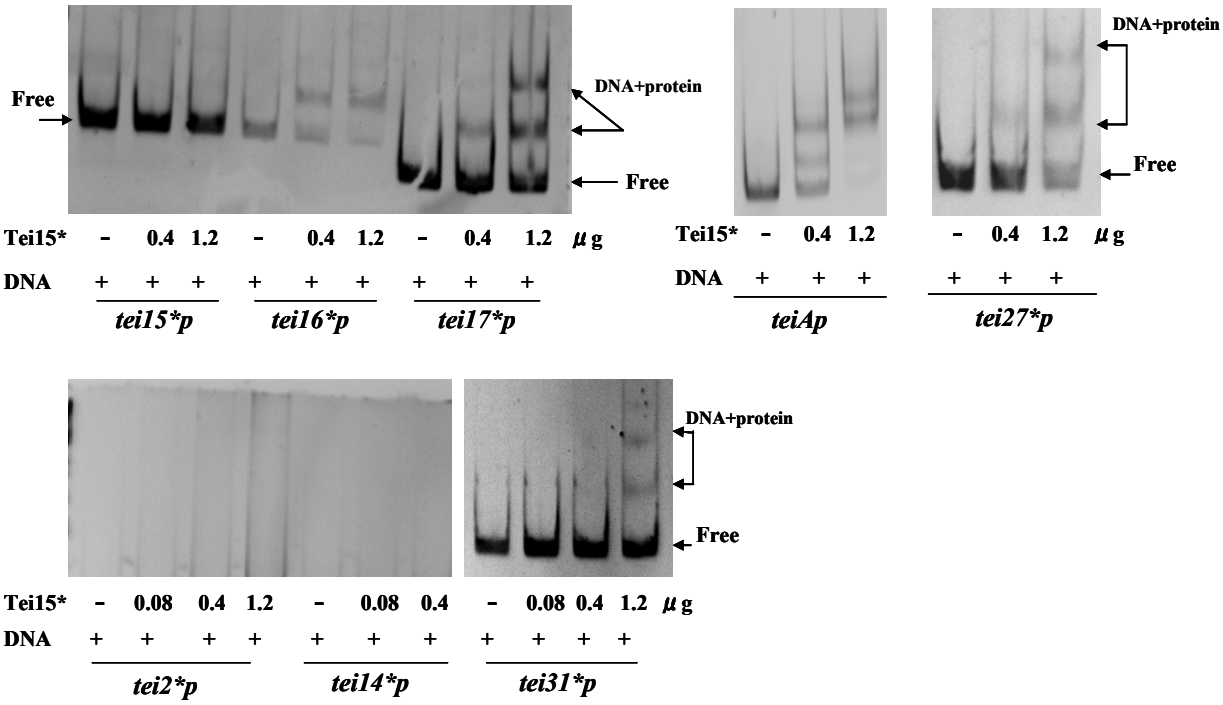

Δtei16*pSETAtei16hyg
Δtei16*
Δtei15*pKCAtcp28hyg
Δtei15*
wt
0
20
40
60
80
100
120
140
T-A2 (mg/L)

Fig. 3

A


Free
DNA+protein
Free
Tei15* - 0.4 1.2 - 0.4 1.2 - 0.4 1.2 μg
DNA + + + + + + + + +
tei15*p
tei16*p
tei17*p
Tei15* - 0.4 1.2 - 0.4 1.2 μg
DNA + + + + + +
teiAp
tei27*p
DNA+protein
Free
Tei15* - 0.08 0.4 1.2 - 0.08 0.4 - 0.08 0.4 1.2 μg
DNA + + + + + + + + + +
tei2*p
tei14*p
tei31*p
DNA+protein
Free

B

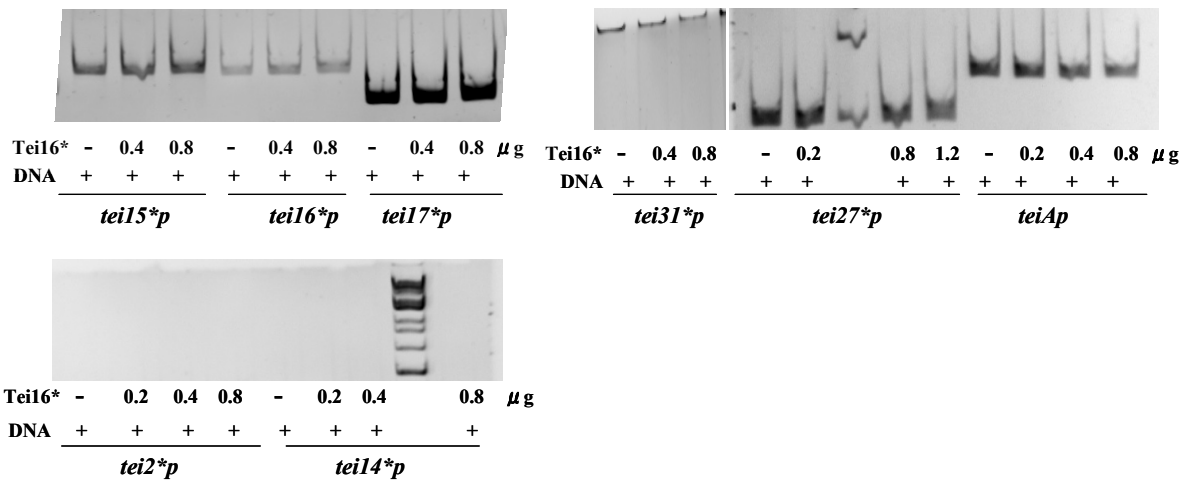

Tei16* - 0.4 0.8 - 0.4 0.8 - 0.4 0.8 μg
DNA + + + + + + + + +
tei15*p
tei16*p
tei17*p
Tei16* - 0.4 0.8 - 0.2 0.8 1.2 - 0.2 0.4 0.8 μg
DNA + + + + + + + + + + + +
tei31*p
tei27*p
teiAp
Tei16* - 0.2 0.4 0.8 - 0.2 0.4 0.8 μg
DNA + + + + + + + +
tei2*p
tei14*p

Fig. 4

A

teiA	GTCCAAGTGTCGACAACGTTCGCTTGGAC
*tei2**	GTCCAGCTGCTCAATGCGGCCGGTTGGAC
*tei16**	GTCCAACCGGCCGGAACCGGCGGTTGGAC
*tei17**	GCAGCCGATCCCGGGACGGGCGGTTGGAC
*tei27**	GTCCAACTGTCATGACCGGCGCCGTCGGC
*tei31**	CGACAACCTTGAGGTTCCTCATCTTGGAC
	6 — 17 — 6
Bbr consensus sequence	GTCCAaNNNNNNNNNNNNNNNNTtGGAC

B

tei2	GTCCAACCGCCCGAT-CGAGCGGCTGGAC
*tei5**	GTCCACCCGCCCGATCCGGACGGTTGGAC
*tei8**	CTGGTAGTGCTCTGCCCTCTCCATTGGAC
*tei23**	GGCCGGGCGCAGTCGACAGTCAGTTGGAC
*tei28**	GCGCCGTCGTGGCCGGTTCTCGGTTGGAC
*tei30**	CGACGAGACGCCGACCGGCCGCGTTGGAC

C

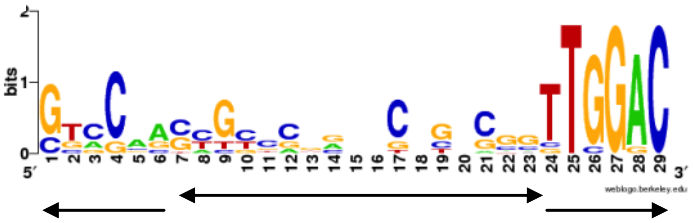


Fig. 5

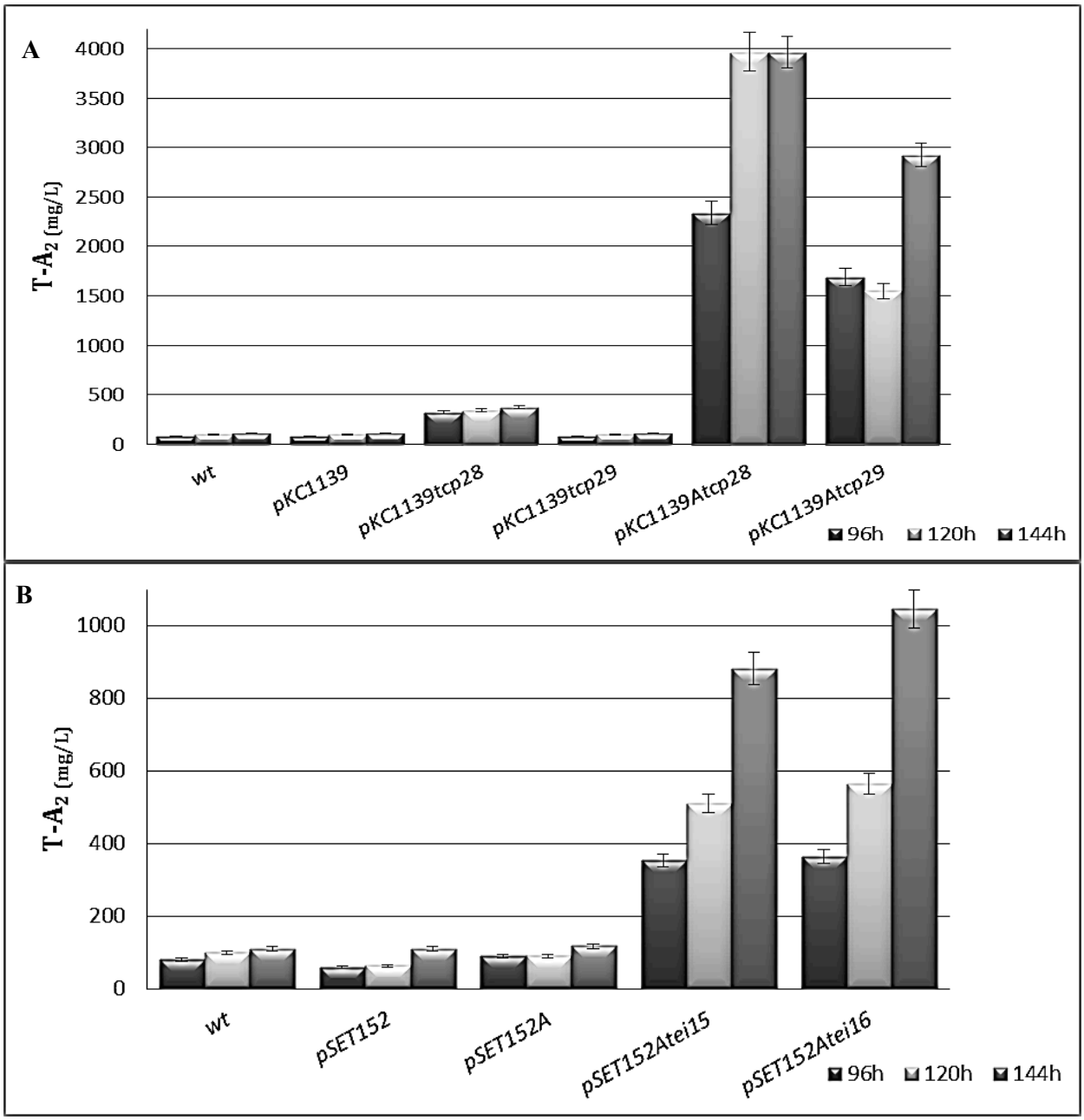

A
T-A2 (mg/L)
4000
3500
3000
2500
2000
1500
1000
500
0
wt
pKC1139
pKC1139tcp28
pKC1139tcp29
pKC1139Atcp28
pKC1139Atcp29
96h
120h
144h
B
T-A2 (mg/L)
1000
800
600
400
200
0
wt
pSET152
pSET152A
pSET152Atei15
pSET152Atei16
96h
120h
144h

Fig. 6

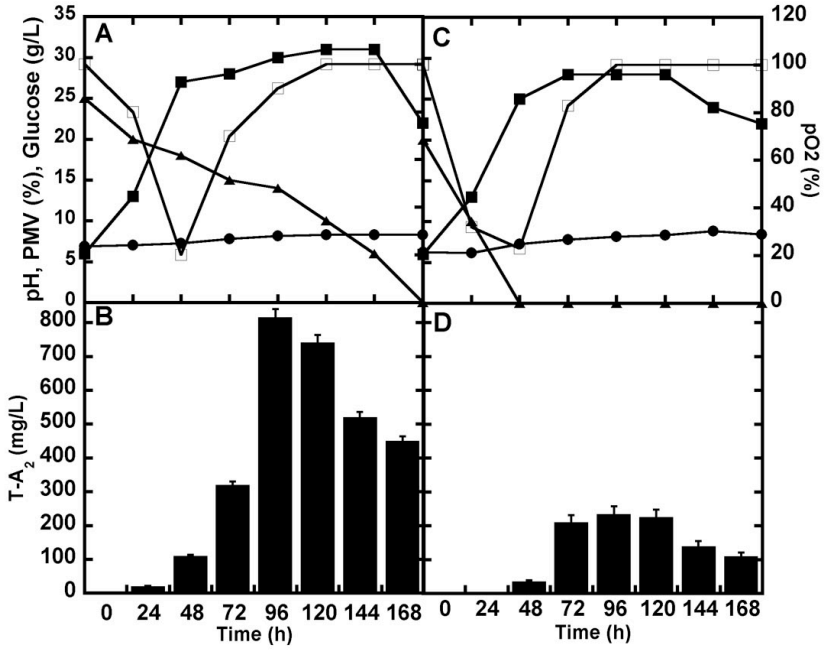
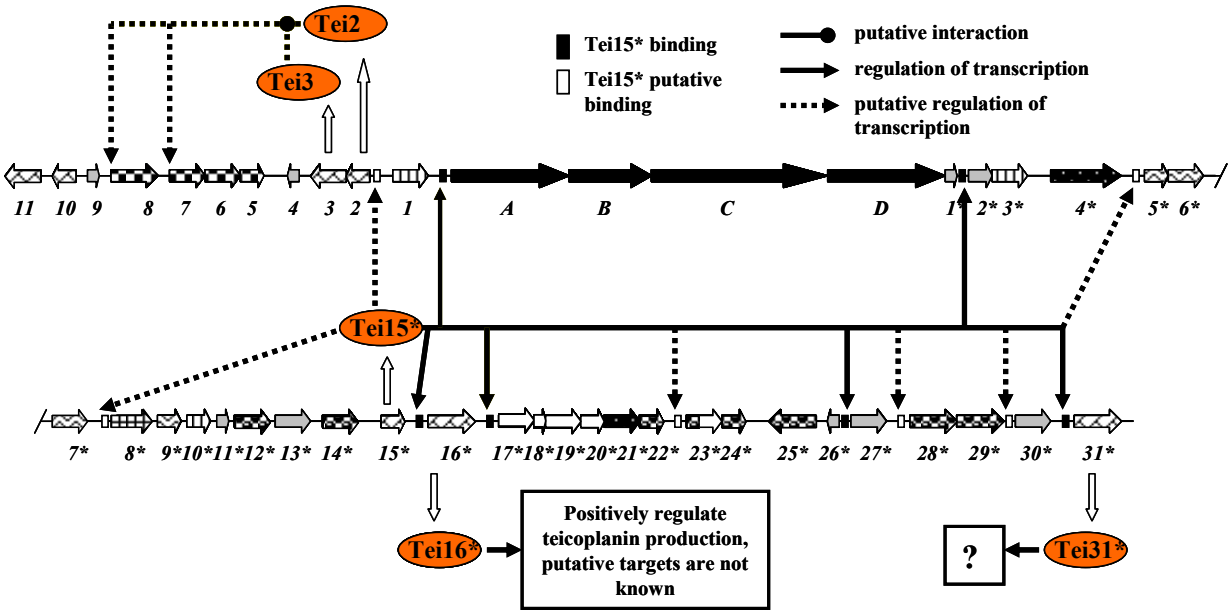

A
B
C
D
pH, PMV (%), Glucose (g/L)
pO2 (%)
T-A2 (mg/L)
Time (h)
Time (h)
0 24 48 72 96 120 144 168
0 24 48 72 96 120 144 168

Fig. 7


Tei2
Tei3
Tei15* binding
Tei15* putative binding
putative interaction
regulation of transcription
putative regulation of transcription
11 10 9 8 7 6 5 4 3 2 1 A B C D 1* 2* 3* 4* 5* 6*
Tei15*
7* 8* 9*10*11*12* 13* 14* 15* 16* 17*18*19*20*21*22* 23*24* 25* 26* 27* 28* 29* 30* 31*
Tei16*
Positively regulate teicoplanin production, putative targets are not known
?
Tei31*