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A Bacterial Transcription Inhibitor of RNA Polymerase Holoenzyme Formation by Structure Based Drug Design: From *In Silico* Screening to Validation

Running title: Transcription inhibitor

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ABSTRACT: Bacterial transcription is a proven target for antibacterial research. However, most of the known inhibitors targeting transcription are from natural extracts or are hits from screens where the binding site remains unidentified. Using an RNA polymerase holoenzyme homology structure from the model Gram positive organism *Bacillus subtilis*, we created a pharmacophore model and used it for *in silico* screening of a publically available library for compounds able to inhibit holoenzyme formation. The hits demonstrated specific affinity to bacterial RNA polymerase, excellent activity using *in vitro* assays, and showed no binding to the equivalent structure from human RNA polymerase II. The target specificity in live cells and antibacterial activity was demonstrated in microscopy and growth inhibition experiments. This is the first example of targeted inhibitor development for a bacterial RNA polymerase, outlining a complete discovery process from virtual screening to biochemical validation. This approach could serve as an appropriate platform for the future identification of inhibitors of bacterial transcription.

Keywords: Transcription, bacteria, protein-protein interaction, antibiotic discovery, *in silico* screening, target validation.

INTRODUCTION

Bacterial infection is a global health problem responsible for numerous deadly and debilitating diseases such as pneumonia, tuberculosis, and many gastro-intestinal diseases including peptic ulcers, that cost health systems \$Billions annually world-wide (1). They are projected to cause >10 million deaths per year (more than the current annual number of deaths due to cancer) and cost the global economy US\$100 trillion per year by 2050 if no new efficient drugs are developed (2). Transcription, together with DNA replication and protein translation, is an essential step in the bacterial cell cycle, which requires RNA polymerase (RNAP) as the core enzyme along with a suite of transcription factors to convert DNA sequences into RNA containing the same genetic information (3). The discovery and development of rifampicin in the 1950s established bacterial transcription as a valid target for antibiotic development (4). Despite the subsequent identification of many further inhibitors of transcription, only fidaxomicin, in 2011, has successfully gained approval for clinical use as a narrow spectrum drug for treatment of *Clostridium difficile* associated diarrhea (5). So far most of the transcription inhibitors reported have been natural products with complex structures requiring arduous fermentation and extraction procedures (6). Furthermore, as they tend to bind at or near the active site of RNAP in a large cleft comprising a substantial surface area made up from the two large subunits (β and β'), point mutations at multiple sites have led to rapid acquisition of resistance which has prevented them being developed as clinically useful drugs (7). Resistance to rifampicin occurs so rapidly it is only used in combination therapy and resistance to fidaxomicin was first reported ~40 years ago (8).

The initiation of transcription requires RNAP holoenzyme, which is formed by association of the RNAP core enzyme ($\alpha_2\beta\beta'\omega$, and ϵ in the Gram positive firmicutes) with an essential σ factor (Figure 1A and B; σ^A in *B. subtilis*) required for specific recognition of promoter DNA sequences (9, 10). Therefore, compounds that prevent holoenzyme formation should be able to inhibit bacterial transcription and could potentially be developed as antibacterials. The SB series of compounds were shown to inhibit RNAP holoenzyme formation (11), and a recent report of anthralinic acid derivatives identified by virtual screening of a pharmacophore model based on alignment to the SB series and other compounds with unknown or diverse binding sites on RNAP, displayed activity against holoenzyme formation and transcription by *in vitro*

binding and functional assays (12). However, the exact binding sites of these compounds are still unknown.

Although σ^A makes extensive contacts with RNAP core, it is the interaction between α -helices in the β' subunit clamp-helix (β' -CH) region and σ region 2.2 ($\sigma_{2.2}$) that is absolutely necessary for the formation of holoenzyme (Figure 1A and B; 13, 14). An incomplete pharmacophore designed to target this interaction has been used to identify compounds that inhibit bacterial transcription *in vitro* and *in vivo* with some success (14, 15). Past studies have also identified inhibitors from natural product extracts using a high throughput screen, but these have not been developed further (16, 17). In order to generate *bone fide* lead molecules it has been necessary to more comprehensively characterize the $\sigma_{2.2}$ - β' -CH interaction to enable construction of a fully effective pharmacophore. The studies outlined in this work established a more comprehensive pharmacophore model based on *in silico* modeling and biochemical verification, and allowed rational high-throughput identification of highly specific new lead compounds from a public drug-like compound library (18) for potential development as a novel class of antibiotic.

RESULTS AND DISCUSSION

In previous reports a pharmacophore model was built incorporating *B. subtilis* σ^A mutagenesis data obtained from far-Western blots (14, 15). However, ELISA based affinity assay of mutant β' -CH with σ^A identified additional interactions that could be used for optimal pharmacophore model construction (14). Therefore, an improved pharmacophore model was created (Figure 1C and D) incorporating all available data and with the following properties: 1. Hit compounds had to map to the region representing β' -CH residue R267 (Figure 1C) as this is the most important residue required for interaction with σ^A (14). 2. Considering that the binding site of β' -CH to σ^A is nearly a flat surface rather than a pocket, exclusion regions present in the original pharmacophore (14) were deleted to allow for more flexible conformational changes of inhibitor compounds. 3. Hit compounds could fit in the region representing residue R264 as binding studies showed that it was important in the β' -CH - σ^A interaction (14).

Using this improved pharmacophore we undertook to identify a drug lead with good solubility and appropriate drug-like properties (18) through *in silico* screening of publically available compound libraries. After screening the mini-Maybridge library (<http://www.maybridge.com/>)

27 hits were identified, from which seven that demonstrated the appropriate distance constraints to the β' -CH region and energy minimized conformations, were shortlisted for further investigation (Figure 2). Figure 1D shows one of the compounds from the 7 selected hits (compound C5; Maybridge No. DSHS00507), fitted into the pharmacophore model.

All 7 compounds were initially tested in an ELISA based binding assay using σ^A and a GST-tagged β' subunit fragment that encompasses the CH region (β' -CH) (14). 3 of the 7 compounds possessed inhibitory activity against the binding of σ^A and β' fragments (Figure 3A). Previously, we demonstrated that σ^A bound to a β' -CH-GST fusion protein in a 1:1 ratio with a $K_d \sim 1 \mu\text{M}$ by isothermal titration calorimetry (14). In similar experiments, C5 bound specifically to the β' -CH-GST fusion (Figure 3B), with a $K_d = 3.10 \pm 0.99 \mu\text{M}$ with a one-site binding mode ($N = 0.986 \pm 0.037$). No non-specific binding was detected to free GST or σ^A proteins (Figure 3B). Titrations using β' -CH constructs where amino acids known to be required for interaction with σ^A were altered to alanine showed no significant binding to C5 (Figure 3C).

Eukaryotic RNA polymerases contain a CH structure similar to the β' -CH but with no sequence similarity (Figure 3D), and in RPB1 of RNA polII it is involved in binding to initiation factor TFIIB (19). Alignment of the amino acid backbones from protein crystal structures of the CH regions from *Escherichia coli* RNAP and *Saccharomyces cerevisiae* RNA polII (grey and brown, respectively, Figure 3D) gave a root mean square deviation of $\sim 0.9 \text{ \AA}$ illustrating how similar these two structures are. However, examination of the surface charge indicated that the electrostatic environment of these two protein interaction interfaces was very different with the eukaryotic CH region being relatively hydrophobic (white-grey, Figure 3E top) and the bacterial CH region being dominated by basic side chains (blue, Figure 3E, bottom). Therefore, despite the similarity in structure it should still be possible to identify compounds that will selectively bind to the bacterial CH region. To this end, we also examined whether compounds 3-5 could bind to a human RPB1 CH-containing fragment. No interaction between human RPB1 CH and compounds C3-5 could be detected by isothermal titration calorimetry (Figure 3F). These results demonstrate that C5 targets the interaction between σ^A and β' -CH with a high level of specificity laying the foundation for future elaboration of a compound with specific antibacterial activity.

The mechanism of transcription inhibition by C5 was tested using *in vitro* transcription assays with *E. coli* RNAP and its cognate essential σ factor, σ^{70} . Initially, C5 was premixed with RNAP followed by the addition of σ^{70} . As shown in Figure 4A, the inhibitory activity positively correlated to the concentration of C5, with a $K_i \sim 0.1 \mu\text{M}$. Next, when the compound was added following the formation of a holoenzyme complex (RNAP + σ^{70}), a similar level of inhibitory activity was observed (Figure 4B), illustrating that the compound could compete with σ for binding to the β' -CH.

When compound C5 was used in bacterial growth inhibition assays, it displayed superior inhibitory activity against Gram positive over -negative bacteria (Figure 5). Although *B. subtilis* was the most sensitive bacterium to the compound with a minimum inhibitory concentration (MIC) of $\sim 25 \mu\text{M}$ (Figure 5A), the community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) strain USA300 also showed susceptibility to the compound (Figure 5B; MIC $\sim 100 \mu\text{M}$). We attribute the low activity of C5 against Gram negative bacteria (Figure 5C) to the presence of the impermeable outer membrane of these organisms, since it worked well against purified *E. coli* RNAP in the *in vitro* transcription assay (see Figure 4). C5 didn't show any inhibitory activity against *Saccharomyces cerevisiae* grown under similar conditions at levels of C5 an order of magnitude greater than those used to inhibit bacterial growth (Figure 5D) supporting the isothermal titration calorimetry data with the human RPB1-CH fragment shown in Figure 3 which indicated the compound was specific against prokaryotes.

Epifluorescence microscopy was used to establish the compound's mechanism of action in live bacterial cells using two *B. subtilis* strains. BS23 contains a GFP fusion to the α subunit of the membrane localized ATP synthase (20), while BS1048 contains a GFP fusion to the β' subunit of RNAP that localizes to the central chromosome-containing lumen of the cell (21). As shown in Figure 6, unlike the membrane-targeting compound colistin (Col) that causes readily detectable membrane damage (top middle), C5 used at its MIC did not affect ATP synthase localization patterns (compare top left, control, with top right, + C5), indicating it does not affect bacterial membrane integrity. In contrast, RNAP-GFP localization in rifampicin (Rif) and C5 treated cells was very similar with chromosomes becoming elongated and decondensed compared to the control cells (Figure 6, bottom left, arrow showing nucleoid). Mechanistically, rifampicin inhibits transcription initiation through binding to the active site of RNAP, and this leads to loss of supercoiling in DNA and decondensed chromosomes (22). Following C5 treatment, chromo-

somes decondensed (as judged by localization of the RNAP-GFP fusion), similar to that seen on rifampicin treatment. However, aggregation of the GFP signal was also visible which may possibly be a result of chromosome fragmentation prior to cell death. Nevertheless, these results are consistent with C5 targeting the transcription apparatus in live cells, resulting in a broadly similar phenotype to that observed on rifampicin treatment.

Antibiotic development through modification of existing drugs is hampered by the spread of existing resistance mechanisms that often are also effective against these derivatized compounds (23). New classes of antibiotic are needed to increase the arsenal of drugs available to clinicians to effectively treat infections. By targeting the binding surface between RNAP and a transcription factor essential for cell viability we have developed pharmacophore models rationally designed based on protein structure and mutagenesis data. The refined *in silico* screen provided small molecules that were highly specific for the CH region of bacterial RNAP, with no evidence that they were able to target the equivalent structure of eukaryotic RNAPs and that showed no inhibitory activity to the growth of *S. cerevisiae* cultures at concentrations as high as 1 mM. Compound C5 could inhibit transcription initiation in *in vitro* transcription assays and demonstrated antibacterial activity. Most importantly, C5 exhibited the ability to inhibit the growth of the antibiotic-resistant strain, CA-MRSA USA300. Together with C3 and C4, the diverse structures of these compounds will help in the study and optimization of the structure-activity relationship potential for further development of drug leads. Furthermore, the high hit rate (27 hits, 7 shortlisted, 3 with activity) from a public compound library demonstrated that this high-throughput *in silico* screening method using a carefully constructed and experimentally verified pharmacophore model will provide a solid platform for drug development targeting essential protein-protein interactions.

Bacterial transcription is a valid but under-developed target for antibiotic discovery. Here, we demonstrated the feasibility of structure based design and validation for the discovery of transcription inhibitors. This platform, utilizing high-throughput virtual screening followed by biochemical confirmation of a small number of carefully chosen hits may represent a valid alternative approach for antimicrobial discovery, as well as for the development of chemical probes to study the role of protein-protein interactions in the regulation of transcription.

METHODS

Strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Pharmacophore design and compound screening. A pharmacophore model was generated on *B. subtilis* σ^A residues utilising data generated from mutagenesis of the RNAP β' -CH region using the 'add query feature' in Accelrys Discovery Studio version 2.0 (Biovia). Hydrogen bond acceptors, hydrogen bond donors and hydrophobes were added based on the property of the amino acid residues. Each feature was defined by a central point, a location constraint sphere and a second sphere which signified hydrogen acceptors and donors interacting with the β' -CH region. After generation of the pharmacophore model, the protocol "search 3D database" was chosen to set up the input database as Mini-Maybridge and the input pharmacophore as above, "Align ligand" set to true, "search method" set to fast, and "What to output" set to one conformation. After the results were obtained, the energy minimized conformation of each of the compounds was generated using the software. Default parameter settings were applied, with the following exceptions: Conformation Method BEST, Maximum Conformations 255, Energy Threshold 20.0. Compounds that had to undergo a large conformational change to satisfy the pharmacophore properties were rejected. The remaining compounds were then mapped onto the RNAP β' -CH region model, and default parameter settings applied, with the following exceptions: Conformation Generation NONE, Best Mapping Only TRUE, Maximum Omitted Features 1, Fitting Method RIGID. Hits where the distance of the compound to the protein was less than 2 Å were rejected.

Protein overproduction and purification. Plasmids used for overproduction of protein fragments are listed in Table 1. *B. subtilis* σ^A , *E. coli* σ^{70} , and *B. subtilis* RNAP β' subunit fragments with amino acid coordinates 1-334, and 220-315 were overproduced and purified as described previously (14, 24). *E. coli* BL21 (DE3) was transformed with pNG871 (TFIIB-His) or pNG899 (RPB1 CH-GST) (Table 1) and cultures grown in auto-induction medium for 48 h at 25°C. Following lysis and clarification the His-tagged TFIIB fragment was purified using a 1 ml HisTrap HP column (GE Healthcare), and the GST tagged RPB1 fragment purified using a 1 ml GSTrap column (GE Healthcare) or 1ml of re-hydrated GST-agarose resin (Sigma-Aldrich) in a gravity flow column according to the manufacturer's instructions. Purified proteins were dialyzed into 20 mM KH_2PO_4 , 150 mM NaCl, 30% glycerol, pH 7.8 and stored at -80°C.

ELISA-based assays. These assays were performed as described previously (14).

Isothermal calorimetric titration (ITC). ITC experiments were performed as described previously (14). Programme “plate”, mode “control”, reaction temperature 30°C, 19 injections (2 µl/inj.), 150 s interval between injections, stirring speed 1000 rpm. The β' CH-GST or RPB1 CH-GST fusions (40 µM) in 50 mM KH₂PO₄, 150 mM NaCl, pH 7.4 buffer were titrated against 400 µM compound in identical buffer.

Transcription assays. Transcription assays were performed as described previously (14). 1 µl of 1 µM *E. coli* RNAP core enzyme was mixed with C5 (0.02-20 µM final concentration) and incubated on ice for 10 min. 1 µl of 0.5 µM purified σ^{70} was then added and the reaction mixture made up to a final volume of 50 µl with 5 µl 350 nM purified template DNA in DEPC water, 5 µl of 10x transcription buffer (400 mM Tris-HCl, 1.6 M KCl, 100 mM MgCl₂, 10 mM DTT, 50% glycerol, pH 7.9 in DEPC treated water), 1 µl 10 mM each ATP, GTP, and CTP, 2.5 mM UTP, 1 µl of α -³²P UTP (3000 Ci/mmol) and DEPC treated water. The reaction was incubated at 37 °C for 5 min. The reaction was stopped by transferring 10 µl of the reaction mixture into 5 µl of RNA gel loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), followed by heating at 95°C for 2 min and run on a 6% denaturing polyacrylamide gel at 50W and 50°C in 1x TBE buffer. After drying at 60°C for 1.5 h, the gel was imaged and relative band intensity determined using ImageJ software (NIH). To examine the competition of C5 with σ factor in transcription, 1 µl of 1 µM core RNAP and 1 µl of 0.5 µM σ^{70} were premixed and equilibrated on ice for 10 min prior to the addition of inhibitor at 20 nM and 0.2 µM final concentration. All other steps were the same as above.

Fluorescence microscopy. Microscopy was performed as described previously (20, 25). *B. subtilis* strains BS23 (ATP synthase-GFP) and BS1048 (RpoC-GFP) (Table 1) were grown on nutrient agar containing 5 µg/ml chloramphenicol and 0.5% (w/v) xylose. Single colonies were picked and then incubated in LB medium supplemented with 5 µg/ml chloramphenicol and 0.5% (w/v) xylose at 37°C until OD₆₀₀ ~0.6. The compound or antibiotics were then added and the cultures incubated for a further 15 min. 5 µl of cell culture was placed onto 1.2% (w/v) agarose pads, and a cover slip placed on top prior to imaging. A Zeiss Axioscop 2 epifluorescence microscope fitted with a CoolLED pE-4000 lightsource and Hamamatsu Orca II cooled CCD camera was used to capture GFP fluorescence images which were processed using Meta-

Morph version 7.01 (UIC). After background subtraction, final images were assembled using Adobe Photoshop.

Antibacterial activity test. 50 mM of compound C5 in DMSO was serially diluted in 100 μ l of LB medium in the range 3.125 μ M to 100 μ M into individual wells in a 96 well plate. *E. coli* DH5 α , *S. aureus* USA300, and *B. subtilis* EU168 cells were incubated at 37°C in 5 ml LB until OD₆₀₀ ~0.6, and 5 μ l of the culture was added to each well. The plate was incubated in a FLUOstar Optima plate reader (BMG Labtech) at 37°C shaking at 600 rpm. *S. cerevisiae* Red Star was grown under identical conditions except the temperature was adjusted to 30°C and compounds were added in the range 31.25 μ M to 1000 μ M. The OD₆₀₀ of each culture was taken every 10 min over an 18 h period. All assays were performed in triplicate and the growth curve compared to cells exposed to medium containing equivalent amounts of DMSO.

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Notes

The authors declare no conflict of interest.

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ABBREVIATIONS

RNAP: RNA polymerase; MRSA: methicillin-resistant *Staphylococcus aureus*; GFP: green fluorescent protein

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Figures and Legends

Figure 1. A) Homology model of *B. subtilis* RNAP HE. RNAP core subunits shown in grey, σ^A in pale green. B) An expanded view of the interaction between RNAP and σ^A showing the β' CH region and $\sigma_{2.2}$ regions. C) New pharmacophore model utilizing mutagenesis data overlaid on the β' CH region with hydrogen bond acceptors in green; cyan, hydrophobic interactions in cyan and hydrogen bond donors in magenta. D) Compound C5 fitting to the pharmacophore model.

Figure 2. Seven hits, labelled C1-C7, identified from the *in silico* screen of the Mini-Maybridge library. Purple sphere: hydrogen bond donor; Green sphere: hydrogen bond acceptor; Cyan sphere: hydrophobic interaction. C1, N'-[(1*E*)-(3,4-dichlorophenyl)methylidene]-3-(4-hydroxyphenyl)propanehydrazide (Maybridge No: BTB01033); C2, 3-(4-hydroxyphenyl)-N'-[(1*E*)-(3,4,5-trimethoxyphenyl)methylidene]propanehydrazide (Maybridge No: BTB01034); C3, 2-{4-[(2-aminophenyl)sulfanyl]-3-nitrobenzoyl}benzoic acid (Maybridge No: BTB02887); C4, 2-(4-chlorophenyl)-3-(4-hydroxyphenyl)-5-(2-methoxyphenyl)-hexahydro-2H-pyrrolo[3,4-d][1,2]oxazole-4,6-dione (Maybridge No: BTB14348); C5, (2*S*,4*bS*,7*R*)-7-hydroxy-2,4*b*-dimethyl-1-[[2(*E*)-3-oxo-1*H*-indol-2-ylidene]methyl]-dodecahydrophenanthrene-2-carboxylic acid (Maybridge No: DSHS00507); C6, 4-chloro-N'-{2-[(2-methyl-1*H*-indol-3-yl)sulfanyl]acetyl}benzohydrazide (Maybridge No: KM07812); C7, 2-[[2-(2-hydroxyphenyl)methyl]amino]-3-phenylpropanoic acid (Maybridge No: RH00596).

Figure 3. A) Assay of hit compound activity determined by ELISA of the β' -CH- σ^A interaction. Inhibitor compounds were used at 500 μ M; B) ITC titration of compound C5 against GST-tagged β' -CH, σ^A and free GST. C) Titration of C5 against GST-tagged β' -CH with R267A and N283A substitutions, respectively. D) Comparison of CH motifs from *S. cerevisiae* (brown, PDB ID 3K1F) and *E. coli* (grey, PDB ID 4IGC). Sequence alignments are shown below with identical residues highlighted in black and similar residues in grey. Asterisks above the alignment indicate amino acids determined from previous studies to be important for interaction with σ . E) Surface electrostatic representations of *S. cerevisiae* (top) and *E. coli* (bottom) CH motifs. White, hydrophobic; red, acidic; blue, basic. E) ITC titration of compounds C3-C5 against GST tagged human RPB1 CH.

Figure 4. A) Determination of the inhibition constant (K_i) of *E. coli* RNAP transcription by compound C5 in an *in vitro* transcription assay. Percent inhibition of transcription was determined from the amount of product band produced on a transcription gel from a multi-round assay and was plotted against concentration of C5 (μM). The inset shows an expanded view of the curve from 0-0.2 μM compound C5; B) Inhibitory activity of compound C5 in transcription assays when it was added before (complex + σ^{70}) and after (Holoenzyme + C5) holoenzyme formation. In both panels plots are averages of triplicate experiments with standard deviations shown.

Figure 5. Antibacterial activity of compound C5 against A) *B. subtilis* strain 168, B) *S. aureus* CA-MRSA strain USA300, C) *E. coli* DH5α. Light blue, control (no compound), dark blue 12.5 μM, yellow 25 μM, grey 50 μM, and orange 100 μM compound C5. D) *S. cerevisiae*. Light blue, control (no compound), dark blue 125 μM, yellow 250 μM, grey 500 μM, and orange 1000 μM compound C5. All plots are averages of triplicates.

Figure 6. Fluorescence images of *B. subtilis* strain BS23 (GFP fusion to ATP synthase, top) and BS1048 (GFP fusion to RNAP, bottom). Ctrl: control, Col: colistin, Rif: rifampicin. Cartoon schematics are shown next to cells in the control panel illustrating GFP localization (green) and cell outline (white). Arrows indicate ATP synthase localisation to the cell membrane (top) and RNAP localization to the nucleoid (bottom). Scale bar, 4 μm .

Table 1. Strains and plasmids used and created in this study

Strains	Genotype	Source/Construction
<i>E. coli</i>		
DH5 α	<i>F⁻ endA1 hsdR17 supE44 thi-1 λ^- re- cA1 gyrA96 relA1 $\Delta(lacZYA-$ <i>argF)</i>U169 ϕ80 dlacZ ΔM15</i>	Gibco BRL
BL21 pLysS	(DE3) <i>F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) (26) λDE3 pLysS(<i>cm^R</i>)</i>	
<i>B. subtilis</i>		
EU168	<i>Prototroph</i>	(27)
BS23	<i>trpC2 chr::pNG24 (atpA–gfp P_{xyI}–‘atpA (20) cat)</i>	
BS1048	<i>trpC2 chr::pST3 (rpoC – gfp cat P_{xyI}– (21) ‘rpoC)</i>	

S. cerevisiae

Red Star *Prototroph* Lesaffre, WI, USA

Plasmids

pNG209 *bla P_{φ10} – ‘ 6xHis -*
(24)
T_φ

pNG651 *bla P_{φ10} – 3CGST -*
(14)
T_φ

Vectors for protein over- production

pNG590 *bla P_{φ10} – 6xHis –*
(15)
sigA-T_φ

pNG786 *bla P_{φ10} –rpoC_{(aa1-}
(14)
_{334)- 3CGST -T_φ}*

pNG871 *bla P_{φ10} - TFII B_{(aa1-} This work. *TFII B_(aa1-315)* cloned into *NdeI*
_{315)- 6xHis -T_φ}* and *Acc65I* cut pNG209

pNG899 *bla P_{φ10} -* This work. *RPB1_(aa236-347)* cloned into *NdeI*

*RPB1*_(aa236-347)-3C- and *Acc65I* cut pNG651

GST-T_φ

*bla P_{φ10} -rpoC*_{(aa220-} (14)

pNG908

315)-PKA- 3CGST -

T_φ

bla P_{φ10} - 6xHis - (14).

pNG997

rpoD-T_φ

bla, *cat*, ampicillin and chloramphenicol resistance gene; P_{φ10}, phage T7 promoter; P_{xyI}, xylose inducible promoter, T_φ, T7 transcription terminator; 3C, the recognition sequence of 3C protease; GFP, green fluorescence protein; GST, Glutathione S-transferase; PKA, protein kinase A recognition site.