Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter know, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Jung-hoon Lee 04/20/2011

RNase III regulates actinomycin production in *Streptomyces antibioticus*

by

Jung-hoon Lee

George H. Jones
Adviser

Department of Biology

George H. Jones

Adviser

Kathleen C. Campbell

Committee Member

John M. Nickerson

Committee Member

4/20/2011

RNase III regulates actinomycin production in *Streptomyces antibioticus*

By

Jung-hoon Lee

George H. Jones

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2011

Abstract

RNase III regulates actinomycin production in *Streptomyces antibioticus*

By Jung-hoon Lee

Streptomyces plays an important role in the production of antibiotics; almost two- thirds of all known natural antibiotics come from the secondary metabolites of *Streptomyces*. Using genetic modifications, production of these secondary metabolites can be manipulated. Previous studies have shown that the *absB* locus of *Streptomyces coelicolor* encodes RNase III, which regulates the production of antibiotics in *S. coelicolor*. The *absB* locus can also be found in *Streptomyces antibioticus*, which led to the hypothesis that RNase III regulates the production of antibiotics in *S. antibioticus*, just like in *S. coelicolor*. To test this hypothesis, the RNase III gene in the chromosome of *S. antibioticus* was disrupted through the use of disruption plasmids, which contained a truncated fragment of the RNase III gene. Once the RNase III gene was disrupted in the chromosome of *S. antibioticus*, the production of the antibiotic, actinomycin D, by the resulting mutants was measured. This study showed that actinomycin production by the RNase III gene disrupted mutants was significantly reduced. The result indicated that RNase III regulates the production of antibiotics in *S. antibioticus*, just like in *S. coelicolor*. This finding also supported the idea that RNase III globally regulates the production of antibiotics in *Streptomyces* in general.

RNase III regulates actinomycin production in *Streptomyces antibioticus*

By

Jung-hoon Lee

George H. Jones

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2011

Acknowledgements

This study was funded by the grant, MCB 0817177, from the National Science Foundation to G.H.J. Special thanks to Dr. George Jones, Marcha Gatewood and Patricia Bralley for their assistance in this project, as well as Dr. Kathleen Campbell and Dr. John Nickerson for their support.

Table of Contents

Purpose

The main goal of this research was to determine the role of RNase III in regulating the synthesis of antibiotics in *Streptomyces antibioticus.* This was done by disrupting the RNase III gene. If the results indicate that RNase III does regulate the production of antibiotics in *S. antibioticus*, then this research will further support the idea that RNase III globally regulates the synthesis of antibiotics in *Streptomyces* in general.

Introduction

Streptomyces is a genus of Gram-positive, soil-dwelling bacteria, encompassing over 500 species (Bentley et al., 2002). Streptomycetes are among the most numerous and ubiquitous bacteria in soil, and are crucial in this environment for their broad range of metabolic processes and biotransformations (Bentley et al., 2002). These include decomposition of insoluble macromolecules from other organisms like lignocelluloses and chitin, making streptomycetes the central organisms in carbon recycling. Interestingly, streptomycetes also exhibit a complex multicellular development, which involves branching, filamentous vegetative growth, followed by the formation of aerial hyphae bearing long chains of reproductive spores (Bentley et al., 2002). These mycelium branches can range from 0.5 to 1.0 micrometer in diameter (Bentley et al., 2002). Some streptomycetes are noted for their production of a variety of antibiotics through secondary metabolic pathways. *Streptomyces coelicolor*, *Streptomyces aureofaciens*, and *Streptomyces rimosis* are few of the examples that produce antibiotics like actinohordin, chlortetracycline, and oxytetracycline, respectively (Bentley et al., 2002). Overall, *Streptomyces* produces more than two-thirds of all known natural antibiotics in use today, as well as

immunosuppressants and anti-tumor agents like actinomycin (a cyclic polypeptide-containing antibiotic that inhibits RNA synthesis by binding to DNA (Sobell, 1985) (Fig. 1)), which is produced by *Streptomyces antibioticus* (Katz & Weissbach, 1962).

The model organism for streptomycetes is *Streptomyces coelicolor*, which produces four structurally and genetically distinct antibiotics: actinohordin, undecylprodigiosin, methylenomycin, and calcium-dependent antibiotic (Adamis & Champness, 1992). The complete genome of *Streptomyces coelicolor* has been sequenced, showing that it is 8,667,507 base pairs long, as a single linear chromosome with a centrally located origin of replication (*oriC*). Also, the genome showed a strong emphasis on regulation, having 965 proteins (12.3%) that appear to have a regulatory function (Bentley et al., 2002). Furthermore, in order to better understand the production of antibiotics in streptomycetes, studies on the regulation of secondary metabolite pathways in *Streptomyces coelicolor* have been ongoing for many years.

One important regulatory gene was determined to be in *absB*. Previous studies done by Adamidis and Champness discovered an *absB* mutant, C120, in *S. coelicolor*. *S. coelicolor* normally produces four antibiotics (actinohordin, undecyloprodigiosin, methylenomycin, and calcium-dependent antibiotic) as secondary metabolites (Adamis & Champness, 1992), but in C120, the production of all four antibiotics was severely reduced. The production of actinohordin (act) was reduced to 2% of normal production amount, and the production of undecylprodigiosin (red) was reduced to 15%. (Gravenbeek & Jones, 2008). DNA sequencing after the complementation of the *absB* mutation identified an open reading frame that shared significant homology with the bacterial RNase III gene (*rnc*). The overexpression of the *absB* locus and the purification of the product revealed that *absB* encodes RNase III (Price et. al., 1999).

Furthermore, when *rnc* gene was reintroduced to the *absB* mutants, the antibiotic production was fully restored (Price et. al., 1999).

RNase III is a double strand-specific endoribonuclease found in both eukaryotes and bacteria, and it participates in regulating gene expressions (Chang et. al., 2005). For example, in *Escherichia coli*, RNase III is involved in the processing and maturation of viral, ribosomal, and messenger RNAs (Price et. al., 1999), and in *Streptomyces coelicolor*, RNase III regulates antibiotic synthesis (Adamis & Champness, 1992; Gravenbeek & Jones, 2008). The exact mechanism of how RNase III regulates the production of antibiotics has not been elucidated yet, but there are two possible ways. The first method would involve the endoribonuclease activity of RNase III, while the second method would involve the binding of RNase III to genes that are critical for antibiotic production (Gravenbeek & Jones, 2008). Recently, Gravenbeek and Jones showed that the first method is most likely to be true. A mutant form of RNase III (D70A) was made by changing the Asp residue at position 70 to Ala, and it retained its ability to bind to RNA substrates but did not retain the ability to cleave them (Gravenbeek & Jones, 2008). When D70A was introduced to a mutant with a disrupted RNase III gene, its antibiotic production was still not restored. This indicated that the binding activity of RNase III alone is not sufficient to regulate antibiotic production and that RNase III requires its endoribonuclease activity to regulate antibiotic production (Gravenbeek & Jones, 2008). Although the exact target for RNase III, or the exact mechanism of regulation has not been elucidated yet, it was confirmed that endoribonuclease activity of RNase III regulates the production of antibiotics in *S. coelicolor* (Gravenbeek & Jones, 2008).

Interestingly, the sequence of the RNase III gene is well preserved in different species of *Streptomyces*, as indicated in Fig. 2. One particular species, whose RNase III gene shares

significant homology with that of *S. coelicolor,* is *Streptomyces antibioticus*. This significant homology of the RNase III gene led to the hypothesis that RNase III could very well regulate the production of antibiotics in *S. antibioticus*, just like in *S. coelicolor*.

In order to test the hypothesis, the RNase III gene was disrupted in *S. antibioticus* through mutational cloning (Jones, 2000). The RNase III gene in the *S. antibioticus* chromosome was disrupted by using a truncated fragment of the gene that was prepared through PCR and incorporated into a conjugative vector, pKC1132 (Bierman et. al., 1992). The fragment was truncated at both the 5'end and the 3'end, so that its integration through a single crossover with the chromosome, would lead to disruption of the RNase III gene by truncating the 5'end in one copy of the RNase III gene and the 3'end in another copy (Jones, 2000). After confirming that the mutational cloning had worked, the mutant's production of antibiotic, actinomycin, was measured and compared with that of the wild-type to test whether RNase III has any effect on the antibiotic synthesis of *S. antibioticus* (Jones, 2000). If RNase III does regulate antibiotic synthesis, the mutant should produce less actinomycin than the wild-type or even lose its ability to produce actinomycin completely.

In this study, it is shown that RNase III regulates the production of antibiotics in *Streptomyces antibioticus*, just like in *Streptomyces coelicolor*. The production of actinomycin by *S. antibioticus* was decreased when its *rnc* was disrupted, and these findings agree with the hypothesis that RNase III globally regulates the synthesis of antibiotics in *Streptomyces* (Adamis & Champness, 1992).

Materials and Methods

Growth of organisms. The strains and plasmids used or constructed in this study are described in Table 1. In order to prepare mycelium, which were used to measure growth and the actinomycin production, the wild-type and the RNase III gene disrupted *S. antibioticus* IMRU 3720 (3720/pJSE1980) were grown on NZ-amine and galactose-glutamic acid (GGA) media (Jones, 2000). Apramycin was added to these media for 3720/pJSE1980. *E. coli* strains, DH5α and ET12567/pUZ8002, were grown in Luria broth (LB) containing necessary antibiotics. Conjugation mixtures were plated on SFM agar (Flett et. al., 1998).

Construction of the disruption plasmid containing truncated RNase III gene. Four sets of different primers were prepared from the sequence of the *S. antibioticus* RNase III gene, as indicated in Table 2. The primers also contained an *Eco*RI restriction site to facilitate cloning into the pKC1132 vector, a 3.5-kb conjugative plasmid containing an apramycin resistance (Apr^r) gene (Bierman et. al., 1992), as shown in Fig. 3. PCR was performed on the chromosomal DNA of *S. antibioticus* IMRU 3720 with four different sets of primers to produce different lengths of truncated fragments of the RNase III gene at both the 5' end and the 3' end, as indicated in Fig. 4. The PCR products were then digested with *Eco*RI and ligated to *Eco*RI digested pKC1132 with T4 DNA ligase. The ligated samples were purified through phenol/chloroform extraction, and gel electrophoresis (0.8% gel) was used to compare the samples before the ligase was added, and after the ligase was added, and confirmed that the ligation was successful, as shown in Fig. 5. As a result, four different disruption plasmids, each containing a different length of truncated fragment of the *S. antibioticus* RNase III as an insert, and pKC1132 as a vector, were made.

Construction of the strains containing the disruption plasmid. Four different types of the disruption plasmid were introduced into *E. coli* DH5α through chemical transformation. The putative DH5α transformants containing the disruption plasmids were selected by apramycin resistance, and minipreps were done in order to isolate the disruption plasmid. PCR was done on these minipreps with the primers described in Table 2. Agarose gel-electrophoresis (0.8%) was used on the PCR products to confirm the presence of disruption plasmid in the transformants, as shown in Fig. 6. The isolated disruption plasmids were then used to transform ET12567/pUZ8002. ET12567/pUZ8002 is an *E. coli* conjugative strain that contains pUZ8002, which allows vectors to mobilize *in trans* (Gust et. al., 2002). pUZ8002 is also an *oriT*RP4 derivative, containing a kanamycin resistance (Kan^r) gene. The putative transformants of ET12567/pUZ8002 containing the disruption plasmid were selected by apramycin resistance. PCR and gel-electrophoresis was carried out as above to confirm the presence of the disruption plasmid, as shown in Fig. 7. The transformants were grown to OD_{600} = 0.4~0.6 in 5 mL Luria broth (LB) containing 50 µg/mL apramycin. The transformants' cells were collected by spinning them in the IEC clinical centrifuge for 10 min at 4000 rpm, and then washed twice with 10 mL of LB. The pellet of cells after the wash was resuspended in 500 µL of LB. At the same time, 50 µL of *S. antibioticus* IMRU 3720 spores were mixed with 450 µL of 2X YT medium and heat-shocked for 10 min in a 50°C water bath. The two samples were mixed together and centrifuged for 10 seconds at 10 000 x g. The pellet was resuspended in the residual medium and plated onto SFM agar and incubated at 30°C for 16~20 hours. To select for the ex-conjugants, 1 mL of distilled water containing 0.5 mg of nalidixic acid and 1 mg of apramycin was used to overlay the plates (Jones et. al., 1997). After about 7 to 10 days, the melanin-producing colonies from the plates were transferred to GGA agar with 50 µg/mL apramycin and incubated at 30°C until a

confluent sporulation covered the entire surface (Hoyt & Jones, 1999). The spores were then collected and stored at -20°C in a 5X volume of 20% glycerol.

Preparation of the mycelium. 50 mL of NZ-amine containing 50 µg/mL apramycin and springs were inoculated with 100µL of the spores from above, and incubated in a shaker at 30°C for 36 hours. Mycelium from these NZ-amine cultures were collected by centrifugation for 10 min in the IEC clinical centrifuge at 4000 rpm. The pellet was then washed twice with 20 mL of 0.9% NaCl and suspended to a final volume of 25 mL with 0.9% NaCl (Jones, 2000).

Preparation of chromosomal DNA. Five mL of mycelium was centrifuged for 10 min at 4000 rpm, and its pellet was mixed with 4 mL TSE containing 4 mg/mL lysozyme and ~10 glass beads to disperse the mycelium. 4 mL of Modified Kirby mix (0.5 g Tri-isopropylnaphtalenesulphonate; 3 g 4-aminosalicylate; 2.5 mL 1 M Tris-HCl, pH 8.3; 12 mL phenol/chloroform/isoamyl alcohol; RO H_2O to 25 mL)(Kieser et. al., 2000) was added to the mixture which was dispersed for 1 min with a VWR vortex mixer, and centrifuged for 5 min in the IEC clinical centrifuge at 4000 rpm. The aqueous phase (upper layer) was then transferred to a clean Falcon tube, and equal volume of phenol chloroform isoamyl alcohol (PCI) was added and mixed for 1 min with a VWR vortex mixer. This was repeated until the interface was clear, and the aqueous phase was mixed with an equal volume of 3 M sodium acetate and 0.1 volume of isopropanol. The resulting mixture was stored in -20 °C for at least an hour in order to precipitate DNA.

Confirmation of the disrupted RNase III gene in the putative positives. Both PCR and Southern blotting were performed on the chromosomal DNA of 3720/pJSE1980 and *S. antibioticus* 3720 IMRU in order to confirm that homologous recombination occurred and replaced the chromosomal RNase III gene with the truncated fragment of the RNase III gene.

PCR was carried out with the MCF1/R1 primer set and the OUTF1/R1 primer set, which binds both upstream and downstream of the RNase III gene. Southern blotting was carried out with the chromosomal DNA of 3720 and 3720/pJSE1980 that were digested with the following restriction enzymes: *Sph*I, *Spe*I, *Bcl*I, *Kpn*I, and *Cla*I. Probes were derived from pJSE1901 (*S. antibioticus* RNase III gene cloned into pCR 2.1-TOPO(Invitrogen)) by digesting them with *Nde*I/*Bam*HI. *S. antibioticus* 3720 IMRU served as a control.

Measurement of actinomycin production. Four flasks containing 200 mL of 5X GGA-5.2 mM phosphate (5XGGA-LP) were inoculated with 5 mL of 3720/pJSE1980 mycelium. Each flask contained different concentrations of apramycin (0 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL). One flask was inoculated with 5 mL of *S. antibioticus* IMRU 3720 mycelium without apramycin to be used as a control. These flasks were incubated in a 30°C shaker, and 4 mL from each flask was harvested at 0, 12, 18, 24, 36, 48, and 72h after inoculation. The collected sample was then mixed with 4 mL of ethyl acetate and dispersed for 1 min with a VWR vortex mixer, and then centrifuged for 5 min at 4000 rpm in the IEC clinical centrifuge. One mL from the upper phase (ethyl acetate) was used to calculate the actinomycin concentration. This was done by using spectroscopy to measure the absorbance of the extract at 452 nm, and actinomycin concentration was calculated with an extinction coefficient of 24,800 (Hoyt & Jones, 1999).

Measurement of growth. Ten mL from the 5XGGA-LP cultures described above was also harvested at 0, 12, 18, 24, 36, 48, and 72h time points. The samples were collected by vacuum filtration to collect mycelium and dry weights of the samples were measured to calculate growth (Jones, 2000).

Results

Construction of the disruption plasmid containing truncated RNase III gene.

Disruption plasmids containing the truncated fragments of the RNase III gene were constructed in order to disrupt the RNase III gene in *S. antibioticus*. As indicated in Materials and Methods, four primer sets were designed to produce different lengths of the truncated RNase III fragments. PCR with all four primer sets was successful, and produced four different truncated fragments of the RNase III gene. The truncated fragments were then successfully incorporated into the pKC1132 vector, as shown by the digests of the minipreps of DH5α transformants and ET12567 transformants in the next experiment (Fig. 6, Fig. 7)

Construction of strains containing the disruption plasmid. The *E. coli* strain, DH5α, was used to amplify the disruption plasmids, and *E. coli* strain, ET12567/pUZ8002, was used to conjugate with *S. antibioticus* 3720 IMRU. Three of the four disruption plasmids, pJSE1980 (724-bp truncated fragment of the RNase III gene in pKC1132), pJSE1981 (599-bp truncated fragment of the RNase III gene in pKC1132), and pJSE1982 (477-bp truncated fragment of the RNase III gene in pKC1132) (Table 1) were successfully introduced into DH5α (Fig. 6) and ET12567/pUZ8002 (Fig. 7). The strains of ET12567/pUZ8002 containing the three disruption plasmids were conjugated with *S. antibioticus*, and one disruption plasmid, pJSE1980, was successfully transferred via single cross over with the chromosomal RNase III gene through the homologous recombination. No ex-conjugants were obtained from other plasmids. This was most likely due to the fact that as truncated fragments got shorter, their probabilities of going through the homologous recombination with the chromosomal DNA also decreased (Jones, 2000).

Confirmation of the disrupted RNase III gene in the putative positives. PCR and Southern blotting were carried out on the chromosomal DNA of 3720 and 3720/pJSE1980 in order to show that pJSE1980 went through homologous recombination with the chromosomal DNA of 3720. If homologous recombination occurred in 3720/pJSE1980, the normal RNase III gene in its chromosome would be replaced with the truncated fragment of the RNase III gene (724-bp). Theoretically, the result from the PCR with the OUTF1/MCR1 primer set should be identical for both 3720 and 3720/pJSE1980, both producing a 900-bp product while the result from the PCR with the OUTF1/R1 primer set should be different for 3720 and 3720/pJSE1980, producing a 1094-bp product and a 5023-bp product, respectively, as described in Fig. 8. PCR on the chromosomal DNA of 3720 and 3720/pJSE1980 with the OUTF1/MCR1 primer set and the OUTF1/R1 primer set showed that the putative positive of 3720/pJSE1980 was truly a disrupted clone. PCR on the chromosomal DNA of 3720 and 3720/pJSE1980 with the OUTF1/MCR1 primers produced a 900-bp product (lane 2 and 3, respectively, in Fig. 9). On the other hand, PCR on the chromosomal DNA of 3720 with the OUTF1/R1 primers produced a 1089-bp product, while PCR on the chromosomal DNA of 3720/pJSE1980 did not (lane 4 and 5, respectively, in Fig. 9). The theoretical 5082-bp product from the PCR on the chromosomal DNA of 3720/pJSE1980 with the OUTF1/R1 primers was not visible on the gel, possibly due to the fact that only small amount was produced under the conditions used here for the PCR. The result from the Southern blot also showed that the putative positive of 3720/pJSE1980 was a disrupted clone (See Fig. 10). Theoretically, when the chromosomal DNA of 3720 and 3720/pJSE1980 is digested with a same restriction enzyme, the size of the RNase III region in the genome should be different for 3720 and 3720/pJSE1980. Since the disruption plasmid integrated into the chromosome of 3720/pJSE1980, the RNase III region in the genome of

3720/pJSE1980 should be 4.4-kb longer than 3720. The Southern blot result showed that when the chromosomal DNA of 3720 and 3720/pJSE1980 were digested with *Bcl*I, the RNase III region in the genome was at different length for 3720 and 3720/pJSE1980. The band from the 3720/pJSE1980 was higher than the band from 3720, meaning that the RNase III region in the genome of 3720/pJSE1980 was longer than that of 3720, as shown in Fig. 10.

Measurement of actinomycin production. Actinomycin production was measured from the wild-type, 3720, and disrupted clone, 3720/pJSE1980, to test if the disruption of the RNase III gene had any effect on antibiotic synthesis in *S. antibioticus*. Actinomycin production was measured by extracting a sample of the 5XGGA-LP cultures that were inoculated with 5 mL of mycelium with ethyl acetate at various time points. The amount of actinomycin present in the extracts was estimated spectrophotometrically at 452 nm using a molar extinction coefficient of 24,800 (Jones, 2000). The levels of actinomycin produced by 3720 was higher than 3720/pJSE1980, regardless of the amount of apramycin added to the growth medium of 3720/pJSE1980. The maximum amount of actinomycin produced was 7.02 µg/mL for 3720, and 1.23 µg/mL for 3720/pJSE1980, as shown in Fig. 11. The reduction of actinomycin production in the RNase III gene disrupted clone was discernible visually as well, as shown in Fig. 12.

Measurement of growth. The growth characteristics of the wild-type and disrupted clones were measured to ensure that differences in the amount of actinomycin produced were not due to the differences in growth. The growth characteristics were measured by taking a sample of the growth medium at multiple time points and then vacuum filtering it to obtain the dry weight of the mycelium (Jones, 2000). While the growth curve of 3720/pJSE1980 in 5XGGA-LP with 50 µg/mL apramycin was different from others, the rest of the samples exhibited growth curves

that were generally similar. However, at later time points, 3720/pJSE1980 seemed to be growing better than 3720 (See Fig. 13).

Discussion

The above data demonstrated that the RNase III gene was successfully disrupted through homologous recombination between pJSE1980 and the wild type gene in *S. antibioticus* IMRU 3720. While four disruption plasmids were initially prepared, each with a different truncated fragment of the RNase III gene, only one was successfully integrated into the chromosome of *S. antibioticus* IMRU 3720. The reason for the failure of the others was most likely due to the small size of the truncated fragment in those disruption plasmids. In order to disrupt the chromosomal RNase III gene, the disruption plasmid had to undergo homologous recombination with the chromosome of *S. antibioticus*. The likelihood of the homologous recombination event depended on the length of the truncated fragment, such that longer truncated fragments had a higher chance of homologous recombination than the shorter truncated fragments did (Jones, 2000). This was probably the reason that pJSE1980, which contains the longest truncated fragment of the RNase III gene (724-bp), succeeded while the others did not. A similar result was observed in the experiments described by Jones, where the truncated fragment of phenoxazinone synthase gene, *phsA*, was used to disrupt the chromosomal phenoxazinone synthase gene in *S. antibioticus*. The disruption plasmids containing 1,688-bp and 1,325-bp truncated fragments of *phsA* successfully integrated into the chromosome of *S. antibioticus*, while the ones with 1,032-bp and 735-bp fragments did not (Jones, 2000). The consequences of

disrupting the RNase III gene, as well as the implications of the results, are explained in greater detail below. This study demonstrated that RNase III regulates the production of antibiotics in *S. antibioticus*, just as in *S. coelicolor*, and that disruption of the RNase III gene led to decreased production of actinomycin in *S. antibioticus*. This finding therefore agreed with the hypothesis that RNase III globally regulates the production of antibiotics in streptomycetes in general (Adamis & Champness, 1992).

The strain, 3720/pJSE1980, produced by using a 724-bp truncated fragment of the RNase III gene sporulated well and grew well in GGA and NZ-amine media. The 3720/pJSE1980 strain showed similar characteristics to its parental strain, 3720, such as growth and phenotype. However, 3720/pJSE1980's actinomycin production was decreased to 14.3% when compared to its parental strain, as shown in Figs. 11 and 12. Also, the 1 µg/mL of actinomycin produced by the mutants could be due to an inadequate blank when the absorbance readings were taken. This study used ethyl acetate as blank, but ethyl acetate extract of 5XGGA-1 mM phosphate would have been a better blank to account for the medium. Nevertheless, the reduction in the amount of actinomycin produced by the mutants confirmed that RNase III plays a role in the regulation of actinomycin production in *S. antibioticus*. It was also important to note that this was solely due to only about a 100-bp deletion, from an 824-bp gene. Although this study could not show whether the 100-bp deletion was sufficient enough to completely disable the activity of RNase III or not, this study showed that altering RNase III was significant enough to cause a huge drop in the production of actinomycin. It can be reasonably concluded that further truncation of the RNase III gene would have caused an even greater reduction in actinomycin synthesis. Therefore, one possible future direction for this research could be to disrupt the RNase III gene with shorter fragments of *rnc*. Also, in a previous study by Jones, it was shown that actinomycin production

from *S. antibioticus* was increased when the phosphate concentration was decreased in GGA (Jones, 2000). This study initially used 1XGGA-1 mM phosphate, instead of 5XGGA-5.2 mM phosphate, to increase the production of actinomycin from *S. antibioticus*. However, while 3720 did grow well in the 1XGGA-1 mM phosphate, 3720/pJSE1980 did not, and actinomycin production data from a non-growing culture was not applicable. Therefore, 5XGGA-5.2 mM phosphate, which contained higher concentration of nutrients than 1XGGA-1 mM phosphate, was used instead, and allowed 3720/pJSE1980 to grow. It can be reasonably concluded that in 5XGGA-1 mM phosphate, the difference in the amount of actinomycin produced between 3720 and 3720/pJSE1980 would be much greater, based on the study by Jones (Jones, 2000).

While disrupting the RNase III gene did have an effect on actinomycin production, it did not appear to have an effect on the growth of *S. antibioticus*. The growth curve of 3720/pJSE1980 was very similar to 3720, with differences attributable to sampling errors. 3720/pJSE1980 did appear to stay in the exponential growth phase longer than its parental strain however. This was interesting to note, since 3720/pJSE1980 would be expected to grow more poorly than 3720 due to the fitness cost from the apramycin resistance. 3720/pJSE1980 did appear to demonstrate this concept in the early time points, as all the 3720/pJSE1980 derivatives stayed in the lag phase longer than their parental strain did. However, at later time points, 3720/pJSE1980 in 50 μg/mL of apramycin grew the most out of all five samples. This higher growth demonstrated by the 3720/pJSE1980 was most likely due to the fact that the loss of actinomycin production allowed the mutants to utilize more resources toward their growth instead. Multiple samples should be taken in order to confirm this hypothesis.

It is also worthwhile to note that in order to disrupt *rnc* in *S. antibioticus*, several different approaches were attempted other than the one described in this study. Previous approaches

included PCR-targeted mutational cloning via cosmid and via plasmid (Gust et. al., 2002), which was used to knock out genes in *S. coelicolor*. The reason for the failure with these approaches in *S. antibioticus* was most likely biological and/or technical issues rising from the genetic difference between *S. coelicolor* and *S. antibioticus*. The reason for choosing the approach described in this study was due to the fact that this approach was proven to be successful in a previous study, where phenoxazinone synthase gene (*phsA)* was disrupted through the same procedures in *S. antibioticus* (Jones, 2000).

In conclusion, this study was carried out in order to test the hypothesis that RNase III regulated the production of antibiotics in *S. antibioticus*, based on the conservation of RNase III gene sequence in many species of *Streptomyces*, as shown in Fig. 2. The study supported the hypothesis that RNase III regulates the production of antibiotics in *S. antibioticus*, as synthesis of actinomycin was visibly reduced in the *rnc* disrupted clones. This finding further supported the hypothesis that RNase III globally regulates the production of antibiotics in streptomycetes in general. Therefore, one possible future direction for this study could be to repeat the experiment in other species of *Streptomyces*, such as *Streptomyces scabies*, *Streptomyces avermitilis* and etc.

Tables and Figures

TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Properties	Reference or source
Strain		
S.antibioticus IMRU 3720	actinomycin-producing wild-type strain	Jones, 2000
E. coli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Bethesda Research Laboratories
E. coli ET12567/pUZ8002	*dam-13::Tn9 dcm-6 hsdM*	Jones, 2000; Gust et. al., 2002
Plasmids		
pUZ8002	Conjugative plasmid bearing a Kan^r gene	Jones, 2000
pKC1132	Conjugative plasmid bearing Apr^r rep^{pUC}	Gust et. al., 2002
pJSE1980	pKC1132 bearing a 724-bp fragment of the RNase III gene	This study
pJSE1981	pKC1132 bearing a 599-bp fragment of the RNase III gene	This study
pJSE1982	pKC1132 bearing a 477-bp fragment of the RNase III gene	This study
pJSE1983	pKC1132 bearing a 389-bp fragment of the RNase III gene	This study

TABLE 2. Primers used in this study (*Eco*RI sites are underlined)

Primer pair	Primer coordinates	Product coordinates	Product Size	Sequences
MCF1/R1	33-59; 756-782	46-770	724-bp	Forward: 5'-CTCGACCGCCAAGAATTCGGCGGACAA-3' Reverse: 5'-GCGGCCGTGGCCGAATTCGCCGCCTCG-3'
MCF2/R2	105-131; 702-728	117-716	599-bp	Forward: 5'-GCTCGAGTCCGGAATTCTGGTGCGTGC-3' Reverse: 5'-GACCGCACGCGGAATTCGCGGCCTGC-3'
MCF3/R3	165-191; 640-666	177-654	477-bp	Forward: 5'-CGGTCTGCCGACGAATTCACGGCTGGA-3' Reverse: 5'-GCCGTACGAGACGAATTCGACGCGGGC-3'
MCF4/R4	225-251; 612-638	237-626	389-bp	Forward: 5'-CACGGACACGCTGAATTCCACCCACCC-3' Reverse: 5'-GCAGCAGTGAAGGAATTCTCGTGATCG-3'
OUTF1/R1*	$^{-}$176 - $^{-}$158; $^{+}$77 - $^{+}$94	$^{-}$176-$^{+}$94	1094-bp	Forward: 5'-GTTCCGAAGCGGAAGATGT-3' Reverse: 5'-CTCTAGAACTAGTGGATC-3'

*The OUTF1/R1 primers bind to the regions that are upstream and downstream from the *S. antibioticus* RNase III gene. Unlike the other primers on this table, the OUTF1/R1 primers are not used for mutational cloning.

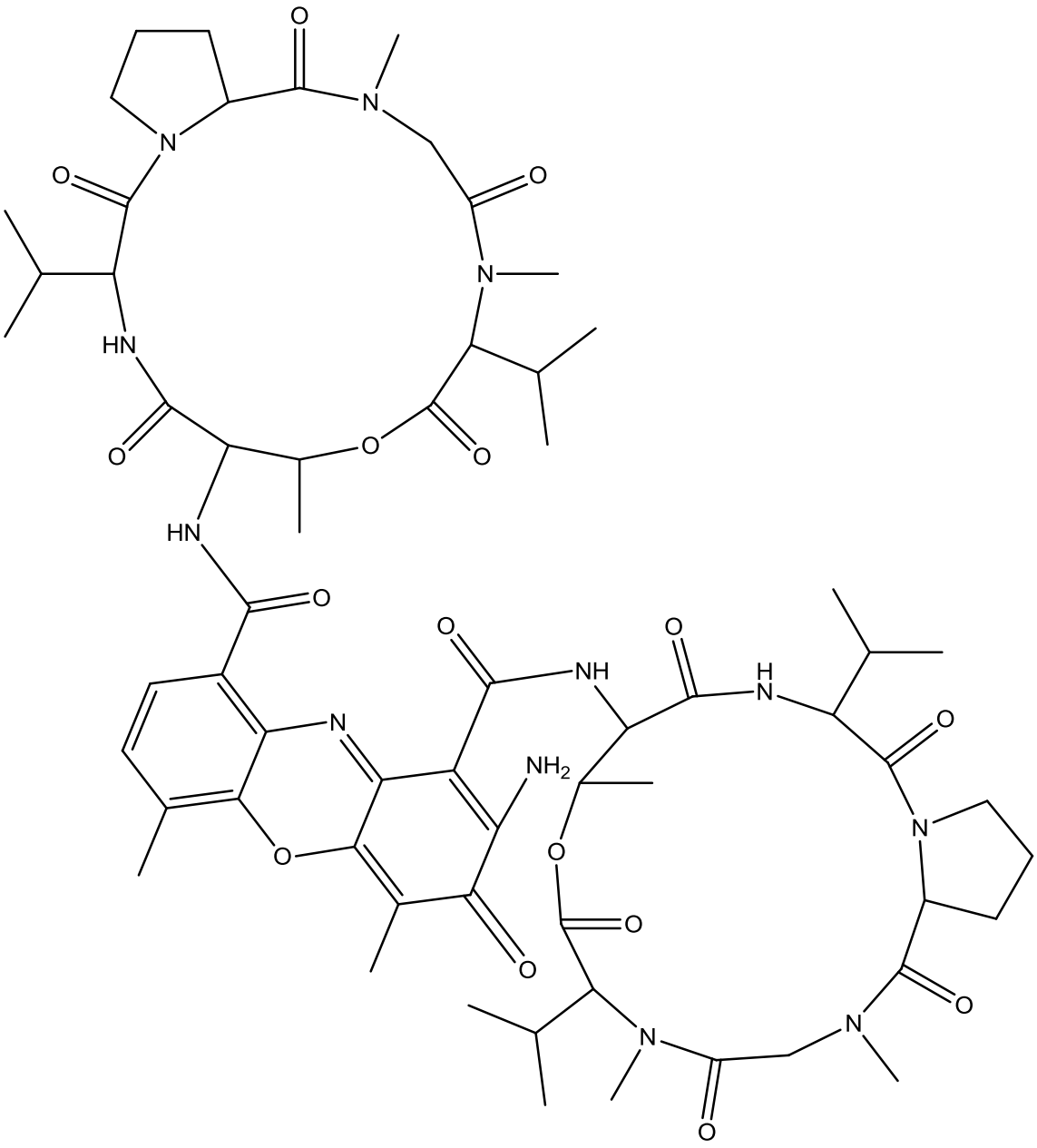

Fig. 1. Structure of Actinomycin D. Actinomycin D is primarily used as an investigative tool in cell biology to inhibit transcription. It binds to DNA at the transcription initiation complex and prevents elongation by RNA polymerase. Actinomycin D has a molecular formula of $C_{62}H_{86}N_{12}O_{16}$ (1255.42g/mol) (Sobell, 1985)

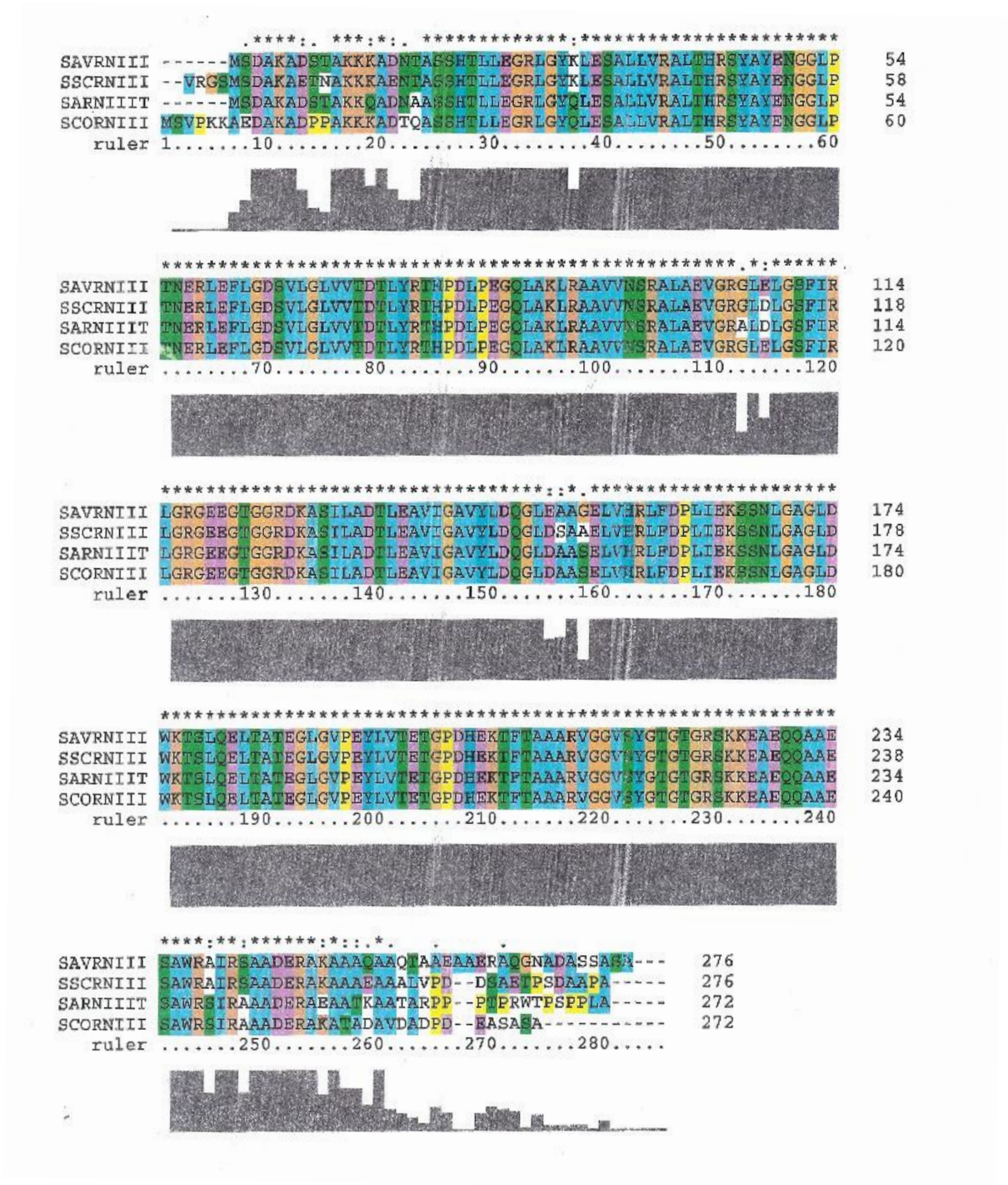

Fig. 2. Comparison of amino acid sequences of RNase III in *S. avermitilis, S. scabies, S. antibioticus, and S. coelicolor.* The asterisks indicate loci where all four species have the same amino acid.

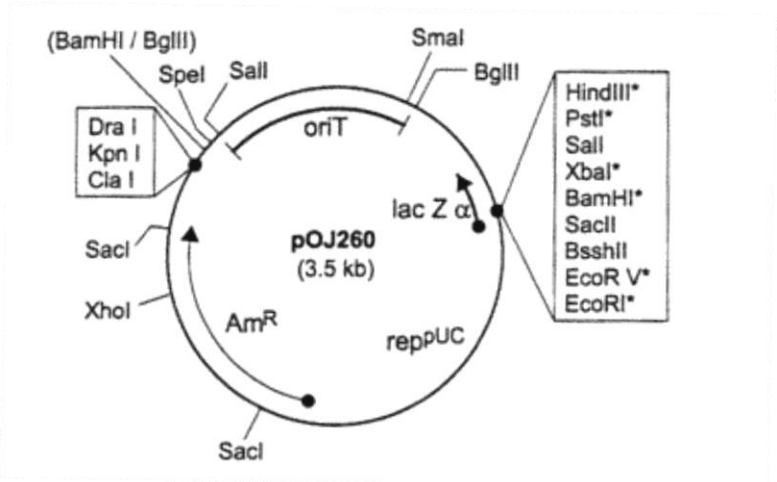

Fig. 3. Schematic diagram of pKC1132. pKC1132 vector is a derivative of pOJ260 in which *Kpn*I to *Spe*I sites are deleted (Bierman et. al., 1992).

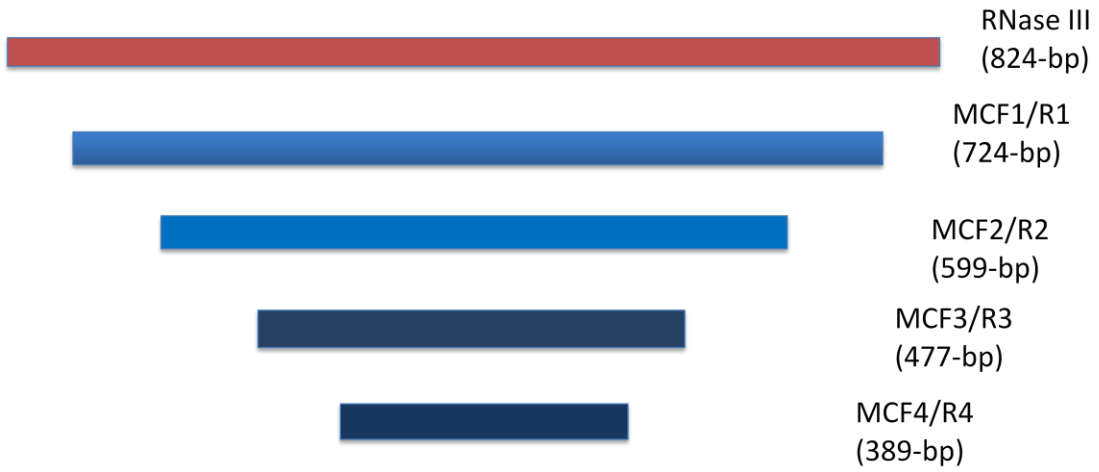

Fig 4. Visual representation of the truncated fragments of the RNase III gene produced by PCR with the primers described in Table 2. (Not drawn to scale)

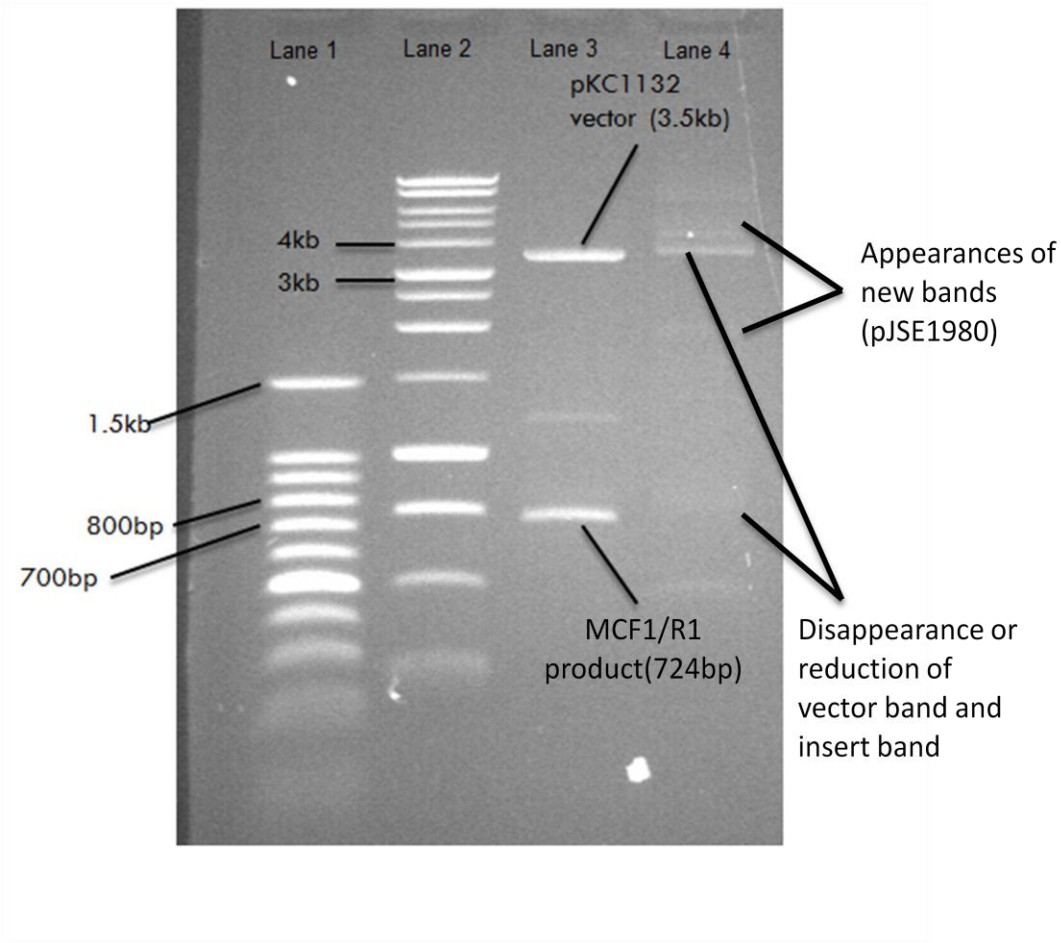

Fig. 5. Picture of the results of agarose gel electrophoresis (0.8% gel) from the pre-ligation and post-ligation of MCF1/R1 product and pKC1132 vector. Lane 1 and 2 are size standards, lane 3 is a pre-ligation, and lane 4 is a post ligation with T4 DNA ligase.

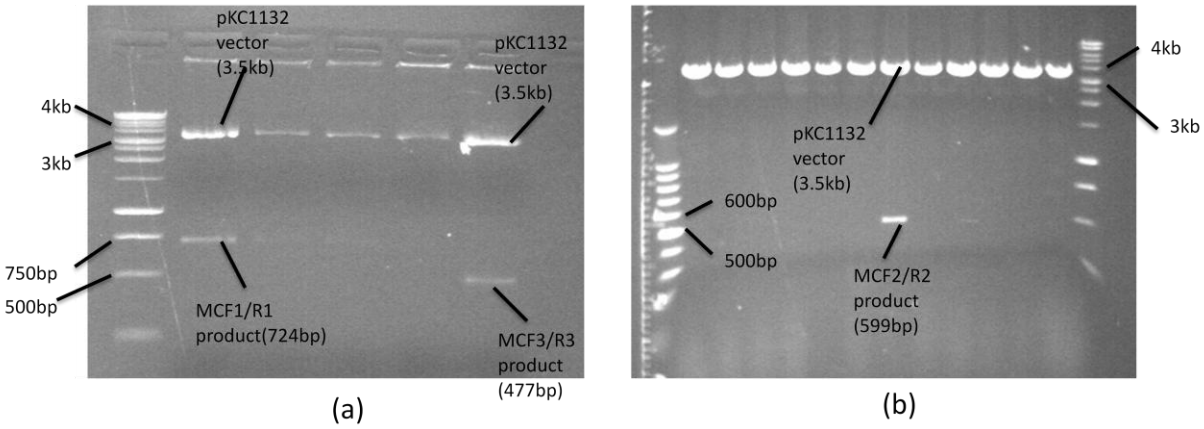

Fig 6. Pictures of the results of agarose gel electrophoresis (0.8% gel) from *Eco*RI digested DH5α transformants. DH5α transformants containing pJSE1980 and pJSE1982 were identified by digesting their minipreps with *Eco*RI (a). DH5α transformants containing pJSE1981 were identified by digesting their miniprep with *Eco*RI also (b).

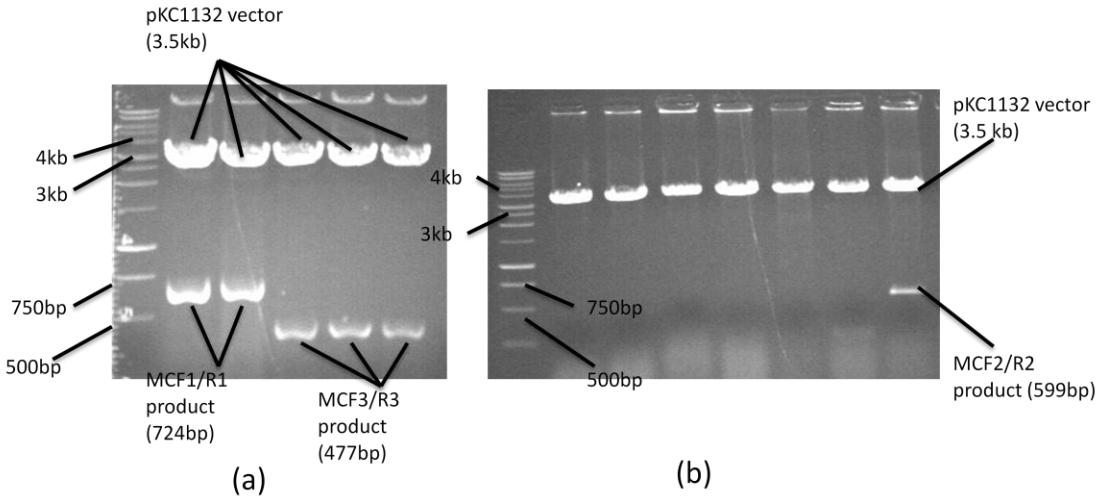

Fig 7. Pictures of the results of agarose gel electrophoresis (0.8% gel) from *Eco*RI digested ET12567 transformants. ET12567 transformants containing pJSE1980 and pJSE1982 were identified by digesting their minipreps with *Eco*RI (a). ET12567 transformants containing pJSE1981 were identified by digesting their miniprep with *Eco*RI as well (b).

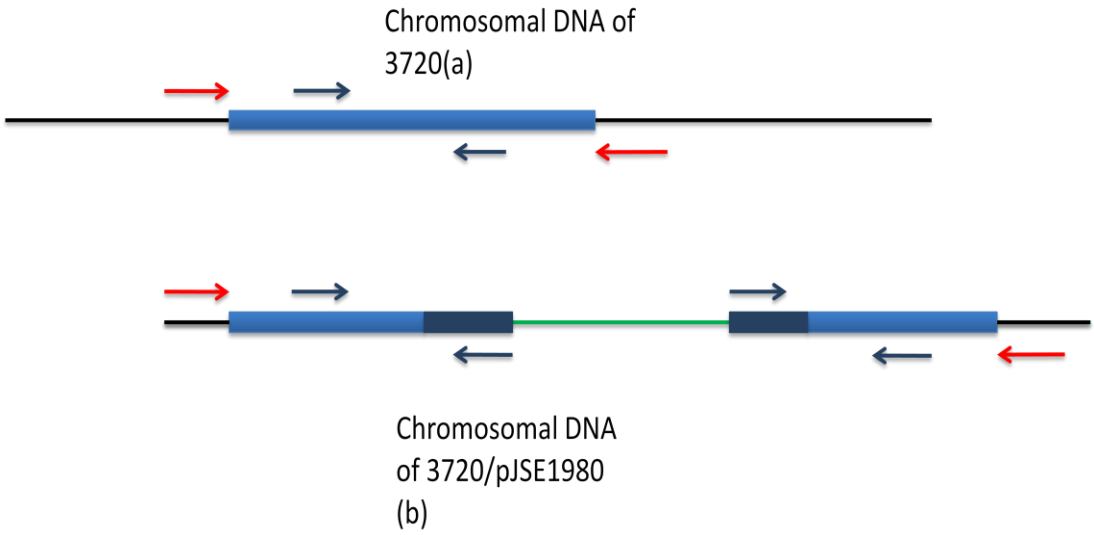

Fig. 8. Schematics of PCR to confirm that pJSE1980 successfully integrated into the chromosome of *S. antibioticus* and disrupted the chromosomal RNase III . Red arrows represent OUTF1/R1 primers that bind to regions that are upstream and downstream from RNase III gene. Blue arrows represent MCF1/R1 primers that bind to regions within RNase III gene. The green line represents vector sequence of pKC1132. Navy regions represent truncated portions of RNase III. The two chromosomes have been aligned in such a way to show that the OUTF1/MCR1 primers will produce identical product (900-bp) for both wild-type (a) and the disrupted clone (b), while the PCR product from OUTF1/R1 primers will be different (The PCR product from OUTF1/R1 primers will be 1094-bp for the wild-type, while it will be around 5023-bp for the disrupted clone).

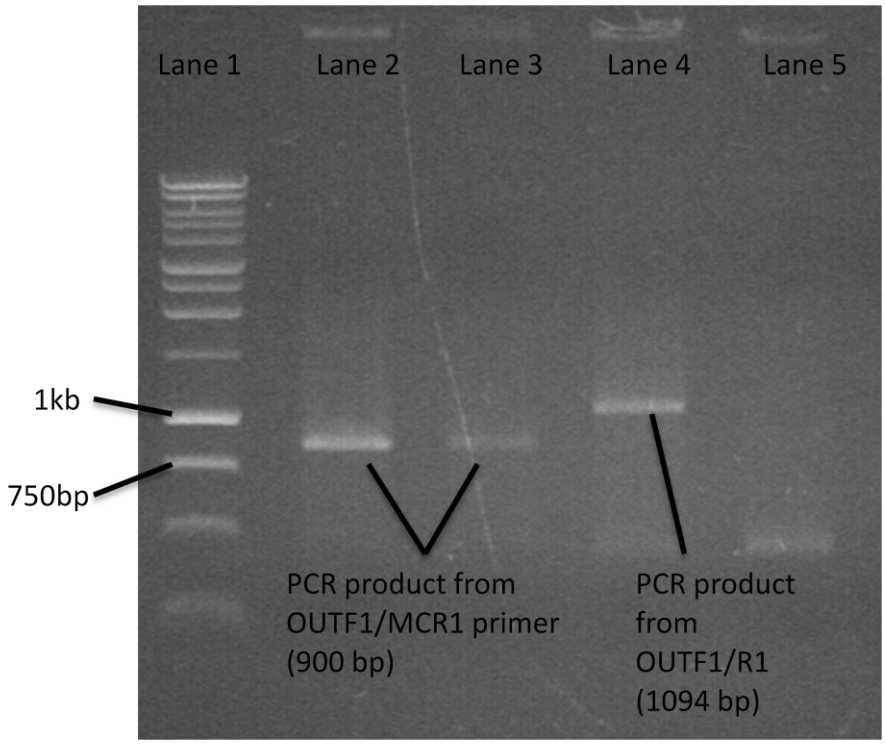

Fig. 9. Picture of the results of gel electrophoresis (0.8% gel) from the PCR on chromosomal DNA of 3720 and 3720/pJSE1980. Lane 1, size standards; PCR on 3720 with the OUTF1/MCR1 primers is in lane 2, PCR on 3720/pJSE1980 with the same primers is in lane 3, PCR on 3720 with the OUTF1/R1 primers is in lane 4, and PCR on 3720/pJSE1980 with the same primers is in lane 5. Lanes 2 and 3 show that PCR with the OUTF1/MCR1 primers produced identical products in 3720 and 3720/pJSE1980, while lane 4 and 5 show that PCR with OUTF1/R1 produced different products in 3720 and 3720/pJSE1980. This suggests that pJSE1980 went through homologous recombination with the chromosomal DNA of 3720, disrupting its RNase III gene.

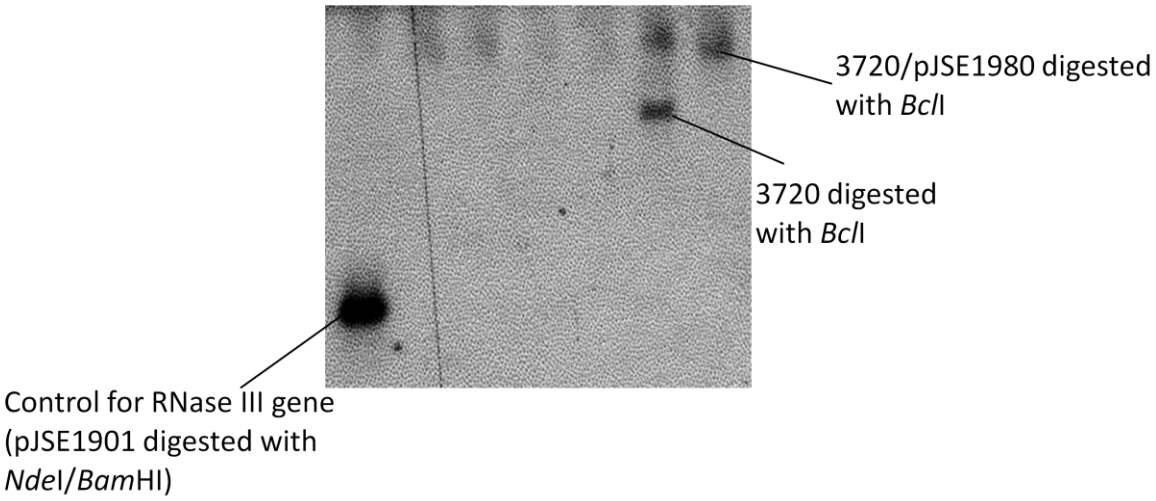

Fig.10. Picture of the results of Southern blot with chromosomal DNA of 3720 and 3720/pJSE1980. The control for the RNase III gene proved that the probe worked and bound to the RNase III gene. The difference between the bands produced by the *Bcl*I digested 3720 and 3720/pJSE1980 can be seen in this picture. Band from the *Bcl*I digested 3720 is lower than the band from the *Bcl*I digested 3720/pJSE1980. This is expected since the vector sequence will add extra 4.4kb to the region containing the RNase III gene.

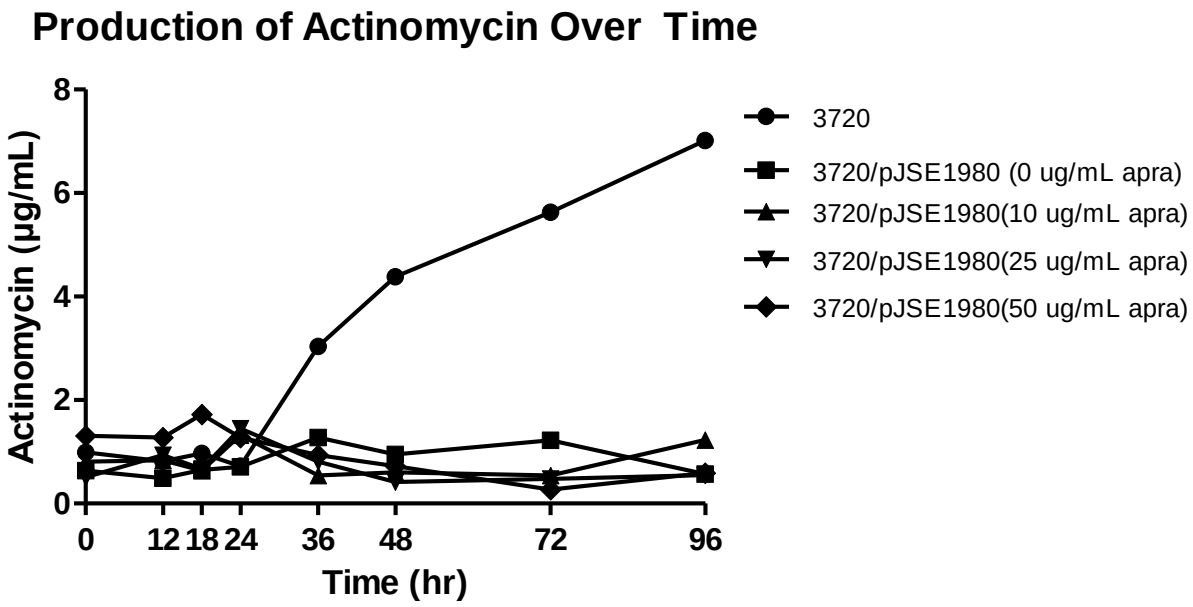

Fig. 11. Graph of actinomycin produced over time by 3720 and 3720/pJSE1980. This graph shows that wild-type *S. antibioticus*, 3720, produces more actinomycin than the clone bearing the disrupted RNase III gene, 3720/pJSE1980.

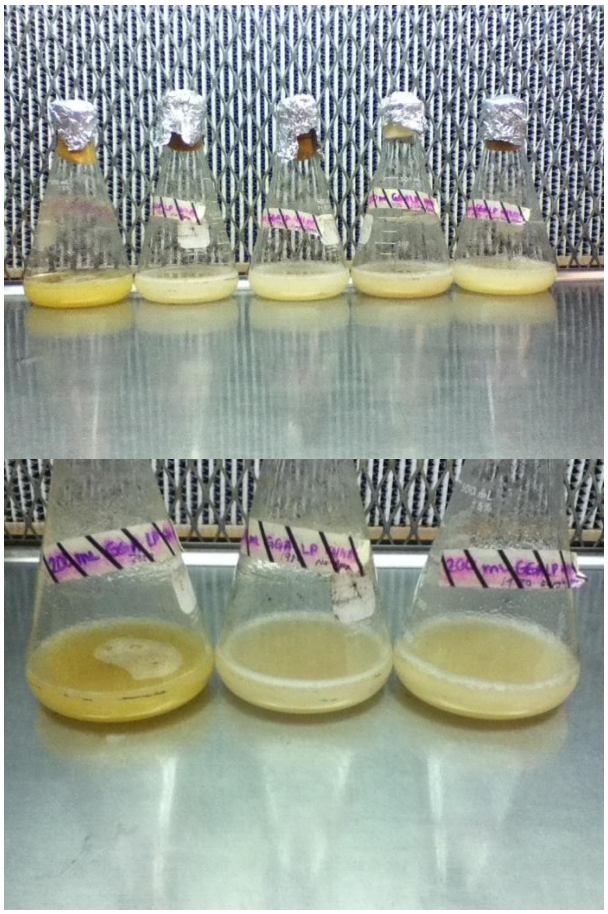

Fig. 12. Comparison of the 5XGGA-LP cultures inoculated with 3720 and 3720/pJSE1980 after 72 hours. From left to right: 3720, 3720/pJSE1980 (0 µg/mL apramycin), 3720/pJSE1980 (10 µg/mL apramycin), 3720/pJSE1980 (25 µg/mL apramycin), 3720/pJSE1980 (50 µg/mL apramycin) (Top). The cultures up close, from left to right: 3720, 3720/pJSE1980 (0 µg/mL apramycin), and 3720/pJSE1980 (50 µg/mL apramycin) (Bottom). The yellow pigment is a characteristic of the actinomycin D, and the straw color is a characteristic of the *S. antibioticus* mycelium.

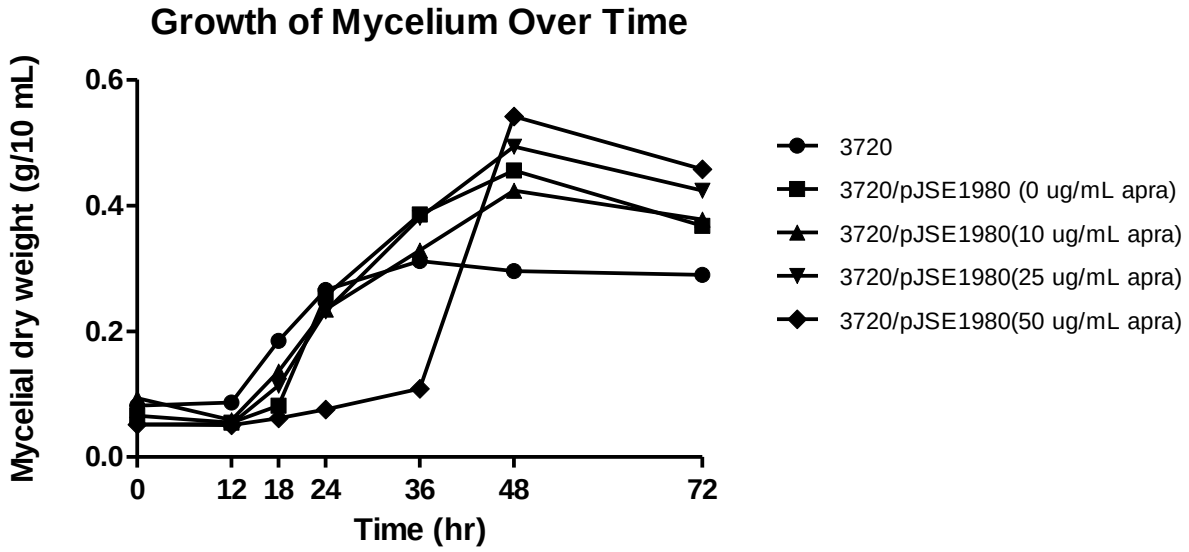

Fig. 13. Graph comparing the growth of 3720 and 3720/pJSE1980 over 72 hours. The graph showed that 3720 and 3720/pJSE1980 grow in a generally similar fashion. However, 3720/pJSE1980 appeared to stay longer in the exponential phase than 3720 did in this experiment. The 3720/pJSE1980 strain in 50 µg/mL apramycin stayed in the lag phase longer than the others most likely due to the high concentration of apramycin.

References

Adamidis, T., Champness, W. (1992). Genetic analysis of *absB*, a *Streptomyces coelicolor* locus involved in global antibiotic regulation. *Journal of Bacteriology, 174*, 4622-4628.

Bentley, S. D., Chater, K. F., Cerdeño-Tárraga, A.-M., Challis, G. L., Thomson, N. R.,. James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.-H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.-A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., Hopwood, D. A. (2002).Complete genome sequence of the model actinomycete: *Streptomyces coelicolor*: A3(2). *Nature, 417*, 141-147.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Nagaraja Rao, R., Schoner, B.E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp., *Gene* 116, 43-49.

Chang, S. A., Bralley, P., Jones, G.H. (2005). The *absB* gene encodes a double strand-specific endoribonuclease that cleaves the read-through transcript of the *rpsO-pnp* operon in *Streptomyces coelicolor*. *Journal of Biological Chemistry, 280*, 33213-33219.

Flett, F., Mersenias, V., Smith**,** C. P. (1998). High efficiency intergenic conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA restricting streptomycetes. *FEMS Microbiology Letters,155*, 223–229.

Gravenbeek, M. L., Jones, G. H. (2008). The endonuclease activity of RNase for the regulation of antibiotic production by *Streptomyces coelicolor*. *Microbiology, 154*, 3547-3555.

Gust, B., Kieser, T., Chater, K. (2002). *PCR Targeting System in Streptomyces coelicolor A3(2)*, Norwich: John Innes Centre

Hoyt, S., Jones, G. H. (1999). *relA* is required for actinomycin production in *Streptomyces antibioticus*. *Journal of Bacteriology, 181,* 3824–3829.

Jones, G. H., Mark S. B., Leony Chamberlin, P., Buttner, M. J. (1997). Sigma-E is required for the production of the antibiotic actinomycin in *Streptomyces antibioticus*. *Molecular Microbiology, 23*, 169-178.

Jones, G. H. (2000). Actinomycin production persists in a strain of *Streptomyces antibioticus* lacking phenoxazinone synthase. *Antimicrobial Agents and Chemotherapy, 44*, 1322-1327.

Katz, E., Weissbach, H. (1962). Biosynthesis of the actinomycin chromophore: enzymatic conversion of 4-methyl 3-hydroxyanthranilic acid to actinomycin. *Journal of Biological Chemistry, 237*,882–886.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A. (2000). *Practical Streptomyces genetics*, Norwich: The John Innes Foundation

Price, B., Adamidis, T., Kong, R., Champness, W. (1999). A *Streptomyces coelicolor* antibiotic regulatory gen, *absB,* encodes an RNase III homolog. *Journal of Bacteriology*, *181*, 6142-6151.

Sobell, H. (1985). Actinomycin and DNA transcription. *Biochemistry*, *82,* 5328-5331.