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이학석사학위논문

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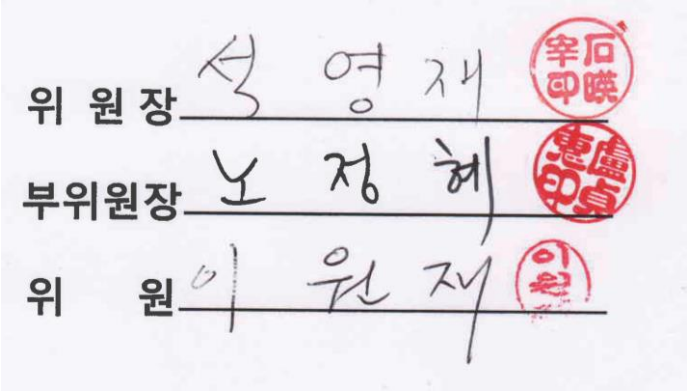

Role of Peroxide sensing transcription regulator OxyR in *Streptomyces coelicolor*

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School of Biological Sciences

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국문초 록

OxyR 은 일반적으로 그람 음성세균과 그람양성 세균 전반에 걸쳐 발견되며 과산화수소를 감지하는 조절자이다. 방선균 *Streptomyces coelicolor* 에서 OxyR 은 자신의 유전자와 alkyl hydroperoxidase 유전자인 *ahpCD* 를 활성자로서 조절한다. 또 다른 과산화수소 감지조절자인 CatR 은 catalase A 유전자를 억제자로서 조절한다. *oxyR* 결손 돌연변이 균주가 예상과 다르게 과산화수소를 처리한 배지에서 야생형균주보다 더 잘 자라고, 분화도 더 잘하며 항생제합성도 더 잘하는 것이 관찰되었다. 이에 대한 원인이 oxyR 결손변이주에서 catalase 의 생성이 더 많아진 것이 아닌가 추정되었다. 효소활성염색을 통해, 돌연변이 균주에서 catalase 효소활성이 야생형보다 더 높은 것이 확인되었다. OxyR 과 CatR 의 기능을 확인하기 위해서는 genome 상에서 이들이 결합하는 유전자 위치를 결정하여야 한다. 이를 위하여, OxyR 과 CatR 의 C-말단에 6 개의 Myc 을 붙이는 재조합체를 제조하였다. Myc 에 대한 항체를 사용한 면역블롯을 통해 Myc tagging 이 성공적으로 되었음을 확인하였다. 이로서, 면역침강을 활용한 유전체차원의 분석이 가능하게 되었다.

주요어: *Streptomyces coelicolor*, OxyR, catalase, Myc tagging, CatR, *ahpCD*

Abstract

OxyR is an H_2O_2 sensing transcriptional regulator of the LysR-family that is generally found in Gram-negative bacteria and some Gram-positive bacteria. In *Streptomyces coelicolor*, OxyR positively regulates its own gene and *ahpCD* encoding alkyl hydro peroxidase. Another peroxide-sensing regulator CatR controls catalase A production in response to H_2O_2. It binds to *catA* and *catR* genes and represses their transcription. Physiological study showed that *ΔoxyR* mutant grew faster in liquid YEME (complex medium) and NMMP (minimal medium). On solid media, *ΔoxyR* mutant exhibited better growth and sporulation even when media contained various concentrations of H_2O_2. It was postulated that *catA* expression may be elevated in *ΔoxyR* mutant to allow better growth phenotype. The amount of catalase A was determined by activity staining and was found to be higher in the mutant. This suggests that compensatory catalase induction may have conferred ΔoxyR with the better growth phenotype. Moreover, antibiotic actinorhodin was produced more in *ΔoxyR* in both solid SFM and R2YE media and liquid minimal medium. To investigate the regulatory role of OxyR and CatR, their target genes need be unraveled on genome scale, possibly by chromatin immunoprecipitation (ChIP). As an initial step to perform ChIP analysis, tagging of OxyR and CatR with detectable probe was performed. Cloning of 6xMyc tag to the C-terminal end of OxyR and CatR were done. Specific detection of tagged proteins by anti-Myc antibody was successful. Further improvement of detection sensitivity, either by changing the tag or solubility optimization, will be useful for genome-wide detection of direct binding sites of OxyR and CatR.

Key words: *Streptomyces coelicolor,* OxyR, catalase, CatR, myc tagging, oxidative stress, actinorhodin

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List of Abbreviations

aa	amino acid
Act	actinorhodin
$Apra^R$	Apramycin resistant cassette
bp	base pair
H_2O_2	Hydrogen peroxide
kDa	kilo dalton
nt	nucleotide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
Red	Undecylprodiosin
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate

CHAPTER 1.

Backgrounds

1.1 Biology of *Streptomyces coelicolor*

Streptomyces are the most widely studied and well-known genus of actinomycete family. They usually inhabit soil and are important decomposers. *Streptomyces* produces many antibiotics and other classes of biologically active secondary metabolites.

Streptomyces is a Gram positive bacterium with high GC content. It undergoes complex multicellular developmental life cycle which starts from germination of spore and formation of highly branched vegetative mycelium. The hyphae are divided into multigenomic compartments by the infrequent formation of vegetative septa. After a period of active growth, aerial mycelium develops from substrate mycelium on the surface of colony, and eventually differentiates into unigenomic spores. *Streptomyces coelicolor* is genetically the best known representative of the genus. The complete DNA sequence of *S.coelicolor M145* has been published (Bentley et al., 2002).

S. coelicolor produces at least four antibiotics; actinorhodin (Act), undecylprodigiosin (Red), methylenomycin (Mmy) and the Ca^{2+}-dependent antibiotic (CDA) (Hopwwood *et al.*, 1985).

1.2 Oxidative stress responses

Living organisms have developed adaptive systems to cope with changing environmental conditions such as nutrient, temperature, osmolarity, pH, and redox state during cellular growth and maintain cellular homeostasis.

Reactive oxygen species (ROS) can be generated as byproducts of enzymatic reactions during respiration, or by exposure to radiation, stimulated macrophages, or redox-active drugs. Because of their high reactivity, the oxidants can damage cellular components such as DNA, proteins, and cell membrane.

1.2.1 Reactive oxygen species

The molecular oxygen has two unpaired electrons in different pi antibonding orbital, and the two electrons have the same spin quantum number. Because of this spin restriction, molecular oxygen is limited to accept electrons.

Its reactivity increases upon serial one electron reduction of oxygen, or a spin flip to become singlet oxygen ($^1\sum_g O_2$). The resulting oxygen derivatives, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\cdot$), and singlet oxygen are collectively defined as reactive as ROS.

Superoxide radical (O_2^-)

The superoxide radical is generated by one electron transfer to one of the two π* orbitals of of the ground state of oxygen. In *Escherichia coli,* autoxidation of membrane-associated respiratory chain enzymes, such as NADH dehydrogenase, succinate dehydrogenase, and D-lactate dehydrogenase or cytosolic glutathione reductase, are major sources of O_2^- generation *in vivo*. Nonenzymatic production of O_2^- occurs by oxidation of several cellular components including ubiquinols, catechols, thiols, and flavins. Electrophilic quinone compounds, both natural cellular constituents (ubiquinone and plastoquinone) and exogenous sources (plumbagin and menadione), are an important group of substrate for flavor proteins catalyzing either two electron reduction to the hydroquinone or one electron reduction to the semiquinone radical (Bellomo *et al.,* 1990), Semiquinones readily reduce O_2 to O_2^-, regenerating the oxidized quinones. The oxidized quinones can repeat this cycle and it thus referred to as a redox cycling agent.

Superoxide radical can oxidize thiols, ascorbate, tocopherol, and catecholamine (Fridovich *et al.*, 1989). The major target of O_2^- damage identified in bacteria is a class of dehydratese

enzymes that utilize [4Fe-4S] clusters to bind their substrate (Imlay *et al.*, 2003). O_2^- is dismutated to H_2O_2 and O_2, spontaneously or enzymatically by superoxide dismutase (SOD).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is generated by dismutation of O_2^- and by autooxidation of flavoenzymes such as sulfite reductase and xanthine oxidase (Imlay *et al.*, 2003; Messner and Imlay *et al.*, 2002). Some carbon sources autooxidize and thereby contribute to H_2O_2 formation (Seaver and Imlay *et al.*, 2001).

It can act as weak oxidizing agent and oxidize cysteinly residues, creating sulfenic acid adducts that can either from disulfide cross-links with other cysteines or be oxidized further to sulfinic acid moieties (Kiley and Storz *et al.*, 2004). It can also oxidize methionine residues to methionine sulfoxide and a variety of carbonyls (Griffiths and Cooney *et al.*, 2002). However, its more significant action is to generate more reactive $OH^{\cdot}$ Radical by reaction with reduced iron or copper ions by the Fenton reaction (Candenas *et al.*, 1989; Imlay and Fridovich *et al.*,, 1991). Since O_2^- can elicit both H_2O_2 production and reduction of Fe^{3+} and Cu^{3+}, it also enhances $HO^{\cdot}$ generation.

Hydroxyl radical ($HO^{\cdot}$)

The only oxygen species that can directly damage most biomolecules is $HO^{\cdot}$ It is formed when ferrous iron transfers an electron to H_2O_2 (Fenton reaction).

The hydroxyl radical oxidize most organic molecules (RNA, DNA, protein, and lipid) at diffusion-limited rates because $HO^{\cdot}$ has high reactivity due to its very high standard electrode potential (Singh and Singh *et al.*, 1982). While the Fenton reaction has been linked to protein carbonylation and membrane peroxidation, its most significant impact is likely to be upon DNA, since even a single DNA lesion is potentially mutagenic or lethal.

Singlet oxygen ($^1\sum_g O_2$)

Singlet oxygen may be generated from hydroxyl radical by the action of certain enzymes, such as decomposition of superoxide or peroxidized glutathione (GSOO), and photosensitization reactions with endogenous sensitizers such as riboflavin and bile pigments. It can be formed by energy transfer to oxygen by excited chromophores and is generated in photosynthetic system.

Almost all reactions involving singlet oxygen with biomolecules are addition of the $^1O_2$ to conjugated bond. The known targets are carotenes, chlorophylls, and fatty acid side chains present in the lipid membrane, suggesting that $^1O_2$ can initiate lipid peroxidation (Kappus *et al.*, 1985).

1.2.2 Biological defense systems to oxidative stress

Cellular defense systems to counter the deleterious effects of ROS include prevention of ROS generation, detoxification of ROS, and repair of damage. Preventation of generation of ROS can be achieved by chelating metal ions such as iron and copper, which promote generation of free radicals.

Both enzymatic and non-enzymatic systems are involved in antioxidant defense by scavenging ROS. Catalase decomposes H_2O_2 to O_2 and H_2O, while peroxide uses intracellular reductants to reduce H_2O_2.

1.3 Hydrogen peroxide sensing transcription regulators

Bacteria adapt to the presence of ROS by increasing the expression of detoxification enzymes and protein and DNA repair functions. These responses are coordinated by transcription factors that regulate target genes in response to ROS. There are known three classes of bacterial hydrogen peroxide sensing regulators in *S.coelicolor*.

1.3.1 OxyR

OxyR is an H_2O_2- sensing transcriptional regulator of the LysR-family that is generally found in Gram-negative bacteria but is also known to occur in a few Gram-positive bacteria (Morikawa, *et al.,* 2006. Oh *et al.,* 2007). Like other regulators of this family it contains a conserved N-terminal helix-turn-helix DNA binding domain, a central co-inducer recognition and response domain which senses the regulatory signal, and a C-terminal domain that functions in multimerization and activation (Kona, *et al.,* 2006. Kullik, *et al.,* 1995) OxyR as a peroxide sensing global regulator maintains intracellular H_2O_2 levels within safe limits. In *Escherichia coli,* OxyR regulates over 20 genes, including genes involved in H_2O_2 detoxification (*katE, ahpCFvx*), heme biosynthesis (*hemH*), reductant supply (*grxA, gor, trxC*), thiol-disulfide isomerization (*dsbG),* Fe-S center repair (*sufA-E, sufS)* (Li *et al.*, 2004, Zheng, *et al.,* 1998).Even though, there are reasonably large differences, OxyR regulons of other organisms includes similar classes of genes.Under oxidizing conditions OxyR acts as a transcription regulator through directly binding α subunit of RNA polymerase. H_2O_2- sensing of OxyR occurs via direct oxidation at a specific "sensing" cysteine residue. Normal intracellular H_2O_2 level is ~20 nM, OxyR is present in its reduced form when intracellular H_2O_2 levels increase to ~100nM rapid oxidation of OxyR occurs in *E.coli* (Seaver *et al.*, 2001).

In *E.coli,* hydrogen peroxide mediated activation of OxyR constitutes a simple on/off switch that occurs through the formation of a specific disulfide bond between the conserved cysteine residues C199 and C208 (Storz *et al.,* 1990).In *Streptomyces coelicolor, oxyR* gene locates 138 nt upstream from the *ahpC* gene, which is the main defense system against endogenously generated hydrogen peroxide, encodes a protein of 313 amino acids 33,096 Da and shows homology to other known OxyR proteins. Two cysteine residues are also conserved in the *Streptomyces coelicolor* OxyR protein C206 and C215.

Most of the LysR family of transcription regulators autoregulate their own expression as negative regulators, in contrast *S.coelicolor* OxyR positively regulates its own gene expression and *ahpCD* gene expression. One of Prof. Roe Jung Hye laboratory members performed S1 mapping analysis using mRNA results. They found that OxyR positively regulates six more genes. Moreover, *S.coelicolor* OxyR does not regulate the production of antioxidant enzymes such as Ni-containing SOD, Fe-containing SOD, or glucose-6-phosphate dehydrogenase (Hahn, *et al.,* 2002).

1.3.2 CatR

CatR (peroxide sensing transcription repressor) is the first fur homologue characterized as one of peroxide sensing repressors found in both Gram-positive and Gram-negative bacteria (Hahn *et al.,* 2000b). PerR was identified in *Bacillus subtilis* as the major peroxide stress response regulator. PerR regulates major vegetative catalase *(KatA),* alkyl hydroperoxide reductase *(ahpCF), fur, perR* and a zinc uptake system *(zosA)* (Herbig and Helmann *et al.,* 2002)

Streptomyces coelicolor CatR encodes a protein of 138 amino acids, 15319 Da, (Hahn *et al.,* 2000c). Δ*catR* mutant over produces CatA, confirming the role of CatR as a negative regulator of *catA* expression. Moreover, CatR represses its own gene transcription. Transcripts of *catA* and *catR* genes were induced within 10 min of H_2O_2 treatment, this suggests that the repressor activity of CatR may be modulated by H_2O_2 (Figure 1). A putative CatR binding site containing an inverted repeat of 23 base pairs was localized up stream of the *catA* and *catR* genes CatR regulon was induced by metal chelators and it contains at least one zinc. Conserved amino acids predicted for metal binding site were important for regulation of *catR* and *catA* expression whose DNA binding ability is directly modulated by redox changes in the cell (Dr. So young Oh thesis, 2006).

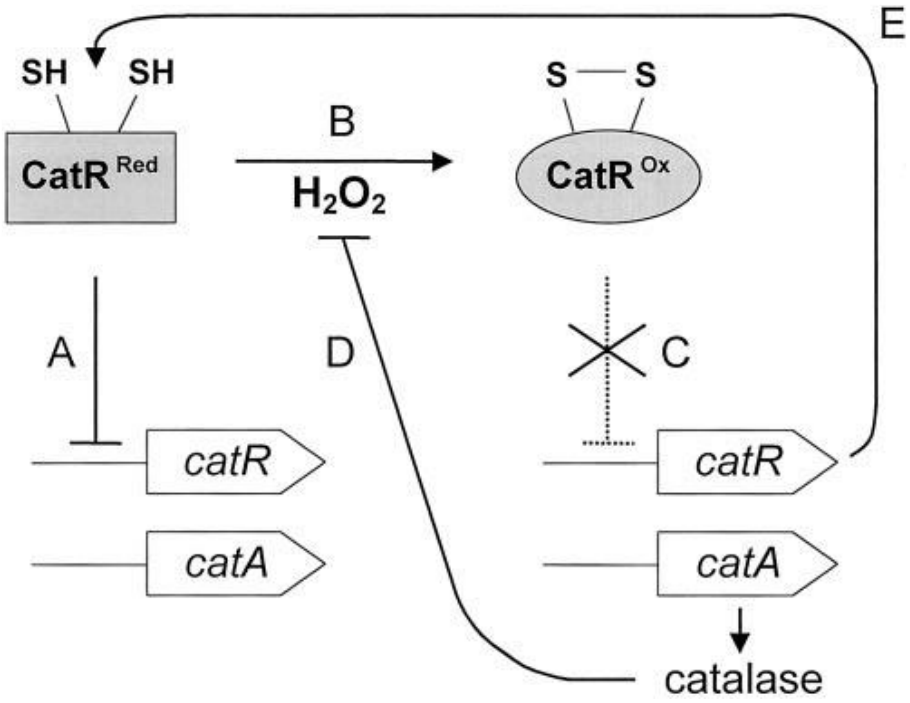


Fig. 1. A model for the rapid H2O2-sensitive regulation by CatR.

Reduced CatR binds to the *catA* and *catR* genes and represses their transcription (*path A*). Upon exposure to H_2O_2, the free cysteine thiols of CatR are oxidized to form disulfide bonds (*B*), causing loss of DNA binding activity and thus derepression of *catA* and *catR* (*C*). The induced catalase removes H_2O_2 (*D*), whereas the induced CatR (coupled with an increase in the proportion of the reduced form as peroxide isremoved) represses both genes (*E*), forming a negative feedback loop(Hahn, *et al.,* 2000b)

1.4 Catalases and peroxidases of *Streptomyces coelicolor*

Catalase plays a crucial role in removing hydrogen peroxide, transfers two electrons to H_2O_2, decomposing it into O_2 and H_2O, while peroxidases uses intracellular reductants to reduce H_2O_2. Regulation of genes for the peroxide-removing system in *S. coelicolor* is achieved by four separate defence enzymes. Alkyl hydroperoxide reductase (AhpC), Catalase A (CatA), Catalase B and Catalase C. Therefore, in *S. coelicolor*, antioxidant genes are regulated by a wider variety of regulators than those observed in other organisms examined so far.

1.4.1 Catalases

Streptomyces coelicolor produces three distinct catalases to cope with oxidative and osmotic stresses and allow proper growth and differentiation. CatA is major catalase which induced by H_2O_2 and its required for efficient aerobic growth (Cho *et al*. 1997) It is regulated by CatR (Hahn *et al*., 2000) *catB* is induced by osmotic stress or at the stationary phase and required for cell differentiation and osmoprotection. (Cho *et al* 2000) while catalase peroxidase (CatC) is expressed transiently at the late exponential to early stationary phase. (Hahn *et al*., 2000b).

CatA is a monofunctional catalase which induced by hydrogen peroxide. It is the main catalase of *Streptomyces coelicolor*. Its gene expression increases during earlier growth phages, but stays constant after mid-exponential growth phase in liquid medium. *catA* gene expression is repressed by reduced CatR but oxidized CatR derepresses *catA* expression (figure 1.).Previous studies suggest that CatA expression is responsible for protection of cells from attack of exogenous H_2O_2.

1.4.1 Peroxidases

Alkyl hydroperoxide reductase system (*ahpC* and *ahpD*) have been isolated from *Streptomyces coelicolor* A3 (2). The *ahpC* and *ahpD* genes constitute an operon transcribed divergently from the *oxyR* gene. Alkyl hydroperoxide reductase (AhpC) is maximally produced during early exponential phase and is induced by exogenous H_2O_2, all under the control of OxyR. The conservation of cysteine residues in the C-X-X-C motif among AhpD proteins from *S. coelicolor* and *Mycobacterium* spp. suggests their function as thioredoxin-like proteins involved in reducing AhpC (Holmgren, A. et al., 1985). Both *ahpCD* and *oxyR* are induced by H_2O_2 in an OxyR-dependent manner.

1.5 Aims of this study

Streptomyces coelicolor crucial in soil because of their broad range of metabolic processes. They are important for medicine because they produces at least four antibiotics; actinorhodin (Act), undecylprodigiosin (Red), methylenomyscin (Mny) and the Ca^{2+} - dependent antibiotic (CDA).It is also a good model system to study the relationship between oxidative stress and differentiation.

Previous studies have been shown that in *Streptomyces coelicolor* OxyR regulates its own gene and *ahpC* gene expression in H_2O_2. And CatR, peroxide sensing repressor, was characterized as a regulator of *catA* and *catR* (Hahn et al., 2000a, b,c; Hahn et al.,2002) Moreover, *catA* transcription levels are higher in *ΔoxyR* in comparison to wild type (Dr. So young Oh thesis 2006).

In order to understand role of peroxide sensing regulators in *Streptomyces coelicolor*.

1-We further examined role of OxyR on growth, morphology, differentiation and antibiotic production in *Streptomyces coelicolor*.

2-Catalase A production of *ΔoxyR* was investigated in comparison with wild type.

3- Positive regulation of some genes by OxyR under treatment of H_2O_2 will be investigated using mRNA sequencing analyses.

4- We aimed to know DNA binding sequence for OxyR and CatR by CHIP analysis. To achieve this goal, we made fusion constructs of *oxyR* and *catR* fused with tags such as 6x myc tagging in *Streptomyces coelicolor*.

CHAPTER 2.

Materials and Methods

2.1 Bacterial strains and culture conditions

Streptomyces and *Escherichia coli* strains used in this study are listed in Table 1.

Streptomyces coelicolor A3 (2) M145 was used as wild type in most studies. *Streptomyces* cells were grown as described previously (Kieser, *et al.,* 2000). For liquid culture, spore suspension was inoculated in YEME medium (1% glucose, 0.5% Bacto Peptone, 0.3% malt extract, 0.3% yeast extract, 10.3% sucrose, 5mM $MgCl_2$), NMMP (0.2% $(NH_4)_2SO_4$, 0.5% Difco casamino acids, 0.06% $MgSO_4.7H_2O$, 5% peg 6000, 15 mM NAH_2PO_4/K_2HPO_4 (pH 6.8), 5% glucose). R2YE (10.3% sucrose, 0.025% K_2SO_4, 1.01% $MgCl_2.6H_2O$, 1% glucose, 0.01% casamino acids, 0.5% yeast extract), NA (0.8% nutrient broth), and SFM (soy flower 2%, mannitol 2%) agar media were used for surface cell growth. Cells were grown in liquid medium by inoculating with spore suspension and incubating at 30°C. *E.coli* strains were grown in LB or SOB. *E. coli* BW25113 was used to propagate the recombination plasmid pIJ790 and *S. coelicolor* cosmids. *E. coli* BT340 carrying pCP20 was used for FLP-mediated site-specific recombination. *E. coli* ET12567/pUZ8002 was the nonmethylating plasmid donor strain for intergeneric conjugation with *S. coelicolor* strain M145. Apramycin (50 μg/mL), chloramphenicol (25 μg/mL) or kanamycin (50 lg/mL) was added to growth media when required.

Table. 1. Strains used in this study

Strains	Genotype of description[a]	Source or reference
***S.coelicolor* A3(2)**		
M145	Prototrophic SCP1$^-$SCP2$^-$Pgl$^+$	Hopwood *et al.*,1985
S12	M145 *catA::*Apra^R	Dr. So Young Oh
S21	M145 *oxyR::*Apra^R	Dr. So Young Oh
S22	M145 *catR::*Apra^R	Dr. So Young Oh
N1	M145 *oxyR 6xmyc::*Apra^R	This study
N2	M145 *oxyR 6xmyc::*Apra^R	This study
N3	M145 *catR 6xmyc::*Apra^R	This study
E.coli		
DH5α	F-*ΔlacU169(Φ*80*lacZ*ΔM15) *endA1 recA1 hsdR17 deoR supE44 thi-1 λ-gyrA96re1A1*	Hanahan, *et al.*, 1983
BW25113	K12 drevative: araBAD, rhaBAD	Datsenko and Wanner, *et al.*, 2000 McNeil et al,.1992
ET12567	F$^-$*dam13::Tn9 dcm6 hsdM hsdR recF143::Tn10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 HİSg4 tsx-78 mtl-1 glnV44*	McNeil et al., 1992

Table.2. Plasmids used in this study Source or reference

	Genotype of description[a]	Source or reference
2st3B6	Cosmid containing *catR* gene cluster	John Innes in UK
SCK7.06	Cosmid containing *oxyR* gene cluster	John Innes in UK
pIJ790	oriR101, repA101^{ts}, RED (araBp-*gam-bet-exo),cat,araC*	Gust, *et al.*, 2003
pIJ773	*AAC(3)IV (*Apr^{R}),*oriT*,Amp^{R}	Gust *et al.*,,2003
pJN1	pUC18:(n)-myc contains FRT sites and Apr^{r} cassette	Ji Nu Kim *et al.*,,2012

Table.3 Primers used in this study

Primer	Sequences	Note*
OxyR myc F	CAGGCGATGGCGGACCTGCCGGTGCGGACGGT GCACGACGAGCTCGAGGAGCAGAAG	For 6xmyc tagging
OxyR myc R	GGACCGAGGGACCGCCCGGCCGGGTGGTCGCC CGGTCCCATTCCGGGGATCCGTCGACC	For 6xmyc tagging
CatR myc F	GTGACGTACCGCGGCACCTGCCCGAACTGCGCG GCGGCGGAGCTCGAGGAGCAGAAG	For 6xmyc tagging
CatR myc R	AACACTACGTCACGATGAAGACGTGAGGCAAA TCCCTGCATTCCGGGGATCCGTCGACC	For 6xmyc tagging
6xmyc dn R	AGCCTACAGGATCCTCATCA	Tagging check
Frt F	CCAACGGCGTCAGCCGGGCAGG	Tagging check
oxyR Mid F	CGCTGGTACAGCTGGTGGCG	Tagging check
oxyR dn R	GTGCCCGACCGGGGCACCGA	Tagging check
6xmyc F	GGCTGGAGCTGCTTCGAA	Tagging check
CatR mid F	GCTGGTCTCGCTCGGCGAGG	Tagging check
CatR dn R	GCGAGCTACCGAGCTGCTCC	Tagging check
AprR	CGCTCGTCATGCCCTCGTGG	Tagging check

*: the purpose of the primers

2.2 General recombinant DNA techniques

General techniques for isolation and manipulation of DNA in *Streptomyces* and *E.coli* were described as previously (Hopwood et al., Sambrook et al., 1989). All plasmids used in this study are listed in Table II-2.

2.2.1 Transformation of *E.coli*

Introduction of DNA into *E.coli* was done by chemical method and electroporation method using gene pulser (BioRad).

2.2.2 Conjugation between *E.coli* and *S.coelicolor*

Conjugal transfer of DNA from *E.coli* to *S.coelicolor* was carried out by a modification of the method of Mazodier et al. (1989). Competent cells of ET12567 containing Puz8002 were prepared under chloramphenicol (12.5 μg/ml) and kanamycin (50 μg/ml) selection and were transformed with oriT-containing conjugation vector selecting for incoming vector only.

2.2.3 PCR-based tandem epitope tagging system for *Streptomyces coelicolor* genome

PUC18 plasmid(pJN1) which contains the flanking FRT sites,6X myc sequence and apramycin resistance gene was provided by Ji-Nu Kim. Linear DNA fragments were amplified using pairs of primers which were 59-bp in length with 39-bp homology extensions overlapping upstream and downstream from stop codon of target genes and 20-bp priming sequences from pJN1 template plasmid series. PCR was carried out as which described in Ji-Nu Kim *at al.,* (2012). Each PCR product was purified, digested with DpnI, repurified, and then electroporated into *E. coli* strain harboring pIJ790 (which expresses the λ Red recombination system under the control of an inducible promoter) and *S. coelicolor* cosmid (which contains a genomic region of interest). The cells were then incubated at 37 ^{o}C for 1 h in 1 mL of LB and spread onto LB-agar medium supplemented with apramycin. The myc-

inserted cosmid was transported into the methylation-deficient *E. coli* strain ET12567 then transferred *S.coelicolor* by conjugation. Single-crossover exconjugants were selected on SFM containing kanamycin and nalidixic acid, to obtain transconjugants. The genomic DNA was then isolated and plasmid integration was confirmed by PCR. 6xmyc tagging strategy is described in figure 2. Primers used in this study are listed in Table II-3.

2.3. DNA analysis

2.3.1 Polymerase chain reaction (PCR)

Each 100 µl of reaction mixture contained the followings; 10 x taq polymerase reaction buffer, 150 µM $MgCl_2$,200 µM dNTP, 5% DMSO, 1 µM of each 5` and 3` primers, 100 ng of template DNA and 5 U of Taq polymerase. Reaction was carried out in Perkin-Elmer thermocycler by denaturation at 94°C for 2 min, annealing at 50-55°C for 45 sec and extending at 72°C for 1 min 30 sec.

2.3.2 DNA sequencing

DNA sequencing was done automatically using automated sequencer (ALF Express, Pharmacia). Deaza-dNTPs were used to minimize sequencing artifacts due to the formation of secondary structure.

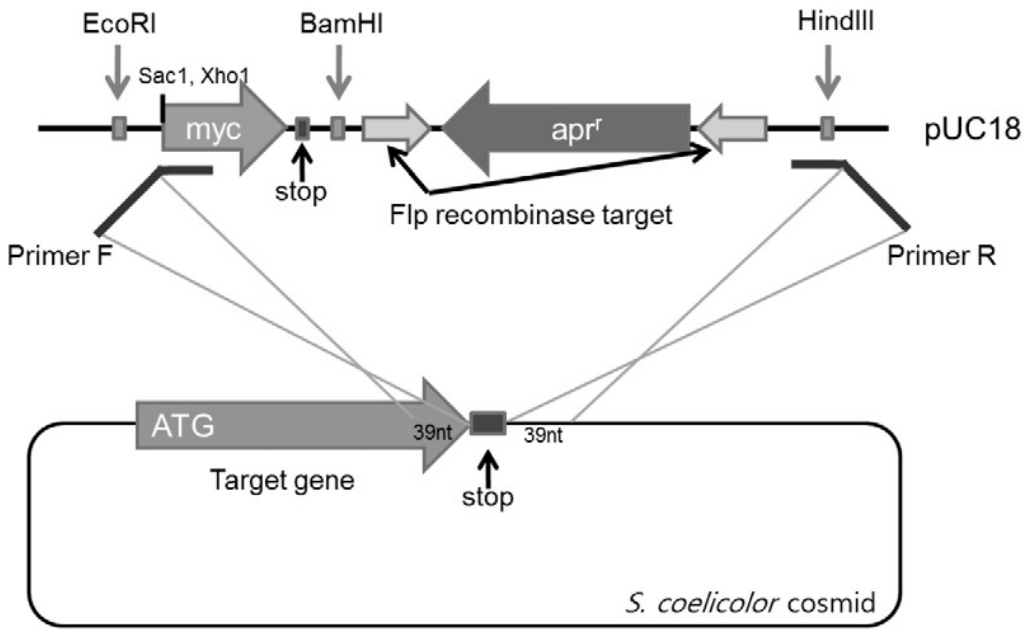
Figure 2. Scheme of 6xmyc tagging strategy.

A. PUC18 plasmid that involves tandem myc sequence and the antibiotic resistance marker (apr^r) is amplified with primers carrying extensions homologous to the upstream and downstream of the translation stop codon of target gene.

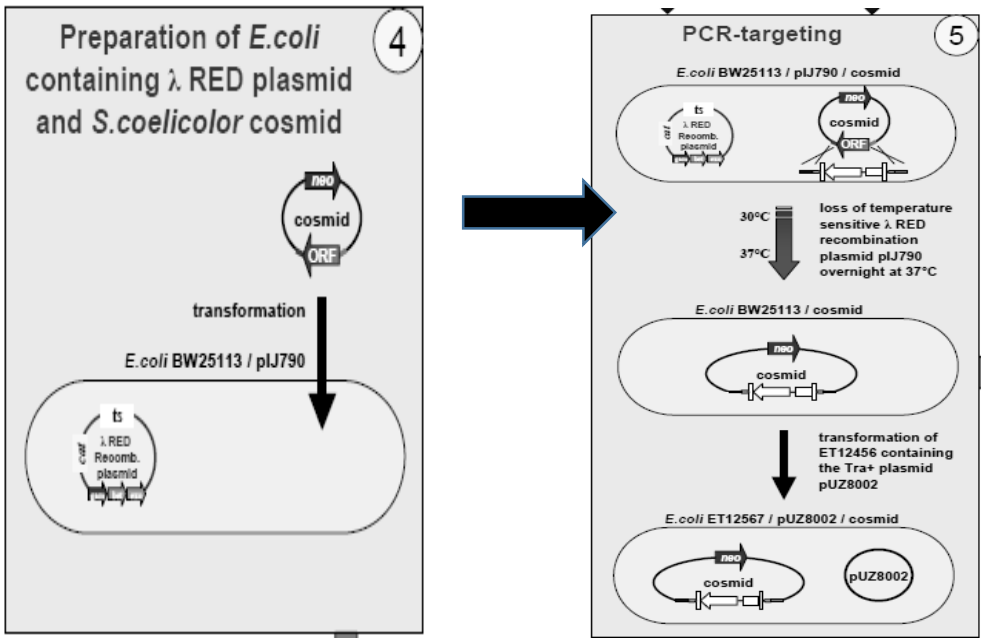
B. *Streptomyces coelicolor* cosmid containing target gene was introduced into *E.coli* BW25113/pIJ790 (contains resistance marker *cat* (chloramphenicol resistance) and a temperature sensitive origin of replication (requires 30°C for replication)). Presence of cosmid in competence cell confirmed by PCR using primers at table II. Then, PCR product of (A) was transformed to *E.coli* containing cosmid with target gene. Again, genomic tagging was confirmed by PCR using control primers. Then, Cosmid DNA of transformants was isolated and it was introduced by transformation into the non-methylating *E.coli* ET12567 containing RP4 derivative Puz8002.

C. The cosmid was transferred to *Streptomyces coelicolor* by intergeneric conjugation. Single-crossover exconjugants were selected on SFM containing kanamycin and nalidixic acid, to obtain transconjugants. The genomic DNA was then isolated and plasmid integration was confirmed by PCR.

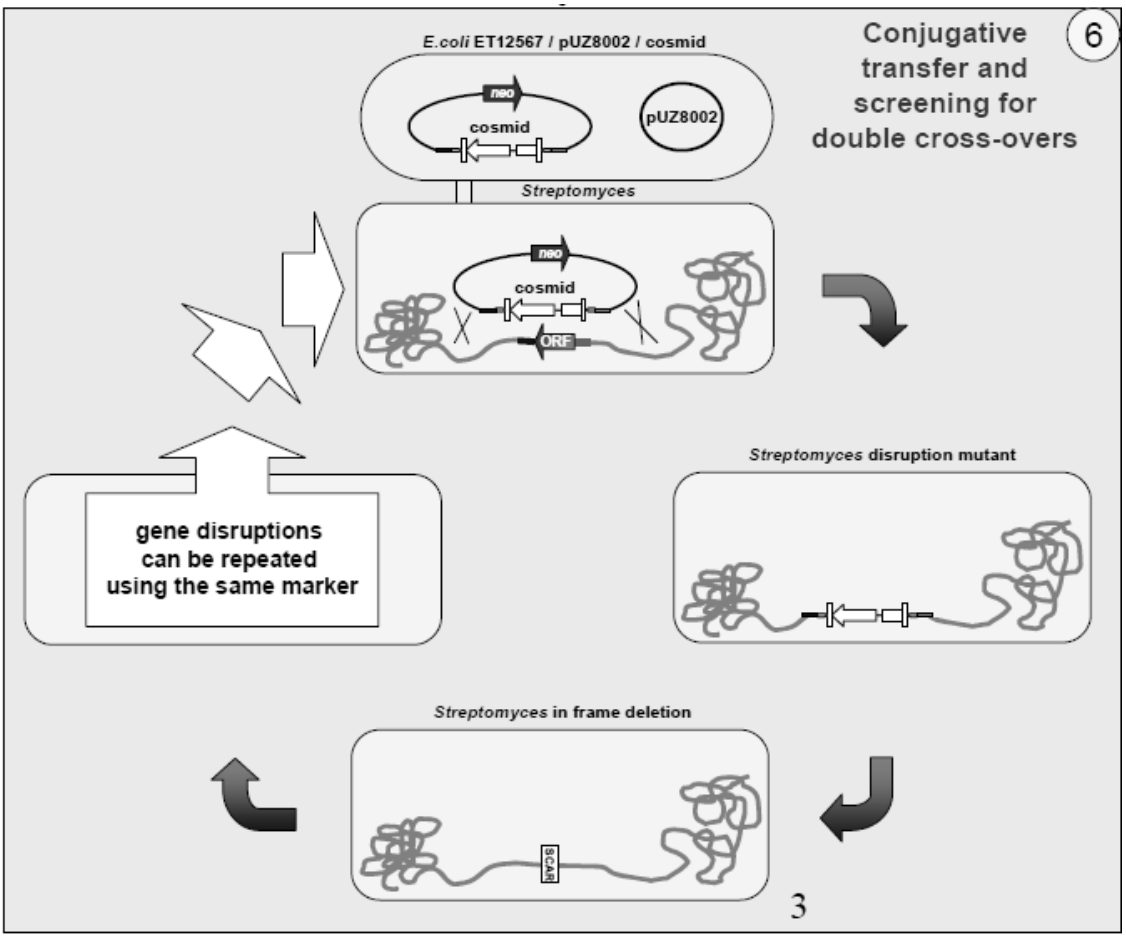
A


EcoRI
BamHI
HindIII
Sac1, Xho1
myc
stop
aprr
pUC18
Flp recombinase target
Primer F
Primer R
ATG
39nt
39nt
Target gene
stop
S. coelicolor cosmid

B


Preparation of E.coli containing λ RED plasmid and S.coelicolor cosmid
4
neo
cosmid
ORF
transformation
E.coli BW25113 / pIJ790
ts
cat
λ RED Recomb. plasmid
PCR-targeting
5
E.coli BW25113 / pIJ790 / cosmid
30°C
37°C
loss of temperature sensitive λ RED recombination plasmid pIJ790 overnight at 37°C
E.coli BW25113 / cosmid
transformation of ET12456 containing the Tra+ plasmid pUZ8002
E.coli ET12567 / pUZ8002 / cosmid
pUZ8002

C



Gust et al, .2003. PCR targeting system in *Streptomyces coelicolor*.

2.4. Protein analysis

2.4.1. Preparation of cell extracts

Harvested cells were resusupended in 50 mM potassium phophate buffer (pH6.8) and disrupted by sonication with ultrasonicator (Sonics and Materials Inc.). The suspension was centrifuged and the protein concentration of soluble supernatant was determined using Bradford reagent (Bio-Rad) and BSA as a standard protein.

2.4.2 Western blot analysis

Following SDS PAGE, the gel was soaked in transfer buffer [25 Mm Tris, 192 mM glycine, 20% (v/v) methanol] for 10 min, and then electrotransferred to PVDF (Roche applied sciences) membrane at 180 Ma for 1.5 hr in Trans-Blot Cell (Biorad). Membrane was blocked in Tris-buffred saline buffer containing 0.5% Triton X-100 (TBST) supplemented with 5% skimmed milk,for 1 hour at room temprature or over night at 4°C. The blocked membrane was incubated with primary antibody for 1 hr and then membrane was washed 3 times for 10 min with TBST. Washed membrane was incubated with anti-mouse IgG secondary antibody 1:10,000 diluted in TBST, and washed with TBST for 10 min 3 times. Detection of signal was done using Western ECL detection system.

2.4.3. Catalase activity staining

Native catalases are electrophoretically separated on 7 % polyacrylamide gel. Staining for catalase activity was done by the method of Kim *et al.,* (1994): the gel was soaked for 45 min in 50 mM potassium phosphate buffer (pH 7.0) containing 50 mg horseradish peroxidase ml^{-1}, followed by addition of 5 mM H_2O_2 for 10 min. The gel was washed twice with distilled water, and then was soaked in 50mM potassium phosphate buffer (pH 7.0) containing 0.5 mg

diaminobenzidine ml^{-1} to develop the background brown colour. No colour would appear in the area where catalase depleted H_2O_2.

2.5.4 Quantification of actinorhodin (Act) and undecylprodigiosin (Red)

10^6 spores of *ΔoxyR* and wild type were inoculated in NMMP (liquid media) and incubated at 30°C with shaking at 180 rpm. Cells were harvested daily for 4 days to measure the content of Act and Red as described previously (Shin et al.,2010). For Act, each 1 ml sample was first treated with 50 µl of 10 N NaOH, gently vortexed and allowed to stand for 5 min, followed by centrifugation at 4,000 × *g* for 5 min. The absorption spectra of supernatants were monitored by UV/VIS spectrophotometer (SHIMADZU model UV- 1650PC). The absorbance at 608 nm was taken to calculate the concentration of actinorhodin based on the extinction coefficient of 25,320 M^{-1} cm^{-1} Undecylprodigiosin (Red) was extracted similarly except that the cell mass was resuspended with 100% methanol and acidified with 10N HCl. The absorbance at 530 nm was taken to calculate its concentration based on the extinction coefficient of 100,500 M^{-1} cm^{-1}.

CHAPTER 3.

Results

3.1 Physiological growth of *ΔOxyR* in comparison with *M145* wild type

In order to further explore the function of deletion of *oxyR* on growth, differentiation and antibiotic production of *Streptomyces coelicolor*. We grew *ΔOxyR* and *M145* wild type on solid media such as NA (nutrient agar), SFM (soy flour mannitol), R2YE and liquid media like YEME, and liquid minimal medium(NMMP) with or without under the various stresses.

3.1.1 Growth comparison in liquid medium

ΔoxyR and M145 wild type strain spores were taken from lab stock. 10^6/µl spores of these two strains were inoculated into YEME (rich medium) and NMMP (minimal medium) containing glucose as carbon source. Then, growth was started to measure spectrofotomectrically after 13 hour incubation at 30°C at OD_{600}. In comparison with M145 wild type *ΔoxyR* mutant grows better in both YEME and NMMP mediums (Figure 3).

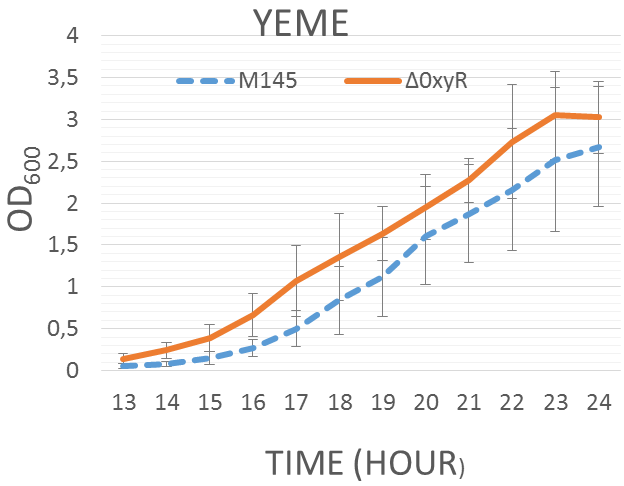
3.1.2 Growth comparison on solid medium

ΔoxyR and M145 wild type strains were tested with various stresses. Such as H_2O_2, tBHP (*tert*- butyl hydro peroxide) which is an organic hydro peroxide, diamide (DA) which is a thiol-specific oxidation agent. NaCl, EtOH, paraquat (PQ) which is often used to catalyze the formation of reactive oxygen species (ROS), more specifically, the superoxide free radical. Paraquat will undergo redox cycling *in vivo*, being reduced by an electron donor such as NADPH, before being oxidized by an electron receptor such as dioxygen to produce superoxide. Different concentration of spores were spotted on NA plates. On NA plate including 200 µM H_2O_2 *ΔoxyR* mutant showed better growth comparing to wild type contrary to our expectations. Under 500 µM diamide (DA) and 100 µM tBHP stress also ΔoxyR mutant showed better growth. On the other hand, under 4% EtOH, 200 µM NaCl and 100 µM PQ stresses wild type M145 and mutant exhibited similar growth (Figure 4).

We wanted to further examine the growth of *ΔoxyR* mutant under various concentrations of H_2O_2 on NA gradient plates. Gradient plates showed graded inhibition of both wild type and *ΔoxyR*. Wild type strain is more sensitive to hydrogen peroxide (Figure 5).

Morphology of M145 wild type, *ΔoxyR* mutant strains on various concentrations of hydrogen peroxide was studied. Spores were streaked and observed on NA and SFM plate. SFM stimulates sporulation of *Streptomyces coelicolor*. The mutant strain showed slightly more antibiotic production on NA plate and exhibited faster spore formation on SFM plates (Figure 6).

A.



B.

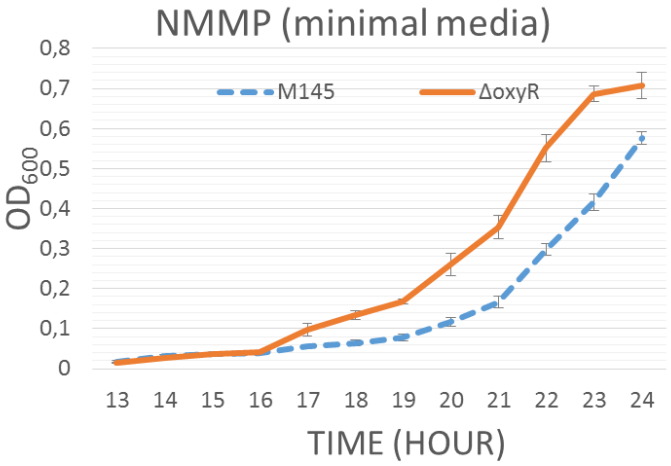


Fig. 3. Growth check in YEME and NMMP liquid media.

Equal numbers of spores were inoculated in 50 ml YEME and NMMP liquid media and O.D was measured during 12 hours after 12 hours from inoculation.

*These figures represents 3 independent experiments

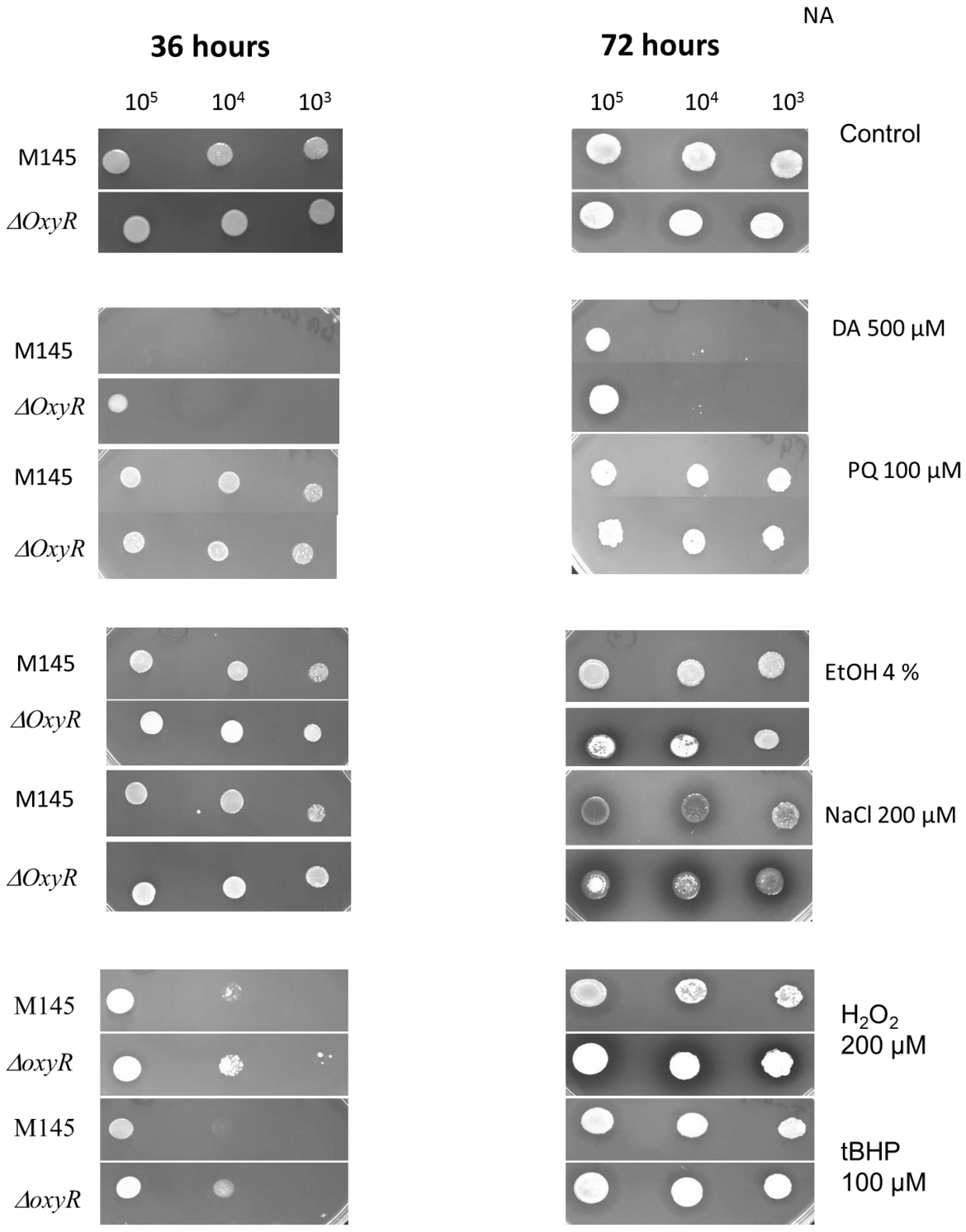


Fig.4. Sensitivity test for wild type and, *ΔoxyR* mutant strains to various stresses.

Wild type and mutant strain spores were equally spotted on each plate with 200 µM H_2O_2, 100 µM tBHP, 200 µM NaCl, 500 µM DA, 100 µM PQ and 4% EtOH. Pictures were taken at 36 and 96 hrs.

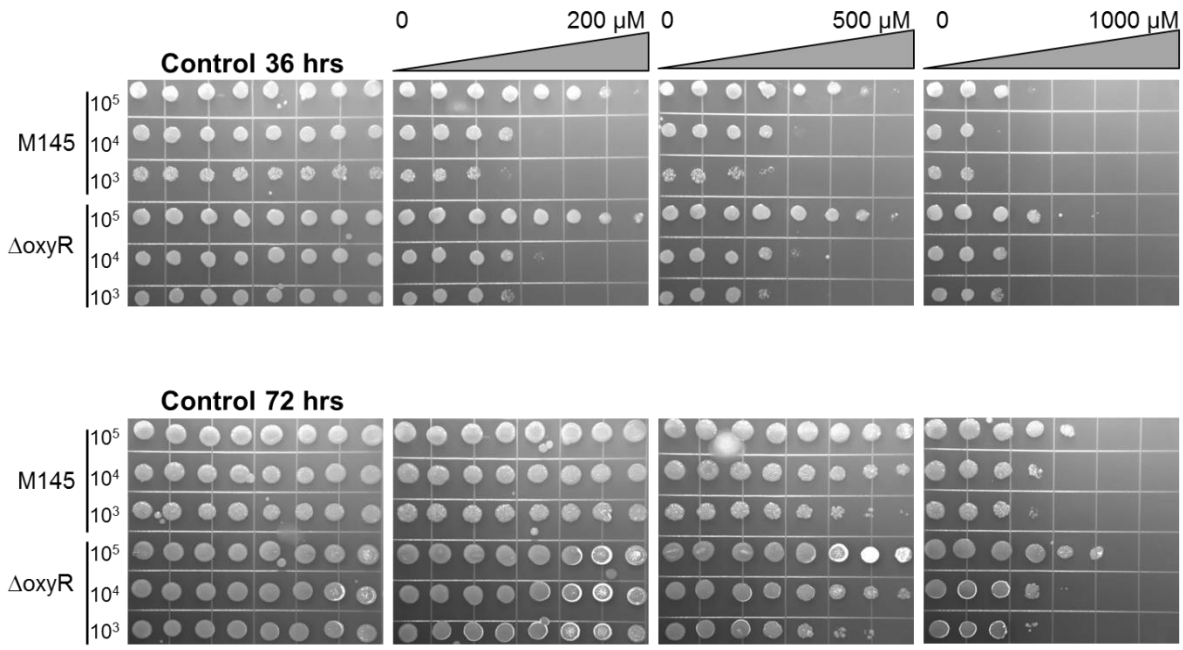


Fig.5. H_2O_2 sensitivity test for wild type and *ΔoxyR* mutant strains.

Wild type and *ΔoxyR* mutant strain spores were equally spotted on NA plate with H_2O_2 and a non- H_2O_2 added NA plate as a control. H_2O_2 concentration increases to the right-hand side with 200, 500 and 1000 µM. Pictures were taken at 36 and 72 hrs. M145- wild type strain.

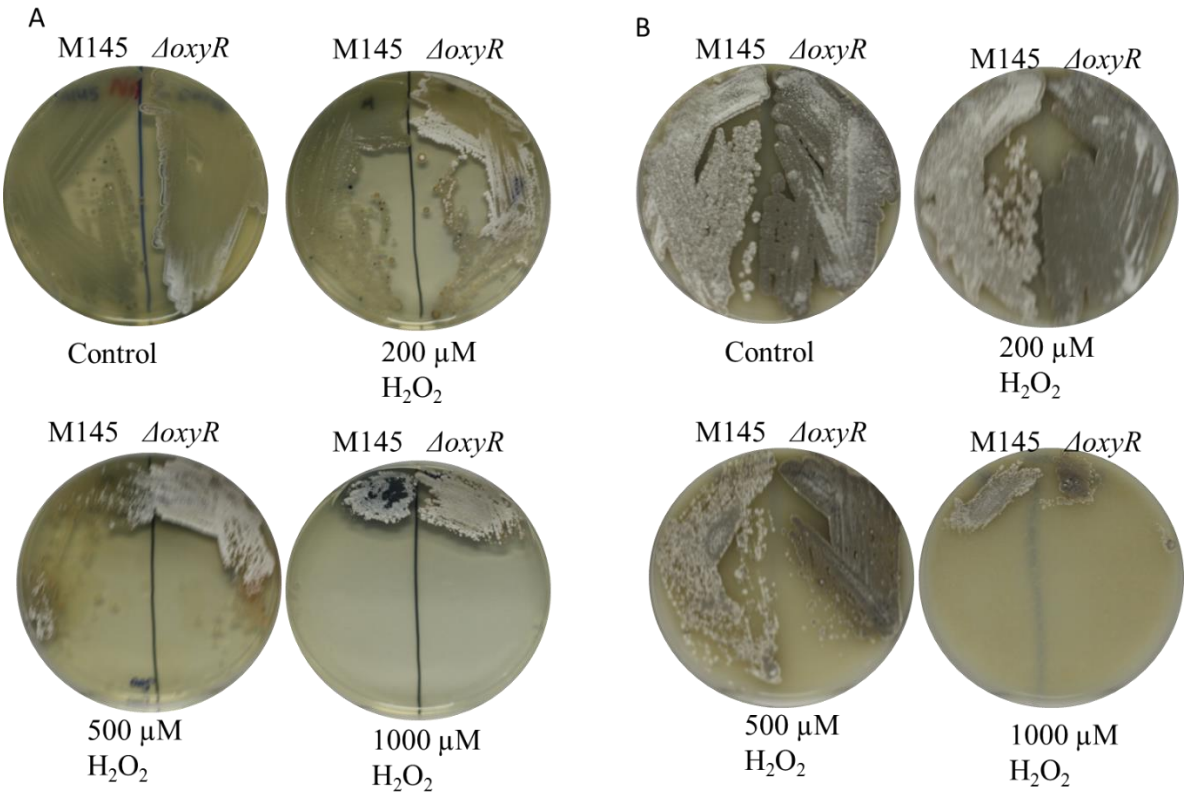


Fig.6. Growth and sporulation of wild type and *ΔoxyR* mutant strains under hydrogen peroxide stress.

A. Wild type and *ΔoxyR* mutant strain spores were streaked on NA plate with or without various concentrations of H_2O_2.

B. Wild type and *ΔoxyR* mutant strain spores were streaked on SFM plate with or without various concentrations of H_2O_2 for sporulation. Pictures were taken at 4^{th} day.

3.1.3 Catalase A production in *ΔoxyR* mutant

Hydrogen peroxide which is generated during aerobic metabolism and also can damage critical biomolecules. *Streptomyces coelicolor* has three kind of known transcription regulation systems to control expression of oxidative defense enzymes. OxyR activates the alkyl hydro peroxide reductase (ahpCD) (Hahn et al., 2002), CatR, a peroxide responsive fur homologue, represses major catalase (CatA)(Hahn et al, 2000b) and RsrA is an anti-sigma factor for SigR, which directs the expression of thioredoxin genes in response to high concentration of H_2O_2 and disulfide stress.

It has been reported that AhpCD is responsible for detoxification of low concentration of H_2O_2 endogenously formed while CatA play an important role in protection from higher concentration of H_2O_2 or in aerial mycelium stage in which AhpCD levels are low. Moreover, in Δ*oxyR* mutant as expected, *ahpCD* expression levels are reduced. On the other hand, *catA* expression levels are high comparing to wild type strain in *Streptomyces coelicolor (*Dr. So young Oh thesis, 2006).

According to our physiological studies, we observed that Δ*oxyR* mutant grows and shows better sporulation than wild type strain on NA and SFM plates with or without treatment of various concentrations of H_2O_2. Then, we hypotised that catalase levels are high in Δ*oxyR* mutant which makes mutant strain more resistance to peroxide and rescue cells from growth defect.

First of all, based on the S1 mapping data which suggested that in Δ*oxyR* mutant *catA* transcription levels are higher than wild type without any treatment of H_2O_2. Then, we further confirmed by catalase activity staining that catalase A activity is elevated in Δ*oxyR* mutant with or without under stress of hydrogen peroxide (Figure 7).

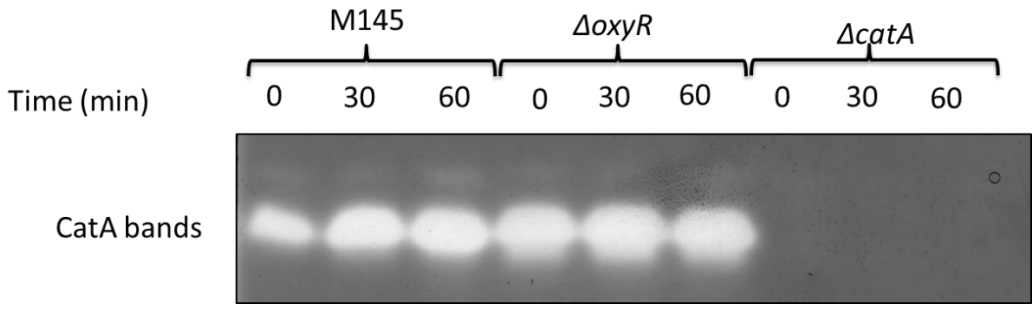


Fig.7. Comparison of catalase A activity of wild type and *ΔoxyR* mutant strains.

Streptomyces coelicolor wild type, *ΔoxyR* and *ΔcatA* cells were grown to exponential phase in YEME and treated with 100 µM H_2O_2. Samples were taken at 30 min intervals over 60 min and 20 µg of crude extract loaded to 7 % native gel the catalase activity staining was done. Catalse A depleted more area on native gel in *ΔoxyR* with or without treatment of hydrogen peroxide comparing to wild type confirming that catalase A levels are higher in *ΔoxyR*. *ΔcatA* was used as negative control proving that bands are CatA bands.

*This figure represents 3 independent experiments.

3.1.4 Antibiotic production in *ΔoxyR* mutant

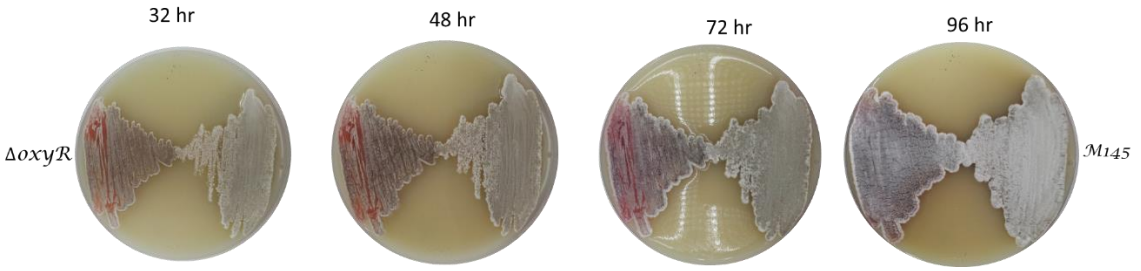
Actinorhodin (Act) and undecylprodigiıosin (Red) are well known antibiotics that *Streptomyces coelicolor* produces. ActII-orf4 and RedD were identified as pathway-specific regulators involved in the production of representative antibiotics, ACT and RED, respectively (Takano et al. 1992). Many regulators are involved in antibiotic production.

We have studied antibiotic production of *ΔoxyR* in comparison with wild type.

On solid media such as SFM and R2YE, *oxyR* deletion strain displayed increasing amount of antibiotic production (Figure 8).

We inoculated same amount of *ΔoxyR* and M145 wild type spore in minimal media (NMMP) containing 5 % glucose as carbon source.Then, quantify the antibiotic production as described in Shin et al, 2011. In NMMP, actinorhodin production was enhanced in oxyR deletion mutant. On the other hand, *ΔoxyR* and M145 wild type strains showed similar amount of Red production (Figure 9).

A.



B.

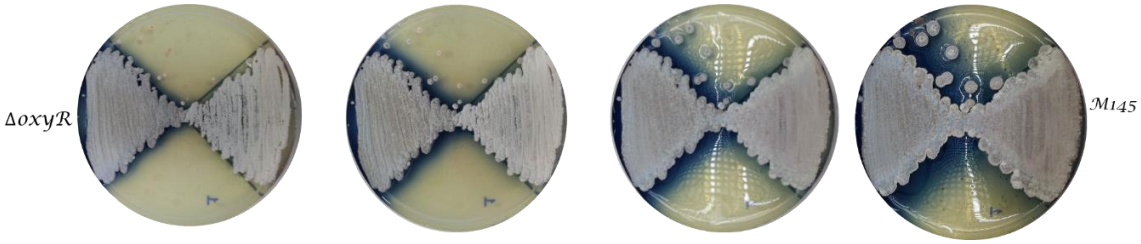


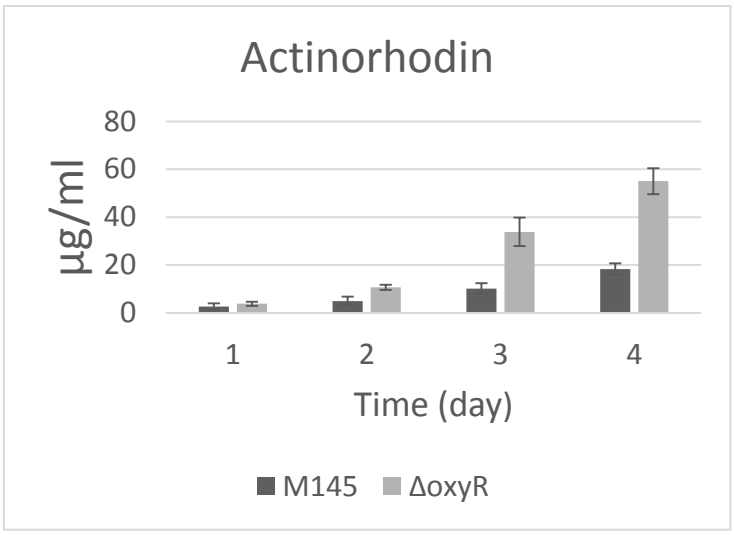
Fig.8. Antibiotic production comparison on solid media.

A. ΔoxyR and M145 wild type spores were streaked to SFM plate.

B. ΔoxyR and M145 wild type spores were streaked to R2YE plate.

Formation of aerial mycelia, spores, and pigmented antibiotics was examined visually by taking photos at indicated time points.

A.



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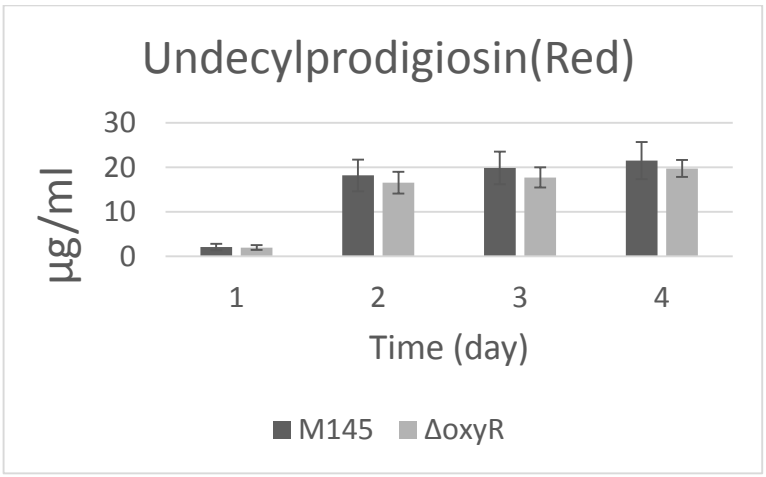


Fig.9. Actinorhodin and Undecylprodigiosin production of *ΔoxyR* in minimal liquid media.

10^6 spore of ΔoxyR and M145 wild type were inoculated in NMMP following incubation at 30°C for five days. Quantification started to be done the day after inoculation and quantify for 4 days.

3.2 PCR-based tandem epitope tagging for CHIP

Streptomyces has complex regulatory systems at transcription and translation levels for sensing and signal transduction to adapt a wide range of nutritional and environmental conditions. OxyR is a peroxide sensing transcription regulator in *Streptomyces coelicolor*. Prof. Roe Jung Hye laboratory has confirmed some new positive target genes for OxyR then we aimed to learn binding sequence for this protein to further study of this transciption regulator. Moreover, we also have tagged other peroxide sensing regulator CatR. Ji-Nu Kim *at al.*,2012 developed a versatile PCR-based tandem epitope tagging for *Streptomyces coelicolor* genome. They combined tagging system with chromatin immunoprecipitation (CHIP). CHIP experiments require antibodies which are highly specific against the target proteins. On the other hand, our anti-OxyR antibody is not specific to OxyR protein. Because of that we tagged our genes of interest by following their procedure.

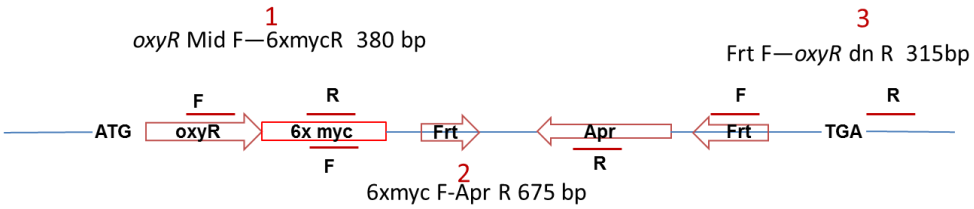
The PCR-based tagging strategy applied here starts with amplifying a DNA segment, which begins with the tandem epitope sequence followed by a drug-resistance gene flanked by FRT sites. In addition, the amplifiable segment has homologous sequences to the last portion and to a downstream region of the targeted gene. The precise insertion of the DNA segment into the cosmid containing the target gene was achieved by electroporating the PCR-amplified DNA segment into *E. coli* BW25113/pIJ790 containing the cosmid followed by λ-Red-mediated recombination. The epitope-inserted cosmid was then transported into the methylation-deficient *E. coli* (ET12567/pUZ8002) and transferred to *S. coelicolor M145* by conjugation. (Gust *at al.*, 2003).

3.2.1 6xmyc tagging of *oxyR* in *Streptomyces coelicolor*

6xmyc tagging of to *S. coelicolor oxyR* has been done as explained at experimental part. Genomic tagging needed to be confirmed by PCR however, for *S. coelicolor* genomic DNA PCR getting whole sequence PCR product is really difficult to overcome this problem we designed 3 pairs of primers (Figure 10-A). Then, genomic DNA has been extracted from each tagged strain as a PCR template. PCR has been carried out using 3 different pairs of primers (Figure 10-B). After confirmation of 6xmyc tagging by PCR, PCR products have been sent to sequencing. DNA sequencing confirmed that tagging was successful. 6xmyc tagging of *oxyR* has been validated by Western blot using anti-myc antibody (Figure 11-A, B).

Ji-Nu Kim *at al.*, 2012 suggests that some epitope-fused proteins lose their in vivo functions. Phenotype of target proteins need to be compared with those of wild type. For comparison we streaked 6xmyc tagged *oxyR*, M145 wild type and *ΔoxyR* mutant strains on to SFM plate. We observed that the tagged strains show wild type behavior as expected (Figure 12).

A.



B.

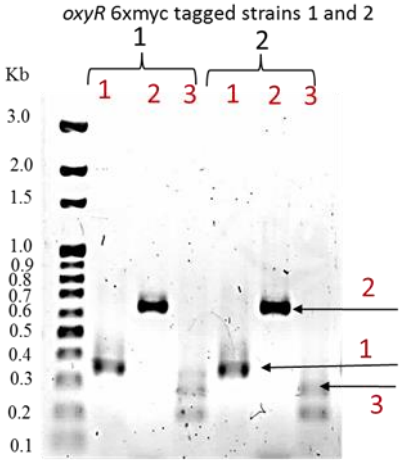


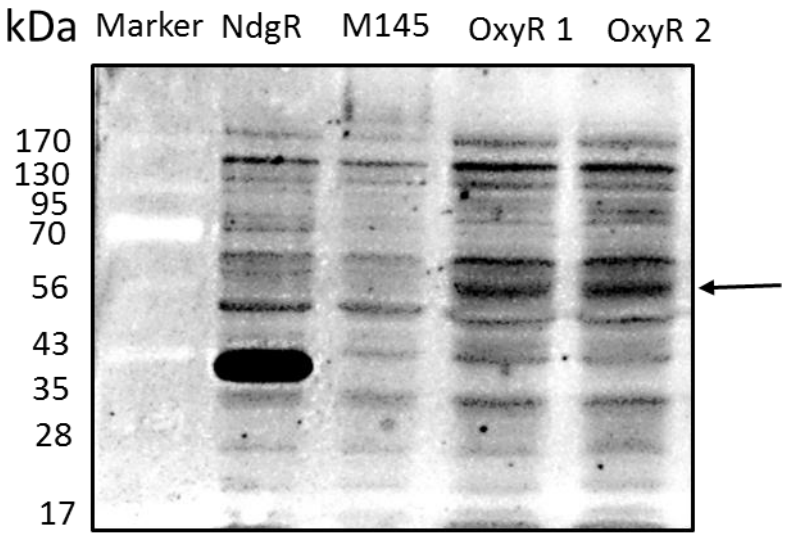
Fig.10. Confirmation of 6xmyc tagging of *oxyR* in *Streptomyces coelicolor* by PCR.

A.Primer design for PCR confirmation of 6xmyc tagging.

Three pairs of primers were designed. First pair forward primer is in *oxyR* ORF, reverse primer homologue to 6xmyc sequence. Second primer pair's forward primer homologues to 6xmyc and reverse primer homologues to Apr^r cassette. Third pair forward primer homologues to FRT cassette and reverse primer homologues to downstream of *oxyR* gene.

B. PCR confirmation of 6xmyc tagging.Genomic DNA of two different tagged strain were amplified using three pairs of primers. First primer pair product is about 380 bp. Second primer product is 675 bp and finally third pair primer product is 315 bp. PCR confirmed that tagging was successful.

A.



B.

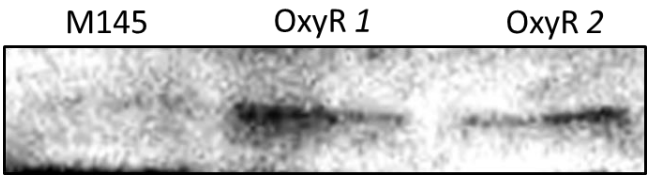


Fig.11. Confirmation of 6xmyc tagging *S.coelicolor* oxyR by western blot.

A. 6xmyc tagged *S.coelicolor* NdgR which is a gift from Ji Nu Kim was used as positive control for myc antibody specificity.Expected band size for NdgR is 32.2 kDa (25 kDa NdgR+7 kDa 6xmyc). M145 wild type was used as negative control.Expected western band size for OxyR 6xmyc tag is 40.2 kDa (33 kDa OxyR+7 kDa 6xmyc). *Arrow shows expected specific bands for 6xmyc tagged OxyR.

B. Same amount of crude extract were subjected to 10% SDS-PAGE gel.Then, we reconfirmed 6xmyc tagging of OxyR with an another independent western blot experiment which shows specific bands.

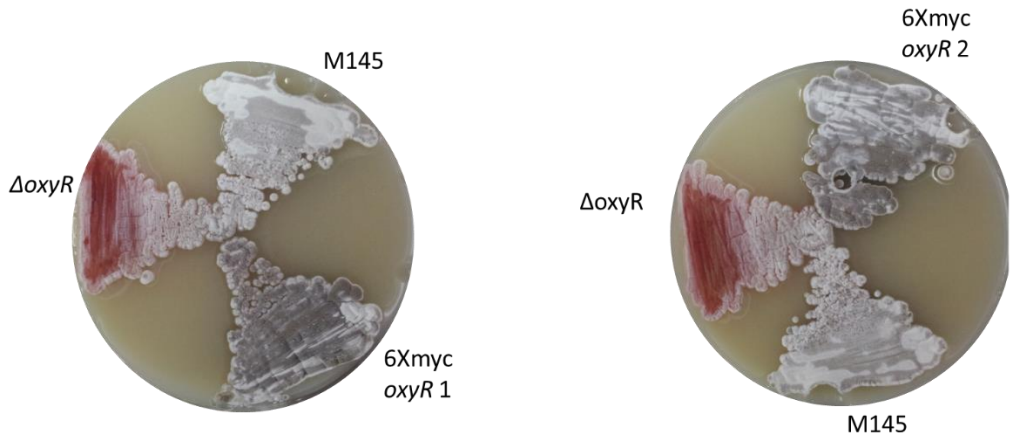


Fig. 12. Phenotype of 6xmyc tagged *oxyR* strains 1 and 2.

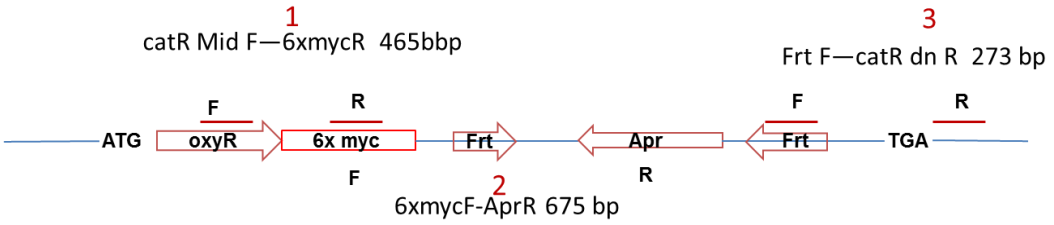
M145 wild type, *ΔoxyR* and 6xmyc tagged *oxyR* strains were streaked on to SFM plate for comparison of phenotypes.6xmyc tagged *oxyR* strains produce spores and antibiotic like wild type but *oxyR* mutant does not.

3.2.1 6xmyc tagging of *catR* in *Streptomyces coelicolor*

6xmyc tagging of to *S. coelicolor catR* has been done as explained at experimental part. Genomic tagging needed to be confirmed by PCR however, for *S. coelicolor* genomic DNA PCR getting whole sequence PCR product was hard to overcome this problem we designed 3 pairs of primers for (Figure 13-A). Then, genomic DNA has been extracted from each tagged strain as a PCR template. PCR has been carried out using 3 different pairs of primers (Figure 13-B). After confirmation of 6xmyc tagging by PCR, PCR products have been sent to sequencing. DNA sequencing confirmed that tagging was successful. 6xmyc tagging of *catR* has been validated by Western blot using anti-myc antibody (Figure 14).

Ji-Nu Kim *at al.*, 2012 suggests that some epitope-fused proteins lose their in vivo functions. Phenotype of target proteins need to be compared with those of wild type. For comparison we streaked 6xmyc tagged *catR*, M145 wild type and *ΔcatR* mutant strains on to R2YE plate. We observed that the tagged strain shows wild type behavior as expected (Figure 15).

A.



B.

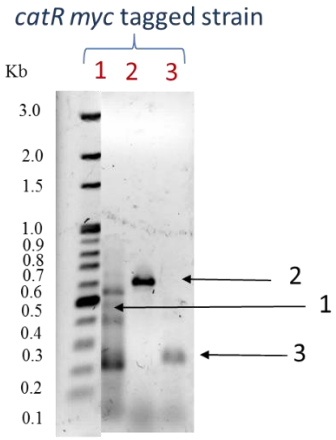


Fig.13. Confirmation of 6xmyc tagging of *catR* in *Streptomyces coelicolor* by PCR.

A.Primer design for PCR confirmation of 6xmyc tagging.Three pairs of primers were designed. First pair forward primer is in *catR* ORF, reverse primer homologue to 6xmyc sequence. Second primer pair's forward primer homologues to 6xmyc and reverse primer homologues to Apr^r cassette. Third pair forward primer homologues to FRT cassette and reverse primer hologues to downstream of *catR* gene.

B. PCR confirmation of 6xmyc tagging.Genomic DNA of tagged strain was amplified using three pairs of primers. First primer pair product is about 465 bp. Second primer product is 675 bp and finally third pair primer product is 278 bp. PCR was confirmed that tagging was successful.

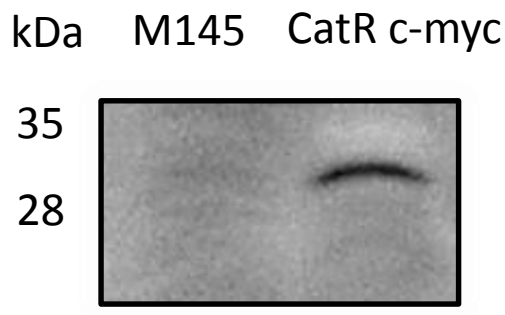


Fig.14. Confirmation of 6xmyc tagging *S.coelicolor* catR by western blot.

Same amount of crude extract were subjected to 13% SDS-PAGE gel.Then, we confirmed 6xmyc tagging of CatR by western blot experiment which shows specific bands. M145 wild type was used as negative control.Expected western band size for CatR 6xmyc tag is 22..2 kDa (15.2 kDa CatR+7 kDa 6xmyc).

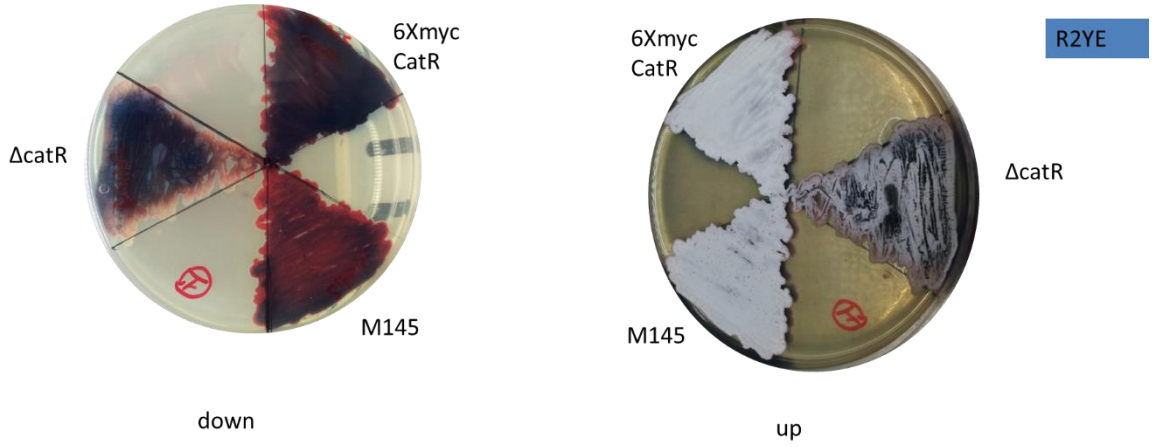


Fig. 15. Phenotype of 6xmyc tagged *catR* strain.

M145 wild type, *ΔcatR* and 6xmyc tagged *catR* strains were streaked on to R2YE plate for comparison of phenotypes.6xmyc tagged *catR* strain produces spores and antibiotic like wild type but *catR* mutant does not.

CHAPTER 4.

Discussion

Oxidative stress, resulting from exposure to reactive oxygen species (ROS) which can damage proteins, DNA, and membranes, is major challenge for all living organisms.*Streptomyces* are soil-dwelling bacteria, which produce a diverse range of secondary metabolites such as natural antibiotics. Members of this genus have complex regulatory systems at transcription and translation levels to adapt changing environmental conditions. *Streptomyces* have developed complicated defense system against ROS resistance.

OxyR and CatR are peroxide sensing transcription regulators in *Streptomyces coelicolor*.

OxyR is a global regulator of the peroxide stress response that maintains intracellular H_2O_2 levels within safe limits.Previous studies have shown that OxyR activates *ahpCD* gene transcription.AhpCD is responsible for detoxification of alkaly hydroperoxides. CatR is a Fur homologue which regulates CatA. CatA is major catalase that play an important role in protection from higher concentration of H_2O_2.

Phenotype of *ΔoxyR* has been studied which suggests that *ΔoxyR* mutant grew faster in liquid YEME rich and minimal media.On solid media, with or without under stress of H_2O_2. *ΔoxyR* displayed faster growth and sporulation comparing to wild type strain.

Actinorhodin and undecylprodigiosin (Red) are antibiotics that *Streptomyces coelicolor* naturally produces. Many regulators have been identified to involve in antibiotic production. On solid SFM, R2YE media and minimal liquid media *oxyR* deletion mutant produced higher amount of actinorhodin whereas no effect on Red production. These results suggest that OxyR might play a role in antibiotic production.

Our study about behaviour of *oxyR* deletion strain against various stresses showed that *ΔoxyR* displayed better growth and sporulation.Previous studies suggests that *catA* transcription elevated in *ΔoxyR* strain. S1 mapping analysis also confirmed this phenomenen.Furthermore, catalase activity staining further proved that *oxyR* deletion strain

produced more catalase A.Here we postulate a possible model to explain this results.This model is expanded version of Hahn et al., 2000b as demonsrated in fig. 16.

Firstly,In *ΔoxyR*, OxyR can not activate *ahpCD* transcription as a result of this AhpCD activity decreases. Low level of AhpCD may not reduce endogenously produced peroxides which may cause oxidation of CatR.Oxidized CatR derepresses *catA* and *catR* gene transcription. Catalase A levels are elevated on the other hand, catalse A decomposes H_2O_2 but other endogenously produces peroxides such as alkaly hydro peroxides still may not be removed. These peroxides may oxidized CatR. In conclusion, catalase A production increases and may rescue *ΔoxyR* from growth defect.

Secondly, our results strongly suggest that *oxyR* deletion mutant produces high levels of catalase A in comparision with wild type strain. Reduced CatR amounts suppose to be lower in *ΔoxyR* which can not repress *catA* transcription as effective as in wild type, resulting production of more catalse A by *oxyR* mutant strain. Further experiments need be done to see CatR levels in *oxyR* mutant to understand and explain the relationship between catalse A production and reduced CatR levels in *ΔoxyR*.

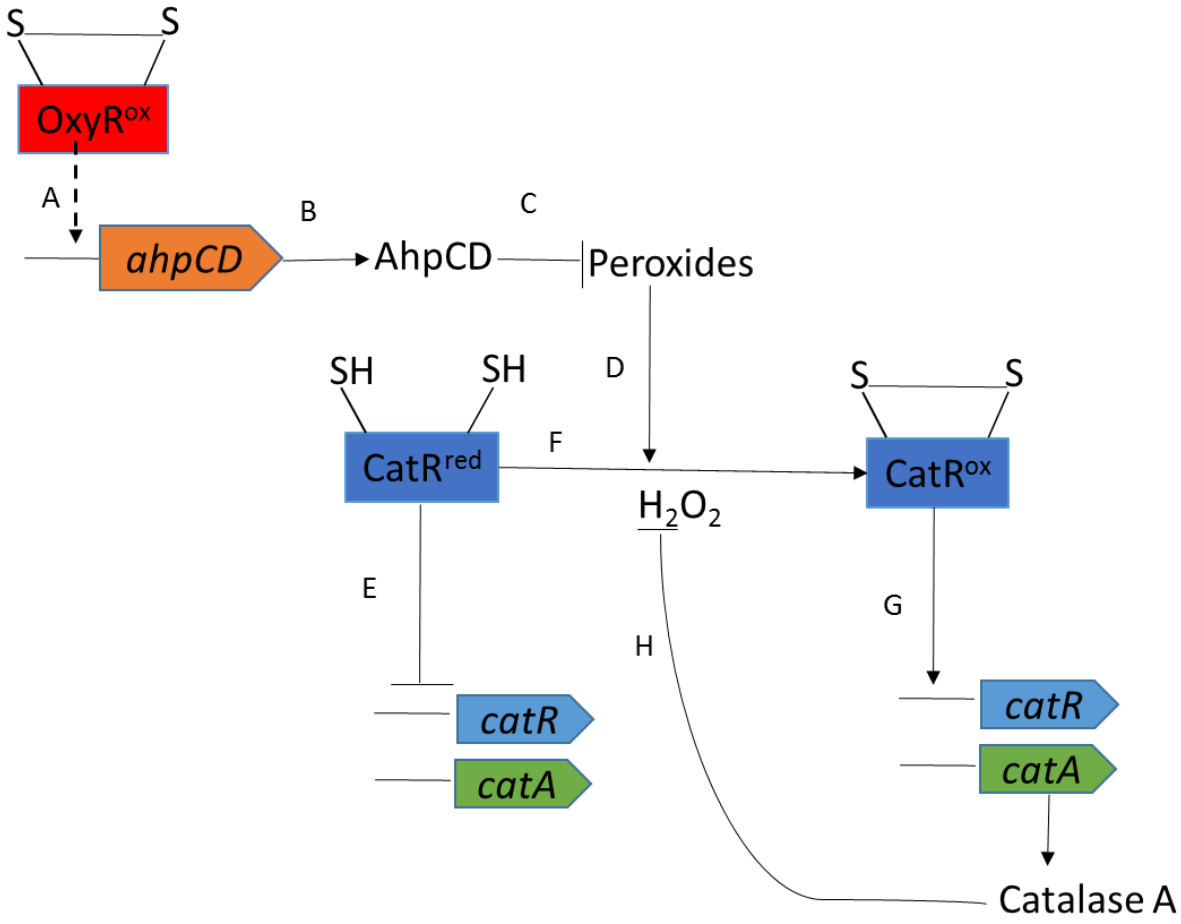


Fig. 16. A possible model for explanation of high catalse A production by *ΔoxyR* strain comparing to wild type strain.

Oxidized OxyR can not bind and induced *ahpCD* transcription (path A).AhpCD production highly decreased resulting increasement of endogenously produced peroxides (B,C). Reduced CatR represses *catA* and catR (E).More endogenously produced peroxides in cell may cause oxidation of CatR (D,F). Oxidized CatR derepresses its own and *catA* transcription (G).As a result of the induced catalase A removes H_2O_2.

According to mRNA sequencing data, 398 genes were positively and 1228 genes were negatively induced by 200 uM H_2O_2.We will reconfirmed by S1 mapping that SCO3132 (putative trans-aconitate methyltransferase), SCO3091(Cyclopropane-fatty-acyl-phospholipid synthase), SCO4409 (Putative RNA polymerase sigma factor), SCO3202 (RNA polymerase principal sigma factor(hrdD), SCO0570 (50S ribosomal protein L33) and SCO1519 (Holliday junction DNA helicase) are positive OxyR target genes.RNA sequencing analyses suggest that OxyR control other genes unrelated to oxidative stress or other oxidative stress gene rather than *ahpC*.In order to learn DNA binding sequence for OxyR and CatR we have tagged *Streptomyces coelicolor oxyR* and *catR* by 6xmyc fusion tag for CHIP.

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2014 년 2 월

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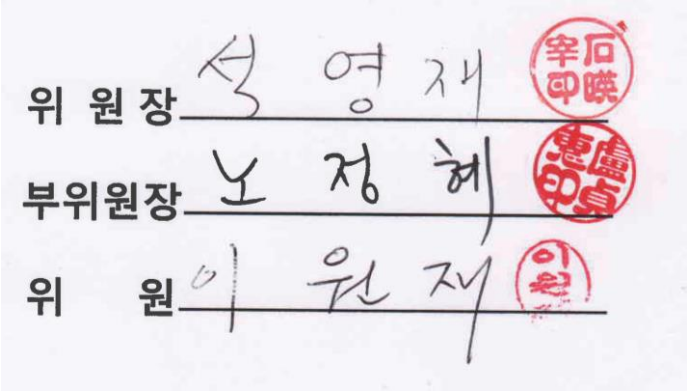

Role of Peroxide sensing transcription regulator OxyR in *Streptomyces coelicolor*

By

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Under the supervision of

Professor Jung-Hye Roe, Ph.D.

A Thesis Submitted in Partial Fulfillment of

the Requirements for the Degree of

Master of science

February, 2014

School of Biological Sciences

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Seoul National University

국문초 록

OxyR 은 일반적으로 그람 음성세균과 그람양성 세균 전반에 걸쳐 발견되며 과산화수소를 감지하는 조절자이다. 방선균 *Streptomyces coelicolor* 에서 OxyR 은 자신의 유전자와 alkyl hydroperoxidase 유전자인 *ahpCD* 를 활성자로서 조절한다. 또 다른 과산화수소 감지조절자인 CatR 은 catalase A 유전자를 억제자로서 조절한다. *oxyR* 결손 돌연변이 균주가 예상과 다르게 과산화수소를 처리한 배지에서 야생형균주보다 더 잘 자라고, 분화도 더 잘하며 항생제합성도 더 잘하는 것이 관찰되었다. 이에 대한 원인이 oxyR 결손변이주에서 catalase 의 생성이 더 많아진 것이 아닌가 추정되었다. 효소활성염색을 통해, 돌연변이 균주에서 catalase 효소활성이 야생형보다 더 높은 것이 확인되었다. OxyR 과 CatR 의 기능을 확인하기 위해서는 genome 상에서 이들이 결합하는 유전자 위치를 결정하여야 한다. 이를 위하여, OxyR 과 CatR 의 C-말단에 6 개의 Myc 을 붙이는 재조합체를 제조하였다. Myc 에 대한 항체를 사용한 면역블롯을 통해 Myc tagging 이 성공적으로 되었음을 확인하였다. 이로서, 면역침강을 활용한 유전체차원의 분석이 가능하게 되었다.

주요어: *Streptomyces coelicolor*, OxyR, catalase, Myc tagging, CatR, *ahpCD*

Abstract

OxyR is an H_2O_2 sensing transcriptional regulator of the LysR-family that is generally found in Gram-negative bacteria and some Gram-positive bacteria. In *Streptomyces coelicolor*, OxyR positively regulates its own gene and *ahpCD* encoding alkyl hydro peroxidase. Another peroxide-sensing regulator CatR controls catalase A production in response to H_2O_2. It binds to *catA* and *catR* genes and represses their transcription. Physiological study showed that *ΔoxyR* mutant grew faster in liquid YEME (complex medium) and NMMP (minimal medium). On solid media, *ΔoxyR* mutant exhibited better growth and sporulation even when media contained various concentrations of H_2O_2. It was postulated that *catA* expression may be elevated in *ΔoxyR* mutant to allow better growth phenotype. The amount of catalase A was determined by activity staining and was found to be higher in the mutant. This suggests that compensatory catalase induction may have conferred ΔoxyR with the better growth phenotype. Moreover, antibiotic actinorhodin was produced more in *ΔoxyR* in both solid SFM and R2YE media and liquid minimal medium. To investigate the regulatory role of OxyR and CatR, their target genes need be unraveled on genome scale, possibly by chromatin immunoprecipitation (ChIP). As an initial step to perform ChIP analysis, tagging of OxyR and CatR with detectable probe was performed. Cloning of 6xMyc tag to the C-terminal end of OxyR and CatR were done. Specific detection of tagged proteins by anti-Myc antibody was successful. Further improvement of detection sensitivity, either by changing the tag or solubility optimization, will be useful for genome-wide detection of direct binding sites of OxyR and CatR.

Key words: *Streptomyces coelicolor,* OxyR, catalase, CatR, myc tagging, oxidative stress, actinorhodin

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List of Abbreviations

aa	amino acid
Act	actinorhodin
$Apra^R$	Apramycin resistant cassette
bp	base pair
H_2O_2	Hydrogen peroxide
kDa	kilo dalton
nt	nucleotide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
Red	Undecylprodiosin
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate

CHAPTER 1.

Backgrounds

1.1 Biology of *Streptomyces coelicolor*

Streptomyces are the most widely studied and well-known genus of actinomycete family. They usually inhabit soil and are important decomposers. *Streptomyces* produces many antibiotics and other classes of biologically active secondary metabolites.

Streptomyces is a Gram positive bacterium with high GC content. It undergoes complex multicellular developmental life cycle which starts from germination of spore and formation of highly branched vegetative mycelium. The hyphae are divided into multigenomic compartments by the infrequent formation of vegetative septa. After a period of active growth, aerial mycelium develops from substrate mycelium on the surface of colony, and eventually differentiates into unigenomic spores. *Streptomyces coelicolor* is genetically the best known representative of the genus. The complete DNA sequence of *S.coelicolor M145* has been published (Bentley et al., 2002).

S. coelicolor produces at least four antibiotics; actinorhodin (Act), undecylprodigiosin (Red), methylenomycin (Mmy) and the Ca^{2+}-dependent antibiotic (CDA) (Hopwwood *et al.*, 1985).

1.2 Oxidative stress responses

Living organisms have developed adaptive systems to cope with changing environmental conditions such as nutrient, temperature, osmolarity, pH, and redox state during cellular growth and maintain cellular homeostasis.

Reactive oxygen species (ROS) can be generated as byproducts of enzymatic reactions during respiration, or by exposure to radiation, stimulated macrophages, or redox-active drugs. Because of their high reactivity, the oxidants can damage cellular components such as DNA, proteins, and cell membrane.

1.2.1 Reactive oxygen species

The molecular oxygen has two unpaired electrons in different pi antibonding orbital, and the two electrons have the same spin quantum number. Because of this spin restriction, molecular oxygen is limited to accept electrons.

Its reactivity increases upon serial one electron reduction of oxygen, or a spin flip to become singlet oxygen ($^1\sum_g O_2$). The resulting oxygen derivatives, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\cdot$), and singlet oxygen are collectively defined as reactive as ROS.

Superoxide radical (O_2^-)

The superoxide radical is generated by one electron transfer to one of the two π* orbitals of of the ground state of oxygen. In *Escherichia coli,* autoxidation of membrane-associated respiratory chain enzymes, such as NADH dehydrogenase, succinate dehydrogenase, and D-lactate dehydrogenase or cytosolic glutathione reductase, are major sources of O_2^- generation *in vivo*. Nonenzymatic production of O_2^- occurs by oxidation of several cellular components including ubiquinols, catechols, thiols, and flavins. Electrophilic quinone compounds, both natural cellular constituents (ubiquinone and plastoquinone) and exogenous sources (plumbagin and menadione), are an important group of substrate for flavor proteins catalyzing either two electron reduction to the hydroquinone or one electron reduction to the semiquinone radical (Bellomo *et al.,* 1990), Semiquinones readily reduce O_2 to O_2^-, regenerating the oxidized quinones. The oxidized quinones can repeat this cycle and it thus referred to as a redox cycling agent.

Superoxide radical can oxidize thiols, ascorbate, tocopherol, and catecholamine (Fridovich *et al.*, 1989). The major target of O_2^- damage identified in bacteria is a class of dehydratese

enzymes that utilize [4Fe-4S] clusters to bind their substrate (Imlay *et al.*, 2003). O_2^- is dismutated to H_2O_2 and O_2, spontaneously or enzymatically by superoxide dismutase (SOD).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is generated by dismutation of O_2^- and by autooxidation of flavoenzymes such as sulfite reductase and xanthine oxidase (Imlay *et al.*, 2003; Messner and Imlay *et al.*, 2002). Some carbon sources autooxidize and thereby contribute to H_2O_2 formation (Seaver and Imlay *et al.*, 2001).

It can act as weak oxidizing agent and oxidize cysteinly residues, creating sulfenic acid adducts that can either from disulfide cross-links with other cysteines or be oxidized further to sulfinic acid moieties (Kiley and Storz *et al.*, 2004). It can also oxidize methionine residues to methionine sulfoxide and a variety of carbonyls (Griffiths and Cooney *et al.*, 2002). However, its more significant action is to generate more reactive $OH^{\cdot}$ Radical by reaction with reduced iron or copper ions by the Fenton reaction (Candenas *et al.*, 1989; Imlay and Fridovich *et al.*,, 1991). Since O_2^- can elicit both H_2O_2 production and reduction of Fe^{3+} and Cu^{3+}, it also enhances $HO^{\cdot}$ generation.

Hydroxyl radical ($HO^{\cdot}$)

The only oxygen species that can directly damage most biomolecules is $HO^{\cdot}$ It is formed when ferrous iron transfers an electron to H_2O_2 (Fenton reaction).

The hydroxyl radical oxidize most organic molecules (RNA, DNA, protein, and lipid) at diffusion-limited rates because $HO^{\cdot}$ has high reactivity due to its very high standard electrode potential (Singh and Singh *et al.*, 1982). While the Fenton reaction has been linked to protein carbonylation and membrane peroxidation, its most significant impact is likely to be upon DNA, since even a single DNA lesion is potentially mutagenic or lethal.

Singlet oxygen ($^1\sum_g O_2$)

Singlet oxygen may be generated from hydroxyl radical by the action of certain enzymes, such as decomposition of superoxide or peroxidized glutathione (GSOO), and photosensitization reactions with endogenous sensitizers such as riboflavin and bile pigments. It can be formed by energy transfer to oxygen by excited chromophores and is generated in photosynthetic system.

Almost all reactions involving singlet oxygen with biomolecules are addition of the $^1O_2$ to conjugated bond. The known targets are carotenes, chlorophylls, and fatty acid side chains present in the lipid membrane, suggesting that $^1O_2$ can initiate lipid peroxidation (Kappus *et al.*, 1985).

1.2.2 Biological defense systems to oxidative stress

Cellular defense systems to counter the deleterious effects of ROS include prevention of ROS generation, detoxification of ROS, and repair of damage. Preventation of generation of ROS can be achieved by chelating metal ions such as iron and copper, which promote generation of free radicals.

Both enzymatic and non-enzymatic systems are involved in antioxidant defense by scavenging ROS. Catalase decomposes H_2O_2 to O_2 and H_2O, while peroxide uses intracellular reductants to reduce H_2O_2.

1.3 Hydrogen peroxide sensing transcription regulators

Bacteria adapt to the presence of ROS by increasing the expression of detoxification enzymes and protein and DNA repair functions. These responses are coordinated by transcription factors that regulate target genes in response to ROS. There are known three classes of bacterial hydrogen peroxide sensing regulators in *S.coelicolor*.

1.3.1 OxyR

OxyR is an H_2O_2- sensing transcriptional regulator of the LysR-family that is generally found in Gram-negative bacteria but is also known to occur in a few Gram-positive bacteria (Morikawa, *et al.,* 2006. Oh *et al.,* 2007). Like other regulators of this family it contains a conserved N-terminal helix-turn-helix DNA binding domain, a central co-inducer recognition and response domain which senses the regulatory signal, and a C-terminal domain that functions in multimerization and activation (Kona, *et al.,* 2006. Kullik, *et al.,* 1995) OxyR as a peroxide sensing global regulator maintains intracellular H_2O_2 levels within safe limits. In *Escherichia coli,* OxyR regulates over 20 genes, including genes involved in H_2O_2 detoxification (*katE, ahpCFvx*), heme biosynthesis (*hemH*), reductant supply (*grxA, gor, trxC*), thiol-disulfide isomerization (*dsbG),* Fe-S center repair (*sufA-E, sufS)* (Li *et al.*, 2004, Zheng, *et al.,* 1998).Even though, there are reasonably large differences, OxyR regulons of other organisms includes similar classes of genes.Under oxidizing conditions OxyR acts as a transcription regulator through directly binding α subunit of RNA polymerase. H_2O_2- sensing of OxyR occurs via direct oxidation at a specific "sensing" cysteine residue. Normal intracellular H_2O_2 level is ~20 nM, OxyR is present in its reduced form when intracellular H_2O_2 levels increase to ~100nM rapid oxidation of OxyR occurs in *E.coli* (Seaver *et al.*, 2001).

In *E.coli,* hydrogen peroxide mediated activation of OxyR constitutes a simple on/off switch that occurs through the formation of a specific disulfide bond between the conserved cysteine residues C199 and C208 (Storz *et al.,* 1990).In *Streptomyces coelicolor, oxyR* gene locates 138 nt upstream from the *ahpC* gene, which is the main defense system against endogenously generated hydrogen peroxide, encodes a protein of 313 amino acids 33,096 Da and shows homology to other known OxyR proteins. Two cysteine residues are also conserved in the *Streptomyces coelicolor* OxyR protein C206 and C215.

Most of the LysR family of transcription regulators autoregulate their own expression as negative regulators, in contrast *S.coelicolor* OxyR positively regulates its own gene expression and *ahpCD* gene expression. One of Prof. Roe Jung Hye laboratory members performed S1 mapping analysis using mRNA results. They found that OxyR positively regulates six more genes. Moreover, *S.coelicolor* OxyR does not regulate the production of antioxidant enzymes such as Ni-containing SOD, Fe-containing SOD, or glucose-6-phosphate dehydrogenase (Hahn, *et al.,* 2002).

1.3.2 CatR

CatR (peroxide sensing transcription repressor) is the first fur homologue characterized as one of peroxide sensing repressors found in both Gram-positive and Gram-negative bacteria (Hahn *et al.,* 2000b). PerR was identified in *Bacillus subtilis* as the major peroxide stress response regulator. PerR regulates major vegetative catalase *(KatA),* alkyl hydroperoxide reductase *(ahpCF), fur, perR* and a zinc uptake system *(zosA)* (Herbig and Helmann *et al.,* 2002)

Streptomyces coelicolor CatR encodes a protein of 138 amino acids, 15319 Da, (Hahn *et al.,* 2000c). Δ*catR* mutant over produces CatA, confirming the role of CatR as a negative regulator of *catA* expression. Moreover, CatR represses its own gene transcription. Transcripts of *catA* and *catR* genes were induced within 10 min of H_2O_2 treatment, this suggests that the repressor activity of CatR may be modulated by H_2O_2 (Figure 1). A putative CatR binding site containing an inverted repeat of 23 base pairs was localized up stream of the *catA* and *catR* genes CatR regulon was induced by metal chelators and it contains at least one zinc. Conserved amino acids predicted for metal binding site were important for regulation of *catR* and *catA* expression whose DNA binding ability is directly modulated by redox changes in the cell (Dr. So young Oh thesis, 2006).

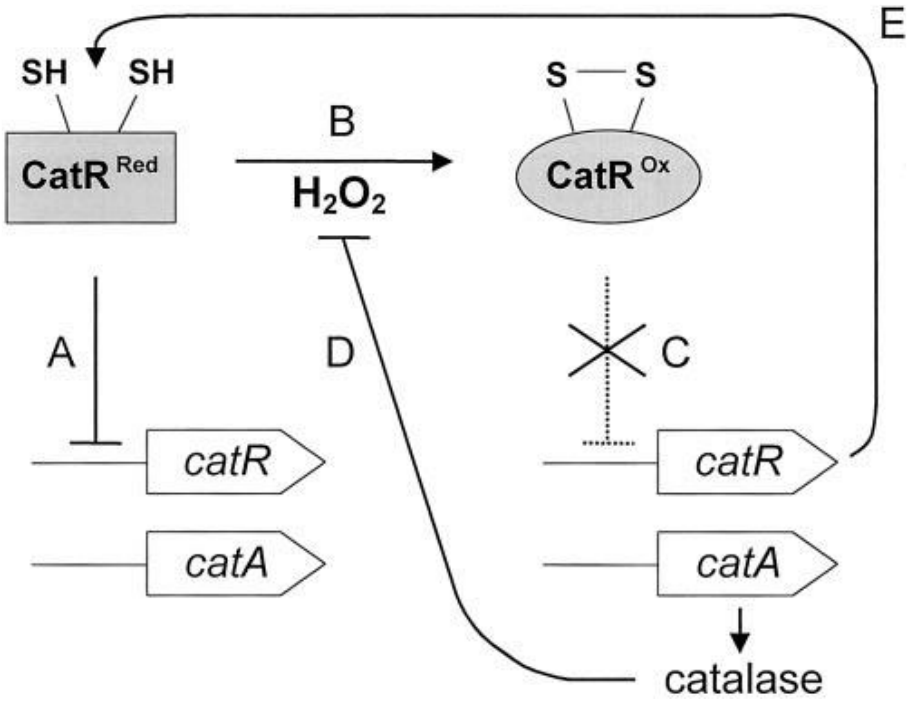


Fig. 1. A model for the rapid H2O2-sensitive regulation by CatR.

Reduced CatR binds to the *catA* and *catR* genes and represses their transcription (*path A*). Upon exposure to H_2O_2, the free cysteine thiols of CatR are oxidized to form disulfide bonds (*B*), causing loss of DNA binding activity and thus derepression of *catA* and *catR* (*C*). The induced catalase removes H_2O_2 (*D*), whereas the induced CatR (coupled with an increase in the proportion of the reduced form as peroxide isremoved) represses both genes (*E*), forming a negative feedback loop(Hahn, *et al.,* 2000b)

1.4 Catalases and peroxidases of *Streptomyces coelicolor*

Catalase plays a crucial role in removing hydrogen peroxide, transfers two electrons to H_2O_2, decomposing it into O_2 and H_2O, while peroxidases uses intracellular reductants to reduce H_2O_2. Regulation of genes for the peroxide-removing system in *S. coelicolor* is achieved by four separate defence enzymes. Alkyl hydroperoxide reductase (AhpC), Catalase A (CatA), Catalase B and Catalase C. Therefore, in *S. coelicolor*, antioxidant genes are regulated by a wider variety of regulators than those observed in other organisms examined so far.

1.4.1 Catalases

Streptomyces coelicolor produces three distinct catalases to cope with oxidative and osmotic stresses and allow proper growth and differentiation. CatA is major catalase which induced by H_2O_2 and its required for efficient aerobic growth (Cho *et al*. 1997) It is regulated by CatR (Hahn *et al*., 2000) *catB* is induced by osmotic stress or at the stationary phase and required for cell differentiation and osmoprotection. (Cho *et al* 2000) while catalase peroxidase (CatC) is expressed transiently at the late exponential to early stationary phase. (Hahn *et al*., 2000b).

CatA is a monofunctional catalase which induced by hydrogen peroxide. It is the main catalase of *Streptomyces coelicolor*. Its gene expression increases during earlier growth phages, but stays constant after mid-exponential growth phase in liquid medium. *catA* gene expression is repressed by reduced CatR but oxidized CatR derepresses *catA* expression (figure 1.).Previous studies suggest that CatA expression is responsible for protection of cells from attack of exogenous H_2O_2.

1.4.1 Peroxidases

Alkyl hydroperoxide reductase system (*ahpC* and *ahpD*) have been isolated from *Streptomyces coelicolor* A3 (2). The *ahpC* and *ahpD* genes constitute an operon transcribed divergently from the *oxyR* gene. Alkyl hydroperoxide reductase (AhpC) is maximally produced during early exponential phase and is induced by exogenous H_2O_2, all under the control of OxyR. The conservation of cysteine residues in the C-X-X-C motif among AhpD proteins from *S. coelicolor* and *Mycobacterium* spp. suggests their function as thioredoxin-like proteins involved in reducing AhpC (Holmgren, A. et al., 1985). Both *ahpCD* and *oxyR* are induced by H_2O_2 in an OxyR-dependent manner.

1.5 Aims of this study

Streptomyces coelicolor crucial in soil because of their broad range of metabolic processes. They are important for medicine because they produces at least four antibiotics; actinorhodin (Act), undecylprodigiosin (Red), methylenomyscin (Mny) and the Ca^{2+} - dependent antibiotic (CDA).It is also a good model system to study the relationship between oxidative stress and differentiation.

Previous studies have been shown that in *Streptomyces coelicolor* OxyR regulates its own gene and *ahpC* gene expression in H_2O_2. And CatR, peroxide sensing repressor, was characterized as a regulator of *catA* and *catR* (Hahn et al., 2000a, b,c; Hahn et al.,2002) Moreover, *catA* transcription levels are higher in *ΔoxyR* in comparison to wild type (Dr. So young Oh thesis 2006).

In order to understand role of peroxide sensing regulators in *Streptomyces coelicolor*.

1-We further examined role of OxyR on growth, morphology, differentiation and antibiotic production in *Streptomyces coelicolor*.

2-Catalase A production of *ΔoxyR* was investigated in comparison with wild type.

3- Positive regulation of some genes by OxyR under treatment of H_2O_2 will be investigated using mRNA sequencing analyses.

4- We aimed to know DNA binding sequence for OxyR and CatR by CHIP analysis. To achieve this goal, we made fusion constructs of *oxyR* and *catR* fused with tags such as 6x myc tagging in *Streptomyces coelicolor*.

CHAPTER 2.

Materials and Methods

2.1 Bacterial strains and culture conditions

Streptomyces and *Escherichia coli* strains used in this study are listed in Table 1.

Streptomyces coelicolor A3 (2) M145 was used as wild type in most studies. *Streptomyces* cells were grown as described previously (Kieser, *et al.,* 2000). For liquid culture, spore suspension was inoculated in YEME medium (1% glucose, 0.5% Bacto Peptone, 0.3% malt extract, 0.3% yeast extract, 10.3% sucrose, 5mM $MgCl_2$), NMMP (0.2% $(NH_4)_2SO_4$, 0.5% Difco casamino acids, 0.06% $MgSO_4.7H_2O$, 5% peg 6000, 15 mM NAH_2PO_4/K_2HPO_4 (pH 6.8), 5% glucose). R2YE (10.3% sucrose, 0.025% K_2SO_4, 1.01% $MgCl_2.6H_2O$, 1% glucose, 0.01% casamino acids, 0.5% yeast extract), NA (0.8% nutrient broth), and SFM (soy flower 2%, mannitol 2%) agar media were used for surface cell growth. Cells were grown in liquid medium by inoculating with spore suspension and incubating at 30°C. *E.coli* strains were grown in LB or SOB. *E. coli* BW25113 was used to propagate the recombination plasmid pIJ790 and *S. coelicolor* cosmids. *E. coli* BT340 carrying pCP20 was used for FLP-mediated site-specific recombination. *E. coli* ET12567/pUZ8002 was the nonmethylating plasmid donor strain for intergeneric conjugation with *S. coelicolor* strain M145. Apramycin (50 μg/mL), chloramphenicol (25 μg/mL) or kanamycin (50 lg/mL) was added to growth media when required.

Table. 1. Strains used in this study

Strains	Genotype of description[a]	Source or reference
***S.coelicolor* A3(2)**		
M145	Prototrophic SCP1$^-$SCP2$^-$Pgl$^+$	Hopwood *et al.*,1985
S12	M145 *catA::*Apra^R	Dr. So Young Oh
S21	M145 *oxyR::*Apra^R	Dr. So Young Oh
S22	M145 *catR::*Apra^R	Dr. So Young Oh
N1	M145 *oxyR 6xmyc::*Apra^R	This study
N2	M145 *oxyR 6xmyc::*Apra^R	This study
N3	M145 *catR 6xmyc::*Apra^R	This study
E.coli		
DH5α	F-*ΔlacU169(Φ*80*lacZ*ΔM15) *endA1 recA1 hsdR17 deoR supE44 thi-1 λ-gyrA96re1A1*	Hanahan, *et al.*, 1983
BW25113	K12 drevative: araBAD, rhaBAD	Datsenko and Wanner, *et al.*, 2000 McNeil et al,.1992
ET12567	F$^-$*dam13::Tn9 dcm6 hsdM hsdR recF143::Tn10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 HİSg4 tsx-78 mtl-1 glnV44*	McNeil et al., 1992

Table.2. Plasmids used in this study Source or reference

	Genotype of description[a]	Source or reference
2st3B6	Cosmid containing *catR* gene cluster	John Innes in UK
SCK7.06	Cosmid containing *oxyR* gene cluster	John Innes in UK
pIJ790	oriR101, repA101^{ts}, RED (araBp-*gam-bet-exo),cat,araC*	Gust, *et al.*, 2003
pIJ773	*AAC(3)IV (*Apr^{R}),*oriT*,Amp^{R}	Gust *et al.*,,2003
pJN1	pUC18:(n)-myc contains FRT sites and Apr^{r} cassette	Ji Nu Kim *et al.*,,2012

Table.3 Primers used in this study

Primer	Sequences	Note*
OxyR myc F	CAGGCGATGGCGGACCTGCCGGTGCGGACGGT GCACGACGAGCTCGAGGAGCAGAAG	For 6xmyc tagging
OxyR myc R	GGACCGAGGGACCGCCCGGCCGGGTGGTCGCC CGGTCCCATTCCGGGGATCCGTCGACC	For 6xmyc tagging
CatR myc F	GTGACGTACCGCGGCACCTGCCCGAACTGCGCG GCGGCGGAGCTCGAGGAGCAGAAG	For 6xmyc tagging
CatR myc R	AACACTACGTCACGATGAAGACGTGAGGCAAA TCCCTGCATTCCGGGGATCCGTCGACC	For 6xmyc tagging
6xmyc dn R	AGCCTACAGGATCCTCATCA	Tagging check
Frt F	CCAACGGCGTCAGCCGGGCAGG	Tagging check
oxyR Mid F	CGCTGGTACAGCTGGTGGCG	Tagging check
oxyR dn R	GTGCCCGACCGGGGCACCGA	Tagging check
6xmyc F	GGCTGGAGCTGCTTCGAA	Tagging check
CatR mid F	GCTGGTCTCGCTCGGCGAGG	Tagging check
CatR dn R	GCGAGCTACCGAGCTGCTCC	Tagging check
AprR	CGCTCGTCATGCCCTCGTGG	Tagging check

*: the purpose of the primers

2.2 General recombinant DNA techniques

General techniques for isolation and manipulation of DNA in *Streptomyces* and *E.coli* were described as previously (Hopwood et al., Sambrook et al., 1989). All plasmids used in this study are listed in Table II-2.

2.2.1 Transformation of *E.coli*

Introduction of DNA into *E.coli* was done by chemical method and electroporation method using gene pulser (BioRad).

2.2.2 Conjugation between *E.coli* and *S.coelicolor*

Conjugal transfer of DNA from *E.coli* to *S.coelicolor* was carried out by a modification of the method of Mazodier et al. (1989). Competent cells of ET12567 containing Puz8002 were prepared under chloramphenicol (12.5 μg/ml) and kanamycin (50 μg/ml) selection and were transformed with oriT-containing conjugation vector selecting for incoming vector only.

2.2.3 PCR-based tandem epitope tagging system for *Streptomyces coelicolor* genome

PUC18 plasmid(pJN1) which contains the flanking FRT sites,6X myc sequence and apramycin resistance gene was provided by Ji-Nu Kim. Linear DNA fragments were amplified using pairs of primers which were 59-bp in length with 39-bp homology extensions overlapping upstream and downstream from stop codon of target genes and 20-bp priming sequences from pJN1 template plasmid series. PCR was carried out as which described in Ji-Nu Kim *at al.,* (2012). Each PCR product was purified, digested with DpnI, repurified, and then electroporated into *E. coli* strain harboring pIJ790 (which expresses the λ Red recombination system under the control of an inducible promoter) and *S. coelicolor* cosmid (which contains a genomic region of interest). The cells were then incubated at 37 ^{o}C for 1 h in 1 mL of LB and spread onto LB-agar medium supplemented with apramycin. The myc-

inserted cosmid was transported into the methylation-deficient *E. coli* strain ET12567 then transferred *S.coelicolor* by conjugation. Single-crossover exconjugants were selected on SFM containing kanamycin and nalidixic acid, to obtain transconjugants. The genomic DNA was then isolated and plasmid integration was confirmed by PCR. 6xmyc tagging strategy is described in figure 2. Primers used in this study are listed in Table II-3.

2.3. DNA analysis

2.3.1 Polymerase chain reaction (PCR)

Each 100 µl of reaction mixture contained the followings; 10 x taq polymerase reaction buffer, 150 µM $MgCl_2$,200 µM dNTP, 5% DMSO, 1 µM of each 5` and 3` primers, 100 ng of template DNA and 5 U of Taq polymerase. Reaction was carried out in Perkin-Elmer thermocycler by denaturation at 94°C for 2 min, annealing at 50-55°C for 45 sec and extending at 72°C for 1 min 30 sec.

2.3.2 DNA sequencing

DNA sequencing was done automatically using automated sequencer (ALF Express, Pharmacia). Deaza-dNTPs were used to minimize sequencing artifacts due to the formation of secondary structure.

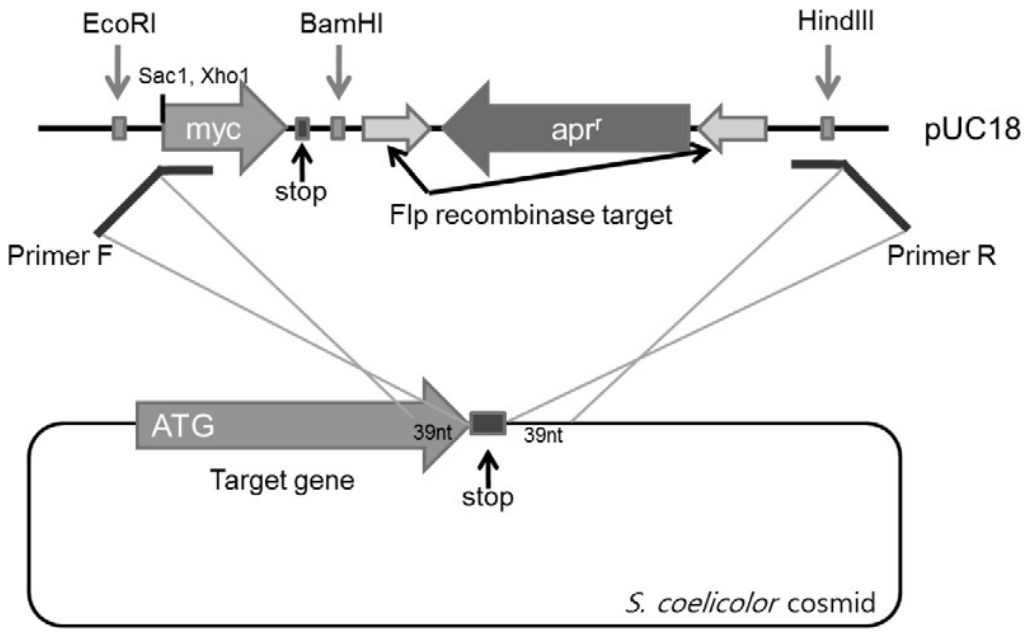
Figure 2. Scheme of 6xmyc tagging strategy.

A. PUC18 plasmid that involves tandem myc sequence and the antibiotic resistance marker (apr^r) is amplified with primers carrying extensions homologous to the upstream and downstream of the translation stop codon of target gene.

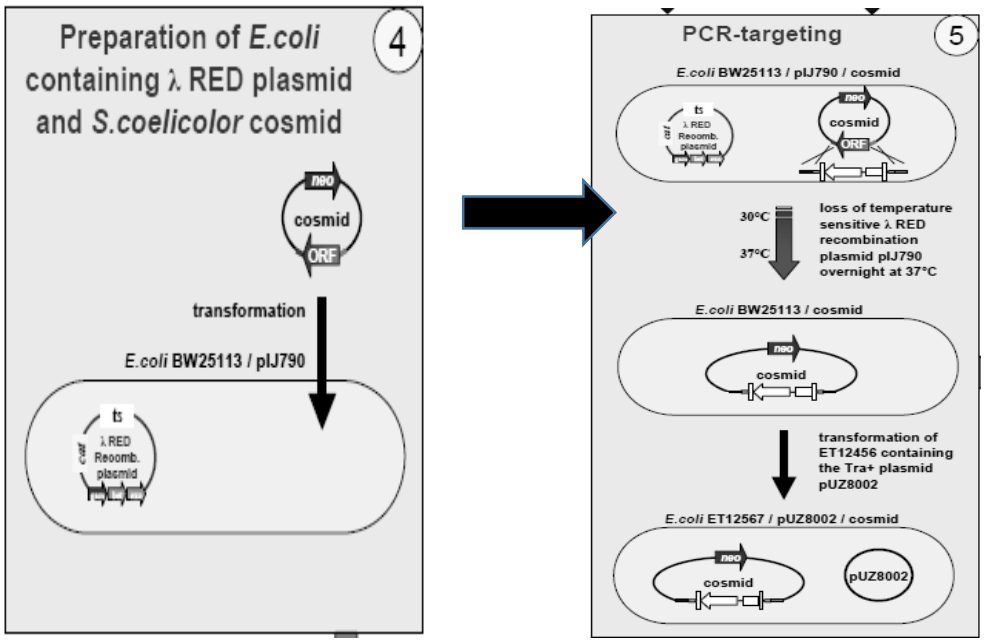
B. *Streptomyces coelicolor* cosmid containing target gene was introduced into *E.coli* BW25113/pIJ790 (contains resistance marker *cat* (chloramphenicol resistance) and a temperature sensitive origin of replication (requires 30°C for replication)). Presence of cosmid in competence cell confirmed by PCR using primers at table II. Then, PCR product of (A) was transformed to *E.coli* containing cosmid with target gene. Again, genomic tagging was confirmed by PCR using control primers. Then, Cosmid DNA of transformants was isolated and it was introduced by transformation into the non-methylating *E.coli* ET12567 containing RP4 derivative Puz8002.

C. The cosmid was transferred to *Streptomyces coelicolor* by intergeneric conjugation. Single-crossover exconjugants were selected on SFM containing kanamycin and nalidixic acid, to obtain transconjugants. The genomic DNA was then isolated and plasmid integration was confirmed by PCR.

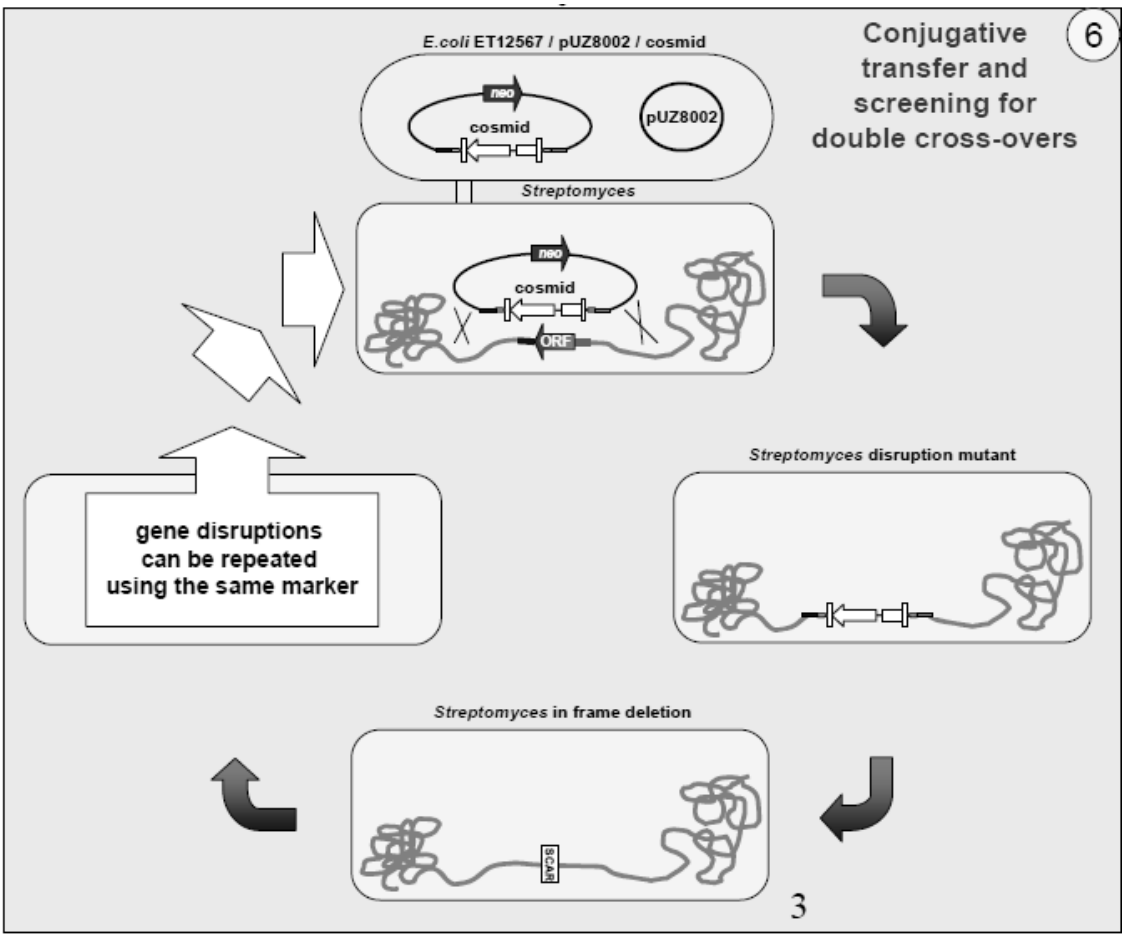
A


EcoRI
BamHI
HindIII
Sac1, Xho1
myc
stop
aprr
pUC18
Flp recombinase target
Primer F
Primer R
ATG
39nt
39nt
Target gene
stop
S. coelicolor cosmid

B


Preparation of E.coli containing λ RED plasmid and S.coelicolor cosmid
4
neo
cosmid
ORF
transformation
E.coli BW25113 / pIJ790
ts
cat
λ RED Recomb. plasmid
PCR-targeting
5
E.coli BW25113 / pIJ790 / cosmid
30°C
37°C
loss of temperature sensitive λ RED recombination plasmid pIJ790 overnight at 37°C
E.coli BW25113 / cosmid
transformation of ET12456 containing the Tra+ plasmid pUZ8002
E.coli ET12567 / pUZ8002 / cosmid
pUZ8002

C



Gust et al, .2003. PCR targeting system in *Streptomyces coelicolor*.

2.4. Protein analysis

2.4.1. Preparation of cell extracts

Harvested cells were resusupended in 50 mM potassium phophate buffer (pH6.8) and disrupted by sonication with ultrasonicator (Sonics and Materials Inc.). The suspension was centrifuged and the protein concentration of soluble supernatant was determined using Bradford reagent (Bio-Rad) and BSA as a standard protein.

2.4.2 Western blot analysis

Following SDS PAGE, the gel was soaked in transfer buffer [25 Mm Tris, 192 mM glycine, 20% (v/v) methanol] for 10 min, and then electrotransferred to PVDF (Roche applied sciences) membrane at 180 Ma for 1.5 hr in Trans-Blot Cell (Biorad). Membrane was blocked in Tris-buffred saline buffer containing 0.5% Triton X-100 (TBST) supplemented with 5% skimmed milk,for 1 hour at room temprature or over night at 4°C. The blocked membrane was incubated with primary antibody for 1 hr and then membrane was washed 3 times for 10 min with TBST. Washed membrane was incubated with anti-mouse IgG secondary antibody 1:10,000 diluted in TBST, and washed with TBST for 10 min 3 times. Detection of signal was done using Western ECL detection system.

2.4.3. Catalase activity staining

Native catalases are electrophoretically separated on 7 % polyacrylamide gel. Staining for catalase activity was done by the method of Kim *et al.,* (1994): the gel was soaked for 45 min in 50 mM potassium phosphate buffer (pH 7.0) containing 50 mg horseradish peroxidase ml^{-1}, followed by addition of 5 mM H_2O_2 for 10 min. The gel was washed twice with distilled water, and then was soaked in 50mM potassium phosphate buffer (pH 7.0) containing 0.5 mg

diaminobenzidine ml^{-1} to develop the background brown colour. No colour would appear in the area where catalase depleted H_2O_2.

2.5.4 Quantification of actinorhodin (Act) and undecylprodigiosin (Red)

10^6 spores of *ΔoxyR* and wild type were inoculated in NMMP (liquid media) and incubated at 30°C with shaking at 180 rpm. Cells were harvested daily for 4 days to measure the content of Act and Red as described previously (Shin et al.,2010). For Act, each 1 ml sample was first treated with 50 µl of 10 N NaOH, gently vortexed and allowed to stand for 5 min, followed by centrifugation at 4,000 × *g* for 5 min. The absorption spectra of supernatants were monitored by UV/VIS spectrophotometer (SHIMADZU model UV- 1650PC). The absorbance at 608 nm was taken to calculate the concentration of actinorhodin based on the extinction coefficient of 25,320 M^{-1} cm^{-1} Undecylprodigiosin (Red) was extracted similarly except that the cell mass was resuspended with 100% methanol and acidified with 10N HCl. The absorbance at 530 nm was taken to calculate its concentration based on the extinction coefficient of 100,500 M^{-1} cm^{-1}.

CHAPTER 3.

Results

3.1 Physiological growth of *ΔOxyR* in comparison with *M145* wild type

In order to further explore the function of deletion of *oxyR* on growth, differentiation and antibiotic production of *Streptomyces coelicolor*. We grew *ΔOxyR* and *M145* wild type on solid media such as NA (nutrient agar), SFM (soy flour mannitol), R2YE and liquid media like YEME, and liquid minimal medium(NMMP) with or without under the various stresses.

3.1.1 Growth comparison in liquid medium

ΔoxyR and M145 wild type strain spores were taken from lab stock. 10^6/µl spores of these two strains were inoculated into YEME (rich medium) and NMMP (minimal medium) containing glucose as carbon source. Then, growth was started to measure spectrofotomectrically after 13 hour incubation at 30°C at OD_{600}. In comparison with M145 wild type *ΔoxyR* mutant grows better in both YEME and NMMP mediums (Figure 3).

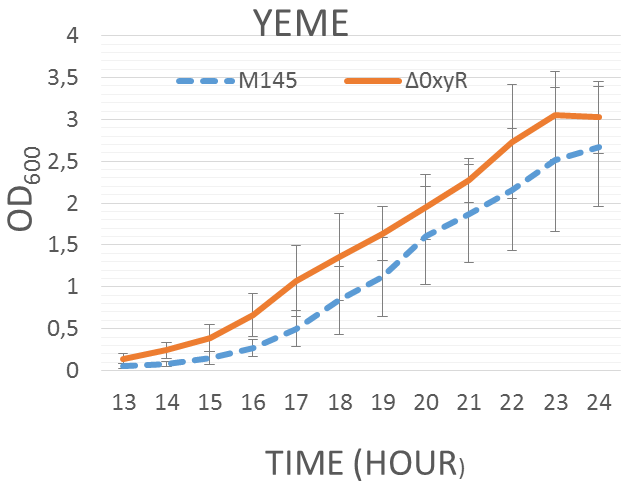
3.1.2 Growth comparison on solid medium

ΔoxyR and M145 wild type strains were tested with various stresses. Such as H_2O_2, tBHP (*tert*- butyl hydro peroxide) which is an organic hydro peroxide, diamide (DA) which is a thiol-specific oxidation agent. NaCl, EtOH, paraquat (PQ) which is often used to catalyze the formation of reactive oxygen species (ROS), more specifically, the superoxide free radical. Paraquat will undergo redox cycling *in vivo*, being reduced by an electron donor such as NADPH, before being oxidized by an electron receptor such as dioxygen to produce superoxide. Different concentration of spores were spotted on NA plates. On NA plate including 200 µM H_2O_2 *ΔoxyR* mutant showed better growth comparing to wild type contrary to our expectations. Under 500 µM diamide (DA) and 100 µM tBHP stress also ΔoxyR mutant showed better growth. On the other hand, under 4% EtOH, 200 µM NaCl and 100 µM PQ stresses wild type M145 and mutant exhibited similar growth (Figure 4).

We wanted to further examine the growth of *ΔoxyR* mutant under various concentrations of H_2O_2 on NA gradient plates. Gradient plates showed graded inhibition of both wild type and *ΔoxyR*. Wild type strain is more sensitive to hydrogen peroxide (Figure 5).

Morphology of M145 wild type, *ΔoxyR* mutant strains on various concentrations of hydrogen peroxide was studied. Spores were streaked and observed on NA and SFM plate. SFM stimulates sporulation of *Streptomyces coelicolor*. The mutant strain showed slightly more antibiotic production on NA plate and exhibited faster spore formation on SFM plates (Figure 6).

A.



B.

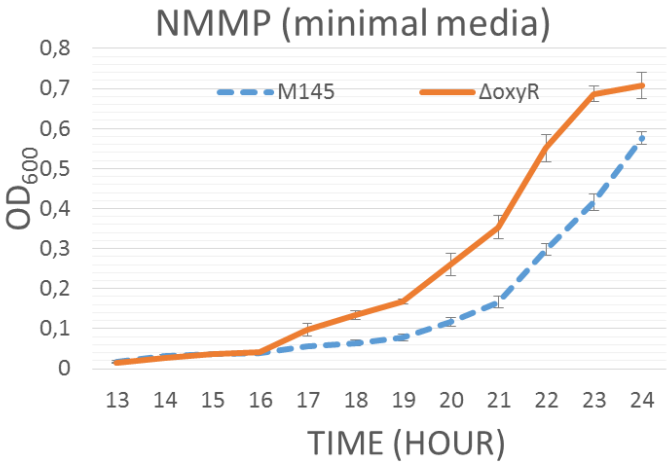


Fig. 3. Growth check in YEME and NMMP liquid media.

Equal numbers of spores were inoculated in 50 ml YEME and NMMP liquid media and O.D was measured during 12 hours after 12 hours from inoculation.

*These figures represents 3 independent experiments

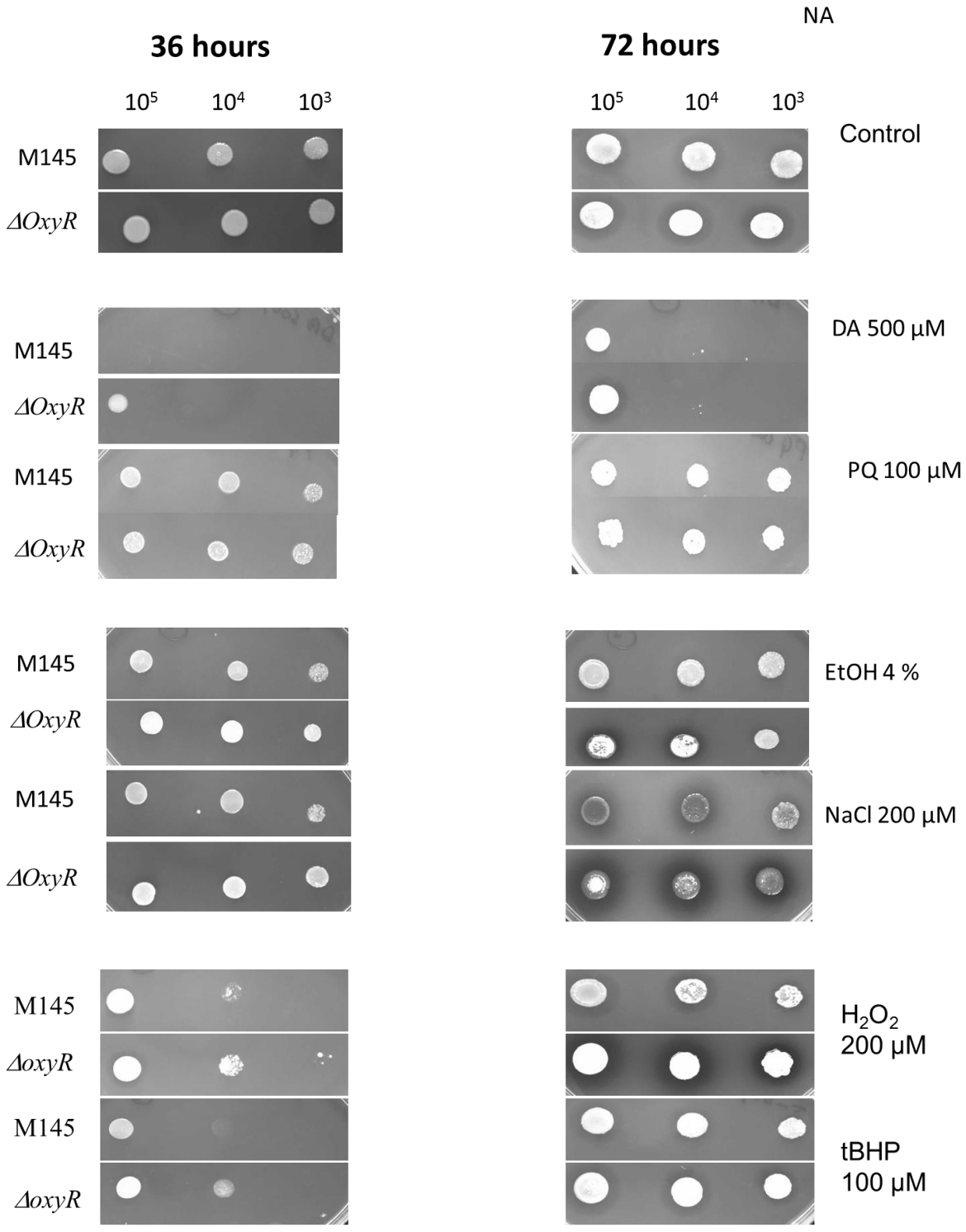


Fig.4. Sensitivity test for wild type and, *ΔoxyR* mutant strains to various stresses.

Wild type and mutant strain spores were equally spotted on each plate with 200 µM H_2O_2, 100 µM tBHP, 200 µM NaCl, 500 µM DA, 100 µM PQ and 4% EtOH. Pictures were taken at 36 and 96 hrs.

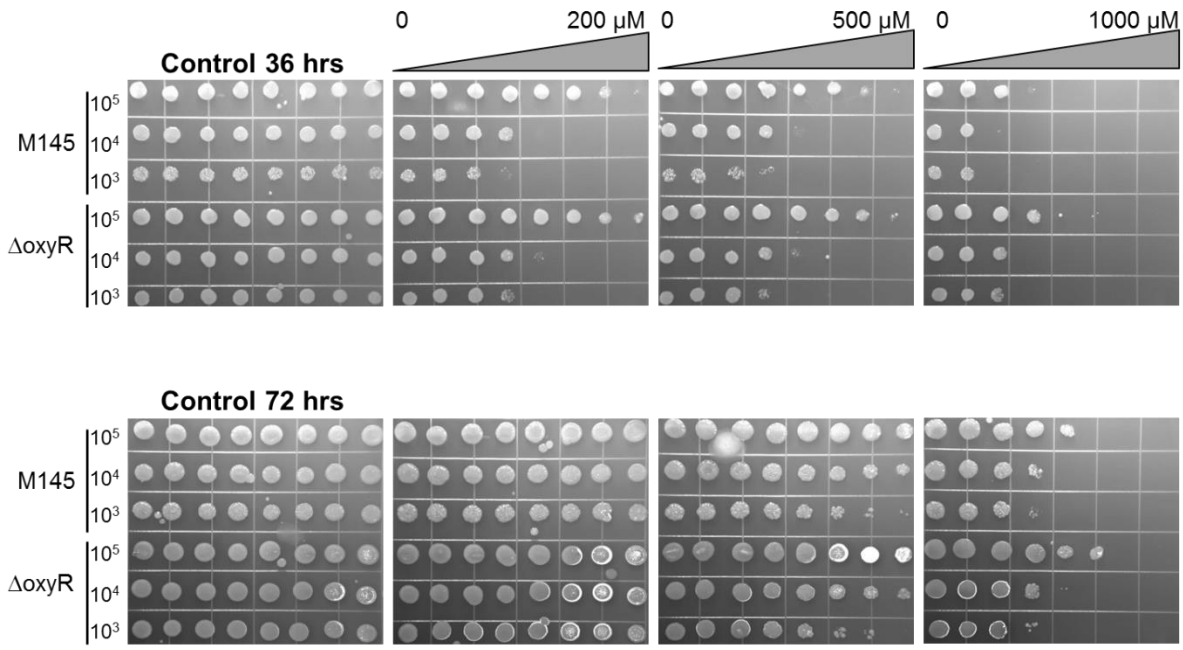


Fig.5. H_2O_2 sensitivity test for wild type and *ΔoxyR* mutant strains.

Wild type and *ΔoxyR* mutant strain spores were equally spotted on NA plate with H_2O_2 and a non- H_2O_2 added NA plate as a control. H_2O_2 concentration increases to the right-hand side with 200, 500 and 1000 µM. Pictures were taken at 36 and 72 hrs. M145- wild type strain.

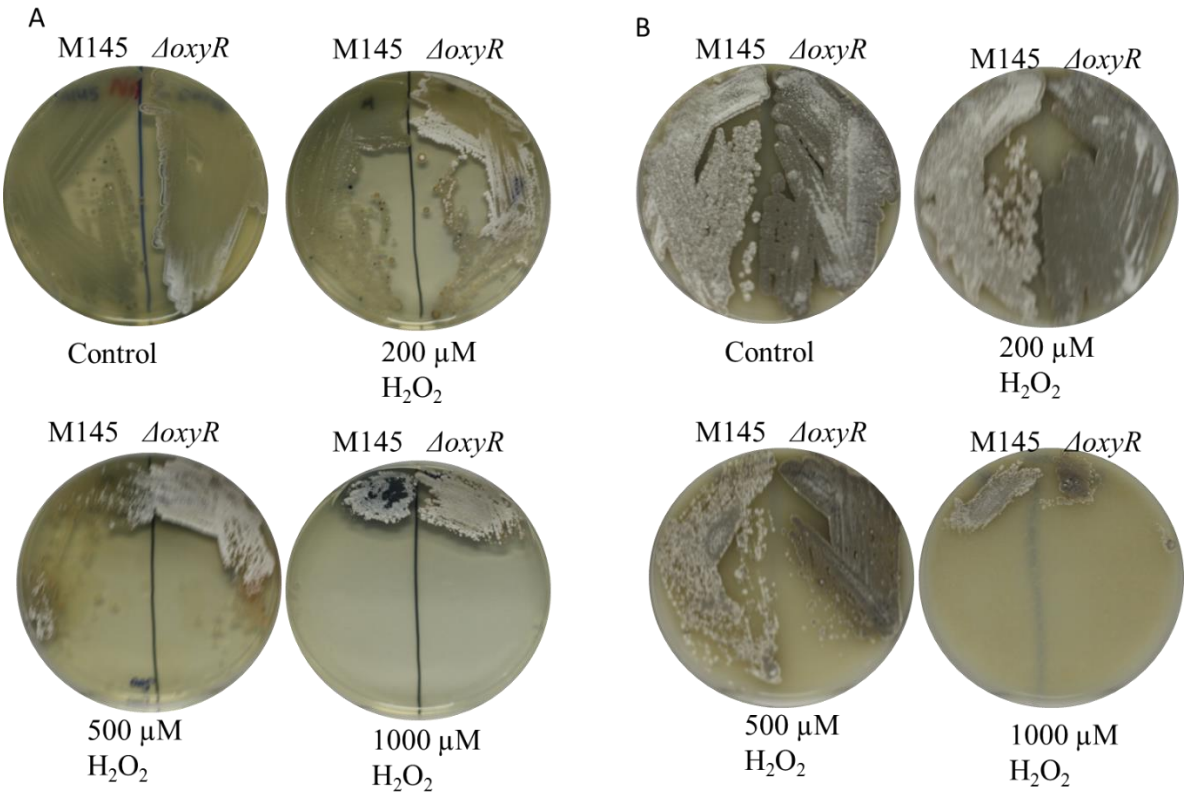


Fig.6. Growth and sporulation of wild type and *ΔoxyR* mutant strains under hydrogen peroxide stress.

A. Wild type and *ΔoxyR* mutant strain spores were streaked on NA plate with or without various concentrations of H_2O_2.

B. Wild type and *ΔoxyR* mutant strain spores were streaked on SFM plate with or without various concentrations of H_2O_2 for sporulation. Pictures were taken at 4^{th} day.

3.1.3 Catalase A production in *ΔoxyR* mutant

Hydrogen peroxide which is generated during aerobic metabolism and also can damage critical biomolecules. *Streptomyces coelicolor* has three kind of known transcription regulation systems to control expression of oxidative defense enzymes. OxyR activates the alkyl hydro peroxide reductase (ahpCD) (Hahn et al., 2002), CatR, a peroxide responsive fur homologue, represses major catalase (CatA)(Hahn et al, 2000b) and RsrA is an anti-sigma factor for SigR, which directs the expression of thioredoxin genes in response to high concentration of H_2O_2 and disulfide stress.

It has been reported that AhpCD is responsible for detoxification of low concentration of H_2O_2 endogenously formed while CatA play an important role in protection from higher concentration of H_2O_2 or in aerial mycelium stage in which AhpCD levels are low. Moreover, in Δ*oxyR* mutant as expected, *ahpCD* expression levels are reduced. On the other hand, *catA* expression levels are high comparing to wild type strain in *Streptomyces coelicolor (*Dr. So young Oh thesis, 2006).

According to our physiological studies, we observed that Δ*oxyR* mutant grows and shows better sporulation than wild type strain on NA and SFM plates with or without treatment of various concentrations of H_2O_2. Then, we hypotised that catalase levels are high in Δ*oxyR* mutant which makes mutant strain more resistance to peroxide and rescue cells from growth defect.

First of all, based on the S1 mapping data which suggested that in Δ*oxyR* mutant *catA* transcription levels are higher than wild type without any treatment of H_2O_2. Then, we further confirmed by catalase activity staining that catalase A activity is elevated in Δ*oxyR* mutant with or without under stress of hydrogen peroxide (Figure 7).

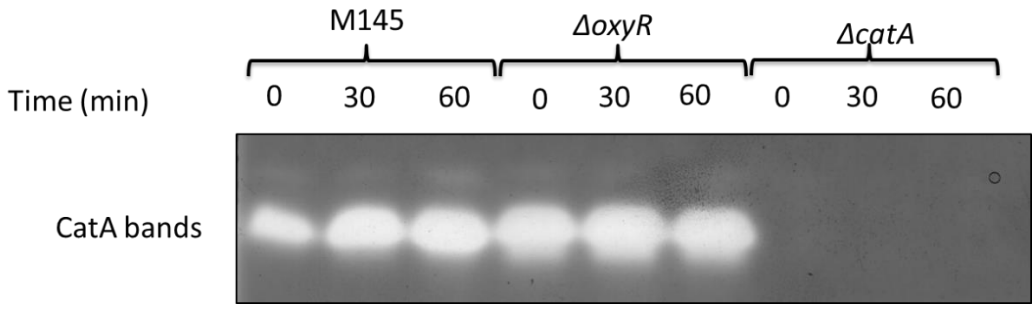


Fig.7. Comparison of catalase A activity of wild type and *ΔoxyR* mutant strains.

Streptomyces coelicolor wild type, *ΔoxyR* and *ΔcatA* cells were grown to exponential phase in YEME and treated with 100 µM H_2O_2. Samples were taken at 30 min intervals over 60 min and 20 µg of crude extract loaded to 7 % native gel the catalase activity staining was done. Catalse A depleted more area on native gel in *ΔoxyR* with or without treatment of hydrogen peroxide comparing to wild type confirming that catalase A levels are higher in *ΔoxyR*. *ΔcatA* was used as negative control proving that bands are CatA bands.

*This figure represents 3 independent experiments.

3.1.4 Antibiotic production in *ΔoxyR* mutant

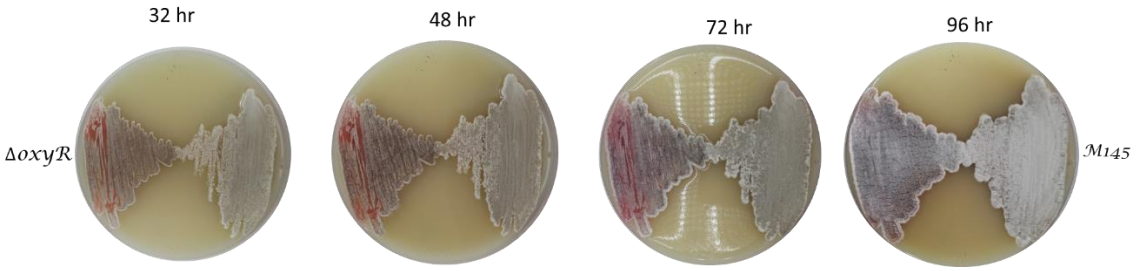
Actinorhodin (Act) and undecylprodigiıosin (Red) are well known antibiotics that *Streptomyces coelicolor* produces. ActII-orf4 and RedD were identified as pathway-specific regulators involved in the production of representative antibiotics, ACT and RED, respectively (Takano et al. 1992). Many regulators are involved in antibiotic production.

We have studied antibiotic production of *ΔoxyR* in comparison with wild type.

On solid media such as SFM and R2YE, *oxyR* deletion strain displayed increasing amount of antibiotic production (Figure 8).

We inoculated same amount of *ΔoxyR* and M145 wild type spore in minimal media (NMMP) containing 5 % glucose as carbon source.Then, quantify the antibiotic production as described in Shin et al, 2011. In NMMP, actinorhodin production was enhanced in oxyR deletion mutant. On the other hand, *ΔoxyR* and M145 wild type strains showed similar amount of Red production (Figure 9).

A.



B.

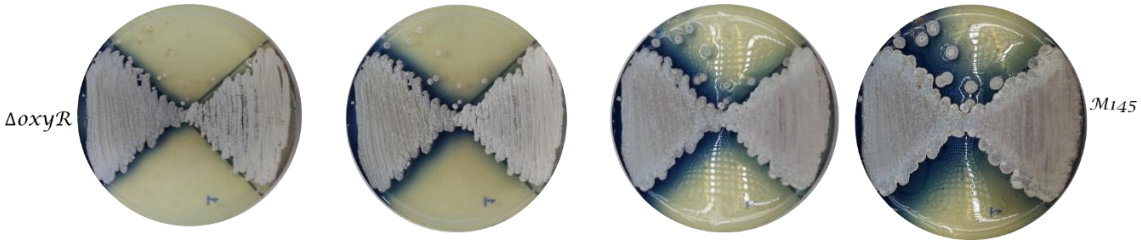


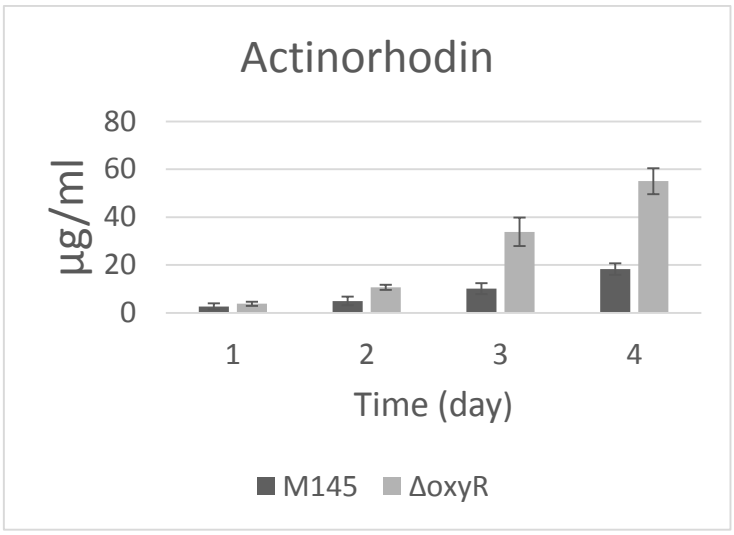
Fig.8. Antibiotic production comparison on solid media.

A. ΔoxyR and M145 wild type spores were streaked to SFM plate.

B. ΔoxyR and M145 wild type spores were streaked to R2YE plate.

Formation of aerial mycelia, spores, and pigmented antibiotics was examined visually by taking photos at indicated time points.

A.



B.

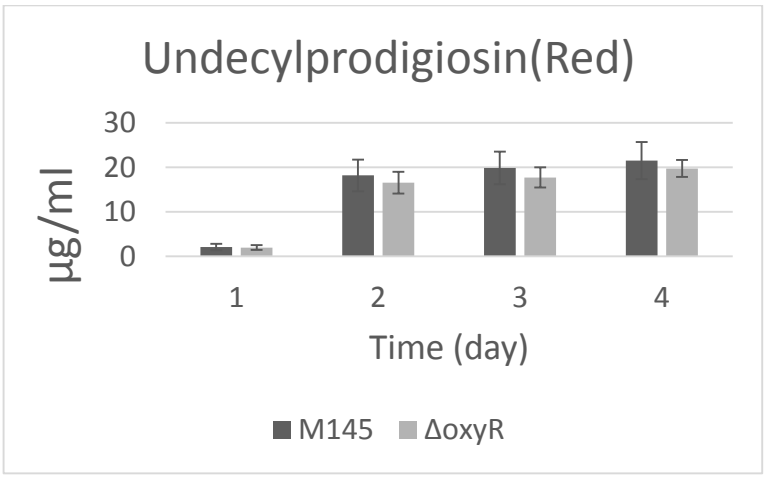


Fig.9. Actinorhodin and Undecylprodigiosin production of *ΔoxyR* in minimal liquid media.

10^6 spore of ΔoxyR and M145 wild type were inoculated in NMMP following incubation at 30°C for five days. Quantification started to be done the day after inoculation and quantify for 4 days.

3.2 PCR-based tandem epitope tagging for CHIP

Streptomyces has complex regulatory systems at transcription and translation levels for sensing and signal transduction to adapt a wide range of nutritional and environmental conditions. OxyR is a peroxide sensing transcription regulator in *Streptomyces coelicolor*. Prof. Roe Jung Hye laboratory has confirmed some new positive target genes for OxyR then we aimed to learn binding sequence for this protein to further study of this transciption regulator. Moreover, we also have tagged other peroxide sensing regulator CatR. Ji-Nu Kim *at al.*,2012 developed a versatile PCR-based tandem epitope tagging for *Streptomyces coelicolor* genome. They combined tagging system with chromatin immunoprecipitation (CHIP). CHIP experiments require antibodies which are highly specific against the target proteins. On the other hand, our anti-OxyR antibody is not specific to OxyR protein. Because of that we tagged our genes of interest by following their procedure.

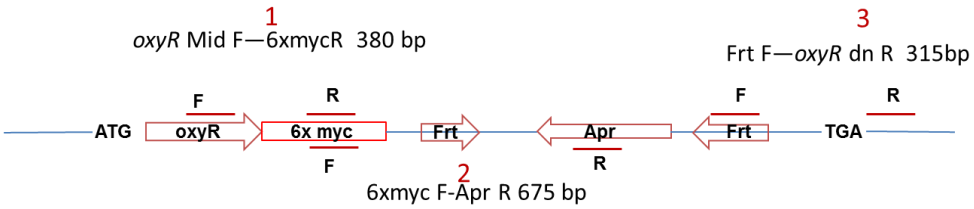
The PCR-based tagging strategy applied here starts with amplifying a DNA segment, which begins with the tandem epitope sequence followed by a drug-resistance gene flanked by FRT sites. In addition, the amplifiable segment has homologous sequences to the last portion and to a downstream region of the targeted gene. The precise insertion of the DNA segment into the cosmid containing the target gene was achieved by electroporating the PCR-amplified DNA segment into *E. coli* BW25113/pIJ790 containing the cosmid followed by λ-Red-mediated recombination. The epitope-inserted cosmid was then transported into the methylation-deficient *E. coli* (ET12567/pUZ8002) and transferred to *S. coelicolor M145* by conjugation. (Gust *at al.*, 2003).

3.2.1 6xmyc tagging of *oxyR* in *Streptomyces coelicolor*

6xmyc tagging of to *S. coelicolor oxyR* has been done as explained at experimental part. Genomic tagging needed to be confirmed by PCR however, for *S. coelicolor* genomic DNA PCR getting whole sequence PCR product is really difficult to overcome this problem we designed 3 pairs of primers (Figure 10-A). Then, genomic DNA has been extracted from each tagged strain as a PCR template. PCR has been carried out using 3 different pairs of primers (Figure 10-B). After confirmation of 6xmyc tagging by PCR, PCR products have been sent to sequencing. DNA sequencing confirmed that tagging was successful. 6xmyc tagging of *oxyR* has been validated by Western blot using anti-myc antibody (Figure 11-A, B).

Ji-Nu Kim *at al.*, 2012 suggests that some epitope-fused proteins lose their in vivo functions. Phenotype of target proteins need to be compared with those of wild type. For comparison we streaked 6xmyc tagged *oxyR*, M145 wild type and *ΔoxyR* mutant strains on to SFM plate. We observed that the tagged strains show wild type behavior as expected (Figure 12).

A.



B.

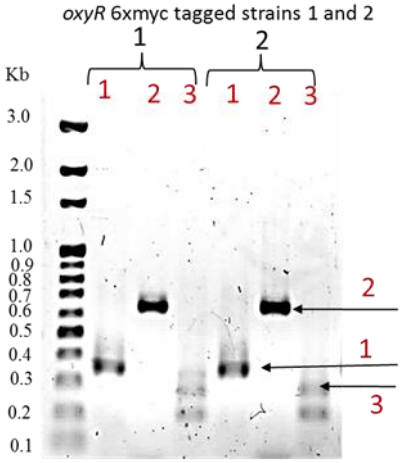


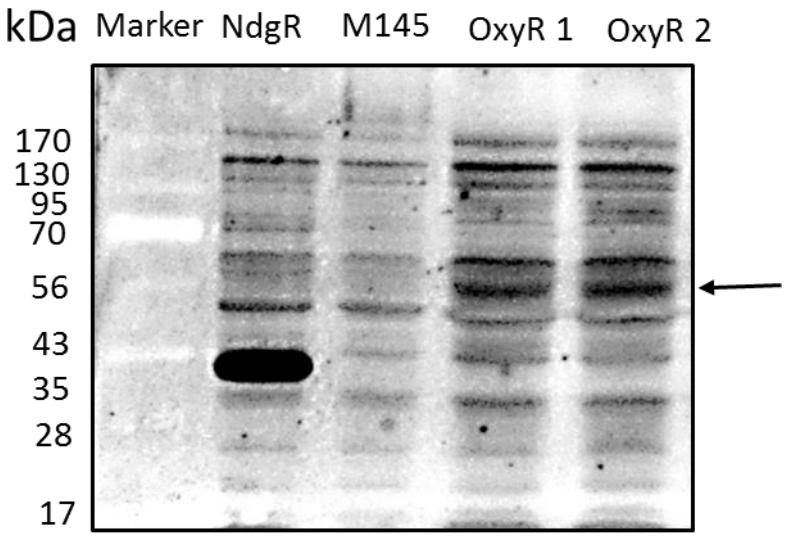
Fig.10. Confirmation of 6xmyc tagging of *oxyR* in *Streptomyces coelicolor* by PCR.

A.Primer design for PCR confirmation of 6xmyc tagging.

Three pairs of primers were designed. First pair forward primer is in *oxyR* ORF, reverse primer homologue to 6xmyc sequence. Second primer pair's forward primer homologues to 6xmyc and reverse primer homologues to Apr^r cassette. Third pair forward primer homologues to FRT cassette and reverse primer homologues to downstream of *oxyR* gene.

B. PCR confirmation of 6xmyc tagging.Genomic DNA of two different tagged strain were amplified using three pairs of primers. First primer pair product is about 380 bp. Second primer product is 675 bp and finally third pair primer product is 315 bp. PCR confirmed that tagging was successful.

A.



B.

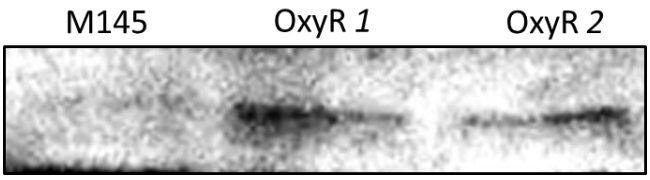


Fig.11. Confirmation of 6xmyc tagging *S.coelicolor* oxyR by western blot.

A. 6xmyc tagged *S.coelicolor* NdgR which is a gift from Ji Nu Kim was used as positive control for myc antibody specificity.Expected band size for NdgR is 32.2 kDa (25 kDa NdgR+7 kDa 6xmyc). M145 wild type was used as negative control.Expected western band size for OxyR 6xmyc tag is 40.2 kDa (33 kDa OxyR+7 kDa 6xmyc). *Arrow shows expected specific bands for 6xmyc tagged OxyR.

B. Same amount of crude extract were subjected to 10% SDS-PAGE gel.Then, we reconfirmed 6xmyc tagging of OxyR with an another independent western blot experiment which shows specific bands.

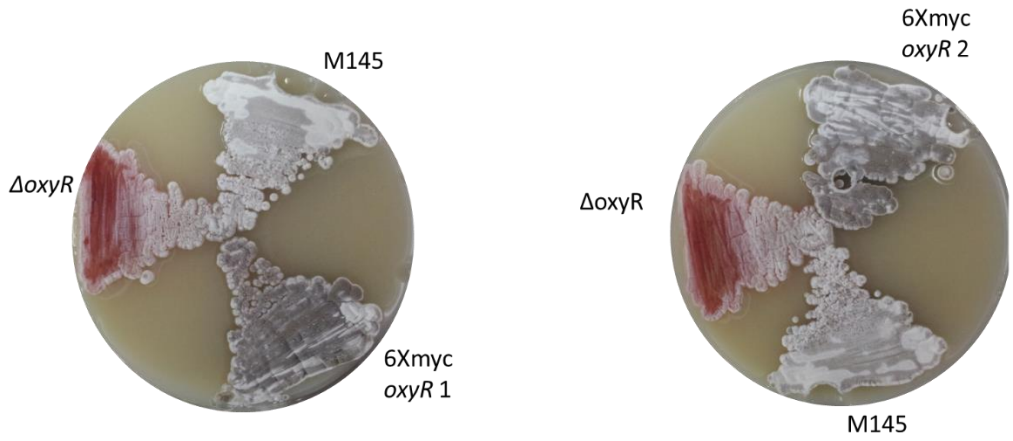


Fig. 12. Phenotype of 6xmyc tagged *oxyR* strains 1 and 2.

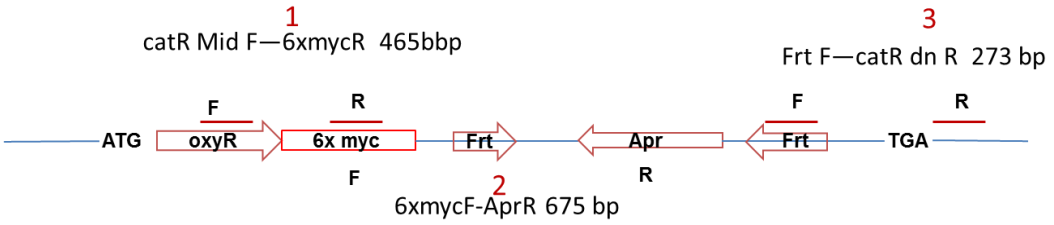
M145 wild type, *ΔoxyR* and 6xmyc tagged *oxyR* strains were streaked on to SFM plate for comparison of phenotypes.6xmyc tagged *oxyR* strains produce spores and antibiotic like wild type but *oxyR* mutant does not.

3.2.1 6xmyc tagging of *catR* in *Streptomyces coelicolor*

6xmyc tagging of to *S. coelicolor catR* has been done as explained at experimental part. Genomic tagging needed to be confirmed by PCR however, for *S. coelicolor* genomic DNA PCR getting whole sequence PCR product was hard to overcome this problem we designed 3 pairs of primers for (Figure 13-A). Then, genomic DNA has been extracted from each tagged strain as a PCR template. PCR has been carried out using 3 different pairs of primers (Figure 13-B). After confirmation of 6xmyc tagging by PCR, PCR products have been sent to sequencing. DNA sequencing confirmed that tagging was successful. 6xmyc tagging of *catR* has been validated by Western blot using anti-myc antibody (Figure 14).

Ji-Nu Kim *at al.*, 2012 suggests that some epitope-fused proteins lose their in vivo functions. Phenotype of target proteins need to be compared with those of wild type. For comparison we streaked 6xmyc tagged *catR*, M145 wild type and *ΔcatR* mutant strains on to R2YE plate. We observed that the tagged strain shows wild type behavior as expected (Figure 15).

A.



B.

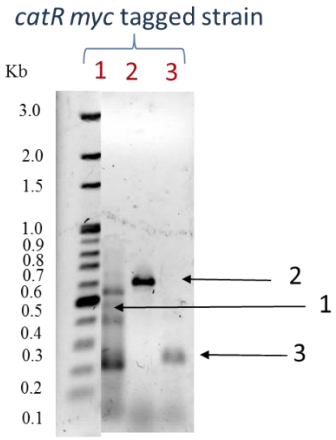


Fig.13. Confirmation of 6xmyc tagging of *catR* in *Streptomyces coelicolor* by PCR.

A.Primer design for PCR confirmation of 6xmyc tagging.Three pairs of primers were designed. First pair forward primer is in *catR* ORF, reverse primer homologue to 6xmyc sequence. Second primer pair's forward primer homologues to 6xmyc and reverse primer homologues to Apr^r cassette. Third pair forward primer homologues to FRT cassette and reverse primer hologues to downstream of *catR* gene.

B. PCR confirmation of 6xmyc tagging.Genomic DNA of tagged strain was amplified using three pairs of primers. First primer pair product is about 465 bp. Second primer product is 675 bp and finally third pair primer product is 278 bp. PCR was confirmed that tagging was successful.

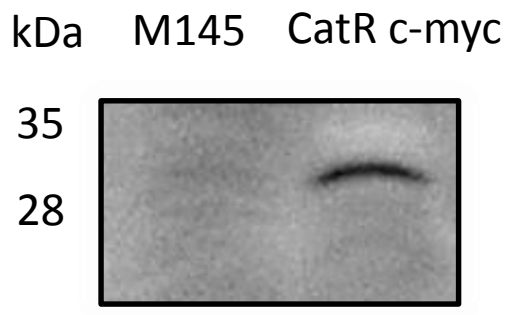


Fig.14. Confirmation of 6xmyc tagging *S.coelicolor* catR by western blot.

Same amount of crude extract were subjected to 13% SDS-PAGE gel.Then, we confirmed 6xmyc tagging of CatR by western blot experiment which shows specific bands. M145 wild type was used as negative control.Expected western band size for CatR 6xmyc tag is 22..2 kDa (15.2 kDa CatR+7 kDa 6xmyc).

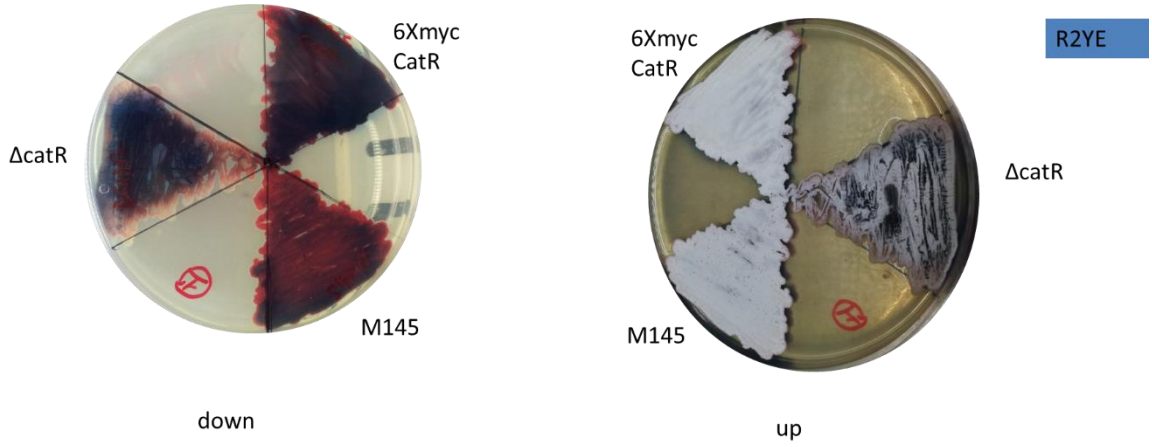


Fig. 15. Phenotype of 6xmyc tagged *catR* strain.

M145 wild type, *ΔcatR* and 6xmyc tagged *catR* strains were streaked on to R2YE plate for comparison of phenotypes.6xmyc tagged *catR* strain produces spores and antibiotic like wild type but *catR* mutant does not.

CHAPTER 4.

Discussion

Oxidative stress, resulting from exposure to reactive oxygen species (ROS) which can damage proteins, DNA, and membranes, is major challenge for all living organisms.*Streptomyces* are soil-dwelling bacteria, which produce a diverse range of secondary metabolites such as natural antibiotics. Members of this genus have complex regulatory systems at transcription and translation levels to adapt changing environmental conditions. *Streptomyces* have developed complicated defense system against ROS resistance.

OxyR and CatR are peroxide sensing transcription regulators in *Streptomyces coelicolor*.

OxyR is a global regulator of the peroxide stress response that maintains intracellular H_2O_2 levels within safe limits.Previous studies have shown that OxyR activates *ahpCD* gene transcription.AhpCD is responsible for detoxification of alkaly hydroperoxides. CatR is a Fur homologue which regulates CatA. CatA is major catalase that play an important role in protection from higher concentration of H_2O_2.

Phenotype of *ΔoxyR* has been studied which suggests that *ΔoxyR* mutant grew faster in liquid YEME rich and minimal media.On solid media, with or without under stress of H_2O_2. *ΔoxyR* displayed faster growth and sporulation comparing to wild type strain.

Actinorhodin and undecylprodigiosin (Red) are antibiotics that *Streptomyces coelicolor* naturally produces. Many regulators have been identified to involve in antibiotic production. On solid SFM, R2YE media and minimal liquid media *oxyR* deletion mutant produced higher amount of actinorhodin whereas no effect on Red production. These results suggest that OxyR might play a role in antibiotic production.

Our study about behaviour of *oxyR* deletion strain against various stresses showed that *ΔoxyR* displayed better growth and sporulation.Previous studies suggests that *catA* transcription elevated in *ΔoxyR* strain. S1 mapping analysis also confirmed this phenomenen.Furthermore, catalase activity staining further proved that *oxyR* deletion strain

produced more catalase A.Here we postulate a possible model to explain this results.This model is expanded version of Hahn et al., 2000b as demonsrated in fig. 16.

Firstly,In *ΔoxyR*, OxyR can not activate *ahpCD* transcription as a result of this AhpCD activity decreases. Low level of AhpCD may not reduce endogenously produced peroxides which may cause oxidation of CatR.Oxidized CatR derepresses *catA* and *catR* gene transcription. Catalase A levels are elevated on the other hand, catalse A decomposes H_2O_2 but other endogenously produces peroxides such as alkaly hydro peroxides still may not be removed. These peroxides may oxidized CatR. In conclusion, catalase A production increases and may rescue *ΔoxyR* from growth defect.

Secondly, our results strongly suggest that *oxyR* deletion mutant produces high levels of catalase A in comparision with wild type strain. Reduced CatR amounts suppose to be lower in *ΔoxyR* which can not repress *catA* transcription as effective as in wild type, resulting production of more catalse A by *oxyR* mutant strain. Further experiments need be done to see CatR levels in *oxyR* mutant to understand and explain the relationship between catalse A production and reduced CatR levels in *ΔoxyR*.

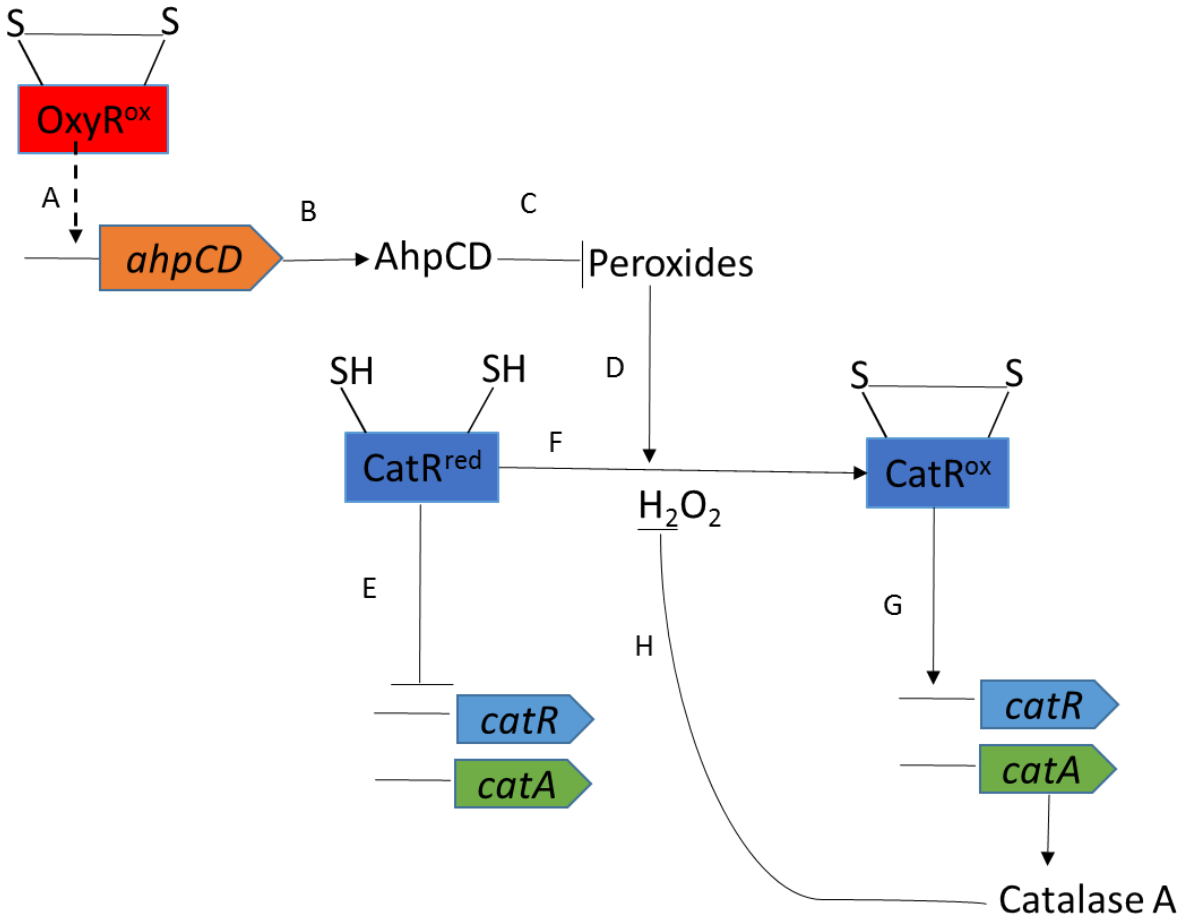


Fig. 16. A possible model for explanation of high catalse A production by *ΔoxyR* strain comparing to wild type strain.

Oxidized OxyR can not bind and induced *ahpCD* transcription (path A).AhpCD production highly decreased resulting increasement of endogenously produced peroxides (B,C). Reduced CatR represses *catA* and catR (E).More endogenously produced peroxides in cell may cause oxidation of CatR (D,F). Oxidized CatR derepresses its own and *catA* transcription (G).As a result of the induced catalase A removes H_2O_2.

According to mRNA sequencing data, 398 genes were positively and 1228 genes were negatively induced by 200 uM H_2O_2.We will reconfirmed by S1 mapping that SCO3132 (putative trans-aconitate methyltransferase), SCO3091(Cyclopropane-fatty-acyl-phospholipid synthase), SCO4409 (Putative RNA polymerase sigma factor), SCO3202 (RNA polymerase principal sigma factor(hrdD), SCO0570 (50S ribosomal protein L33) and SCO1519 (Holliday junction DNA helicase) are positive OxyR target genes.RNA sequencing analyses suggest that OxyR control other genes unrelated to oxidative stress or other oxidative stress gene rather than *ahpC*.In order to learn DNA binding sequence for OxyR and CatR we have tagged *Streptomyces coelicolor oxyR* and *catR* by 6xmyc fusion tag for CHIP.

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