Isolation and Genetic Mapping of Paraquat-Resistant Sporulating Mutants of *Streptomyces coelicolor*

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***S. coelicolor* A3(2) cells were treated with various redox-cycling agents on nutrient agar plates and examined for their effect on the growth and differentiation. When treated with plumbagin, severe effect on cell viability was observed at concentrations above 250 μM. However, the surviving colonies differentiated normally. When treated with 100 μM paraquat, growth rate was decreased and morphological differentiation was inhibited, while the survival rate was maintained at about 100% even at 5 mM paraquat. Menadione or lawsone did not cause any visible changes at concentrations up to 1 mM. The effect of paraquat was also observed when it was added to nutrient agar plate before spore inoculation. Paraquat had no effect on colonies growing on R2YE agar plates. Among the components of R2YE medium selectively added to nutrient agar medium, $CaCl_2$ was found to have some protective function from the inhibitory effect of paraquat. As a first step to study the mechanism of the inhibitory effect of paraquat on differentiation, resistant mutants which sporulate well in the presence of paraquat were screened following UV mutagenesis. Three paraquat-resistant mutants were isolated with a frequency of 3×10^{-5}. Their mutation sites were determined by genetic crossings. All three mutations were mapped to a single locus near *arg4* at about 1 o'clock on the genetic map of *S. coelicolor* A3(2).**

Key words: *S. coelicolor* A3(2), redox-cycling agents, differentiation, paraquat-resistant mutants

The genus *Streptomyces* includes a variety of species which have a complex life cycle of morphological and physiological differentiation. When grown on solid media, they form a network of branching hyphae to maximize the use of various hydrolytic enzymes secreted, continue to develop a mass of aerial hyphae and eventually form uninucleate spores. A variety of secondary metabolites produced by the members of *Streptomyces* are indispensible to human life especially in medical applications. *S. coelicolor* A3(2) is a member of the genus *Streptomyces*, classified into the *S. albidoflavus* group (17). It is genetically the best characterized species, and its genes involved in morphological differentiation (1, 15) and synthesis of antibiotics (2, 5, 6) have been studied extensively (reviewed in ref. 3). In addition to a well-known genetic linkage map, its chromosome has been physically mapped (11). The combined genetic and physical map, with all other characteristics, makes *S. coelicolor* A3(2) one of the model species among the Gram-positive bacteria.

Oxygen metabolism in aerobically growing organisms can induce stressful, and even fatal conditions by generating reactive oxygen species (ROS; 4, 10). It has been of interest for decades to elucidate what are responsible for the generation of these reactive species and how organisms cope with the toxicity and potential damages from them (reviewed in ref. 4, 13). *S. coelicolor*, an obligate aerobe, is bound to encounter ROS throughout its life cycle. Especially when it initiates the growth of hyphae into the air, it could be in a hyperoxidant state. It has been reported that, in *Neurospora crassa* a decrease in the reduced-to-oxidized ratio of NAD and NADP coenzymes was accompanied with the induction of the growth of aerial hyphae (16). Lee *et al.* (14) have observed that there exists an inducible response system against oxidative stress in *S. coelicolor*. Defense enzymes consisting of this system have been purified and characteriz-

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ed in *S. coelicolor* (12) and in another *Streptomyces* sp. (19).

We were interested in observing the effect of redox-cycling agents on the growth and development of *S. coelicolor* A3(2). Redox-cycling agents can generate superoxide radicals *in vivo* by cycles of reduction and autooxidation, conferring single electron to dioxygen (7, 8). Youn has observed the effect of lawsone on the differentiation of a *Streptomyces* sp. isolated from soil (18). We tested the effect of several redox-cycling agents on the differentiation of *S. coeilcolor* and isolated mutants which are resistant to the toxic effect of paraquat and characterized these mutants genetically as a first step toward understanding the effect of reactive oxygen species on the development of *S. coelicolor*.

Materials and Methods

Bacterial strains

The wild type strain used was J1501, a derivative of *S. coelicolor* A3(2). J650 and 1514 strains used for genetic analysis were also derivatives of *S. coelicolor* A3(2) (Table 1). These strains were kindly provided by Drs. D. Hopwood and K. Chater in John Innes Centre, U.K..

Bacterial growth

Solid media used for physiological (R2YE and nutrient agar media) and genetic studies (minimal medium) were prepared as described by Hopwood *et al.* (9). All the strains were incubated at 30°C.

Treatment with redox-cycling agents

Spores were spread on agar plates at about 200 colonies per plate. After 24 to 36 hours of incubation, plates were soaked for 1 hour in 10 ml of aqueous solution containing one of the redox-cycling agents (Fig. 1). Each chemical was freshly dissolved (menadione sodiumbisulfite, 100~1,000 mM in water; lawsone, 25~50 mM in ethanol; plumbagin, 10~50 mM in ethanol; paraquat dichloride, 25~100 mM in water) and diluted in distilled water.

Isolation of paraquat-resistant mutants

Spores of J1501 strain were irradiated with UV light to a survival rate of 0.1 to 0.5% and spread on nutrient agar (NA) plates at 500 to 800 colonies per plate. Colonies were treated with 100~250 μM paraquat for 1 hour after 24 to 32 hours of incubation when they became visible. Colonies sporulated on the primary screening plates were transferred by picking to fresh NA plates as duplicates or triplicates and treated with various concentrations of paraquat at 24 to 32 hours after inoculation, or transferred to NA plates already containing 50~100 μM paraquat (NA-PQ plates). Colonies sporulating on paraquat-treated plates were identified as paraquat-resistant mutants.

Genetic mapping

Mutants were crossed with J650 or 1514 strain and their recombinant progeny were selected and analyzed as described by Hopwood *et al.* (9). Chromosomal recombination was mediated by the plasmid SCP1, which is integrated at 9 o'clock on the genetic map of J650 and 1514 to give the NF fertility type. Parental cultures were mixed on R2YE plates and their spore progeny were plated on minimal media (MM) containing streptomycin (to exclude J650 or 1514 parental genomes) and all growth factor complements except histidine (to ex-

Table 1. Strains of *S. coelicolor* A3(2) used in this study.

Strain	Relevant genotype[a]	Source
J1501	*hisA1 uraA1 strA1* SCP1 SCP2 pgl	Hopwood
J650	*cysD18 mthB2 agaA1* NF SCP2⁻	Hopwood
1514	*proA1 cysA15 argA1 uraA1 nicA1 agaA1* NF SCP2⁻	Hopwood
U501	*hisA1 uraA1 strA1 pqr-501* SCP1 SCP2 pgl (*pqr-501* in J1501)	this study
H5013	*hisA1 strA1 pqr-501* NF	this study
U605	*hisA1 uraA1 strA1 pqr-605* SCP1 SCP2 pgl (*pqr-605* in J1501)	this study
H6054	*hisA1 strA1 pqr-605* NF	this study
U607	*hisA1 uraA1 strA1 pqr-607* SCP1 SCP2 pgl (*pqr-607* in J1501)	this study
H6071	*hisA1 strA1 pqr-607* NF	this study

[a] Abbreviations: SCP1, *S. coelicolor* plasmid 1; SCP2, *S. coelicolor* plasmid 2; NF, SCP1 is integrated into the chromosome at 9 o' clock; pgl , ΦC31 sensitive.

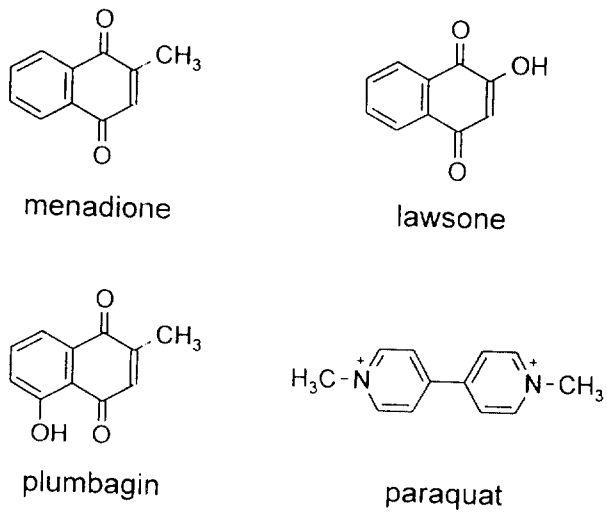


Fig. 1. Structures of redox-cycling agents used in this study.

clude mutant parental genomes, which are derivatives of J1501). Selected *his*$^-$ *strA1* recombinants were transfered to new plates (R2YE or MM) and allowed to sporulate. Spores of recombinants were then patch-plated on MM containing streptomycin and all growth factors required except histidine or histidine and one more amino acid to analyze the frequency of other markers. Spores of recombinants were also pick-plated on NA and NA-PQ plates to test their resistancy to paraquat. J650 and 1514 strains exhibited a paraquat sensitivity comparable to that of J1501, so the paraquat-resistant phenotype of recombinants could be segregated in the same way.

Results and Discussion

Effect of redox-cycling agents on *S. coelicolor*

S. coelicolor (strain J1501) cells growing on NA plates were treated with various redox-cycling agents generating superoxide radical *in vivo*. Quinones such as menadione, lawsone, and plumbagin as well as a bipyridine paraquat were tested for their effect on growth and differentiation (Fig. 1). We found out that menadione and lawsone had no effect either on growth or on differentiation at concentrations up to 1 mM (Fig. 2A and B; data on lawsone not shown). The treatment with plumbagin severely inhibited cell viability. When treated after 24~28 hours of incubation, about 40% of colonies survived with 100 μM of plumbagin, and none with 200 μM. When treated after 32~36 hours, only 20% of colonies survived with 250 μM, and none with 500 μM (Fig. 2C and D). However, in any case, surviving colonies differentiated normally.

Upon treatment with paraquat, we could see a distinguishing effect on the growth of *S. coelicolor* when compared with other redox-cycling agents used. At 100 μM paraquat, growth rate was significantly reduced and morphological differentiation was inhibited. However, the

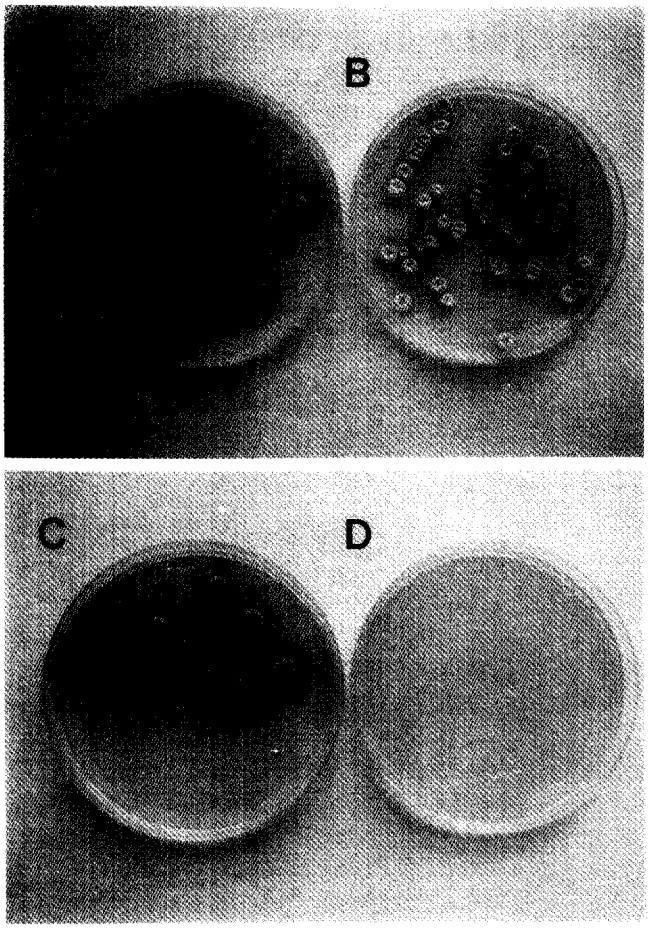


Fig. 2. Effect of menadione and plumbagin on NA plates. Spores of J1501 strain were spread on plates, grown for 32 hours, and treated with the following compounds for 1 hour; (A) none, (B) 1 mM menadione, (C) 250 μM plumbagin, (D) 500 μM plumbagin.

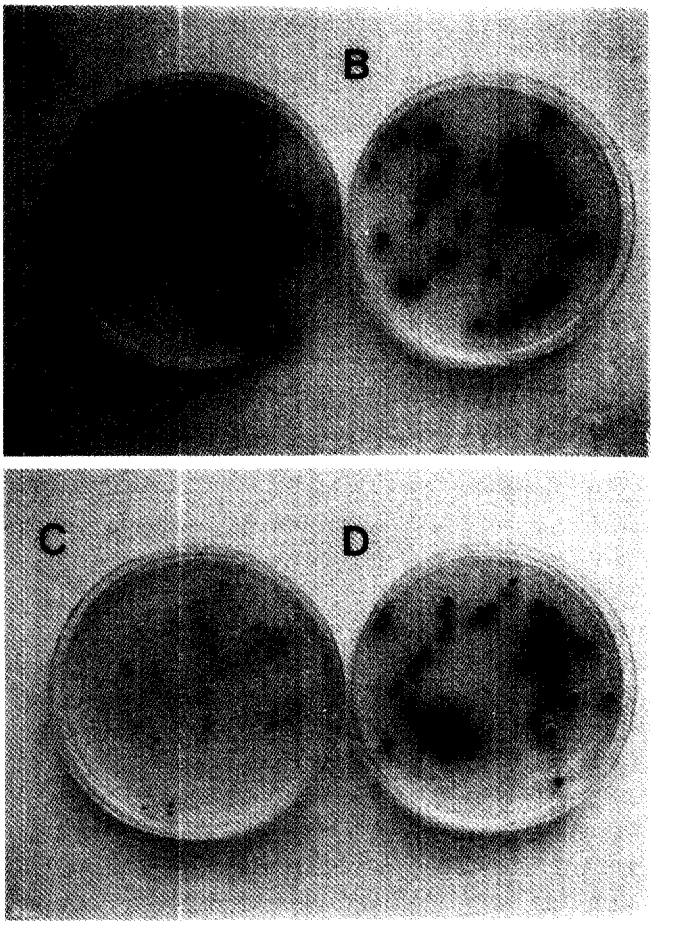


Fig. 3. Effect of paraquat on NA plates. Colonies were grown without any treatment (A), treated with 100 μM paraquat (B), or 5 mM paraquat (C) for 1 hour at 32 hour incubation, or grown on NA plate containing 50 μM paraquat (D).

Table 2. R2YE components tested for their protective effect from the sporulation inhibition by paraquat.

		Concentration in R2YE	Concentration tested in NA	Sporulation in NA-PQ[a]
Carbon source	Sucrose	10.3%	10.3%	−
			5.2%	−
	Glucose	1.0%	1.0%	−
			0.5%	−
Amino acid	Proline	0.3%	0.3%	−
Salts	K_2SO_4	1.4 mM	1.4 mM	−
	KH_2PO_4	0.4 mM	0.4 mM	−
	$MgCl_2$	50 mM	50 mM	−
			20 mM	+/−
			10 mM	−
	$CaCl_2$	20 mM	20 mM	+
			10 mM	+
			5 mM	+/−
Acid/Alkali	TES and NaOH			−

[a] +, good sporulation; −, no sporulation; +/−, poor or nonreproducible sporulation.

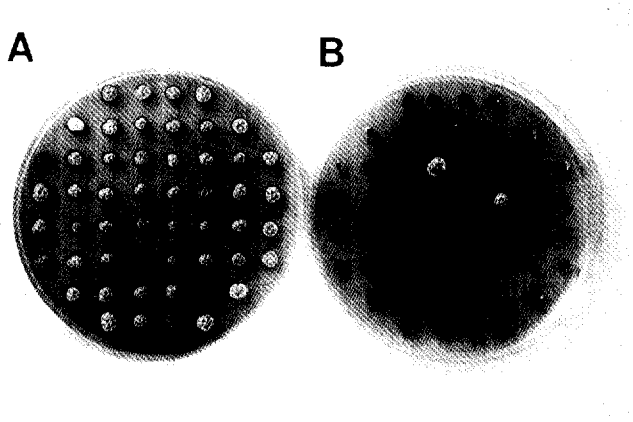


Fig. 4. Isolation of mutants resistant to the inhibitory effect of paraquat. Spores of J1501 strain were irradiated with UV light to a survival rate of 0.1 to 0.5%. Surviving colonies were treated with 100 μM paraquat and sporulating colonies were selectively transferred in duplicate to an NA plate (A) and an NA plate containing 50 μM paraquat (B).

survival was maintained almost 100% even when treated with 5 mM paraquat (Fig. 3A-C). The same effect was observed when paraquat was added to NA plates before inoculating spores (Fig. 3D). The effect of paraquat was dependent on the cell density: when we inoculated more than 500 spores per plate or streaked them using a loop, only well-isolated colonies showed abnormal phenotype, whereas nearby colonies went on normal differentiation together (data not shown). Colonies grown on NA-PQ plates could sporulate normally when transferred to a fresh NA plate (data not shown). Paraquat, however, did not inhibit the production of pigments, even though the blue pigment (actinorhodin) did not diffuse out upon paraquat treatment.

When colonies grown on R2YE plates were treated with paraquat, no such effect was observed at up to 5 mM paraquat (data not shown). To find out what was responsible for this difference, we selectively added various components of R2YE medium to NA and NA-PQ plates at the same concentrations as in R2YE and examined whether cells sporulate normally. Among the added components, only 20 mM $CaCl_2$ was reproducibly found to have some protective function from the inhibitory effect of paraquat (Table 2). Colonies growing on NA-PQ plates complemented with even lower concentrations of $CaCl_2$ (down to 10 mM) sporulated well. It did not seem that $CaCl_2$ in NA-PQ plates had affected the stability of paraquat, for its protection effect could be reproducibly observed when colonies growing on NA plates complemented with $CaCl_2$ were treated with paraquat after 36 hours of incubation.

The effect of each redox-cycling agent might be varied depending on the type of strains and culture media. Lawsone, which showed no effect on the growth of *S. coelicolor* on NA medium, severely inhibited morphological differentiation of a soil isolate *Streptomyces* sp. IMSNU-1 on Bennett agar medium (18). The inhibitory concentration of lawsone was about the same as that of paraquat on NA medium. We could not test the effect of redox-cycling agents on Bennett agar medium because our strain did not grow well on this medium even in the absence of any chemicals.

Isolation of paraquat-resistant mutants

Spores of J1501 strain were irradiated with UV light and screened for mutants which are resistant to the inhibitory effect of paraquat as described above (see Materials and Methods). Three paraquat-resistant mutants which sporulated well in the presence of paraquat were isolated at a frequency of about 3×10^{-5} (Fig. 4).

Phenotypic characterization of paraquat-resistant mutants

Spores of paraquat-resistant mutants were spread on NA plates at less than 200 per plate and treated in the same way to confirm their phenotype as isolated colonies. They grew and sporulated normally on NA plates following treatment with 100 μM paraquat or on NA-PQ plates. Among them, U501 had the strongest resistance to the inhibition by paraquat (Fig. 5). None of the paraquat-resistant mutants, however, became more resistant to the lethal effect of plumbagin (data not shown).

When three mutants were tested for their auxotrophic

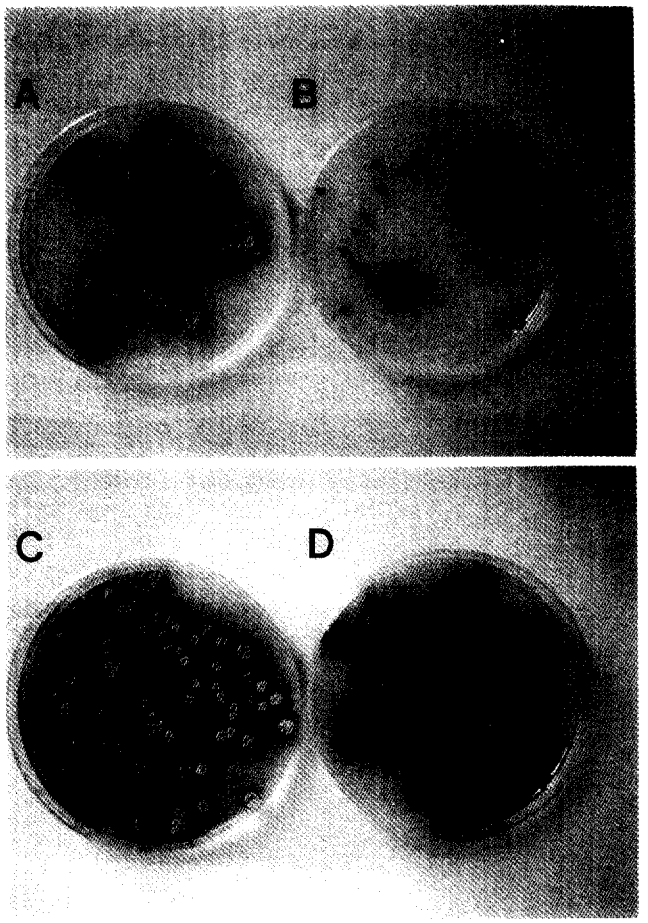


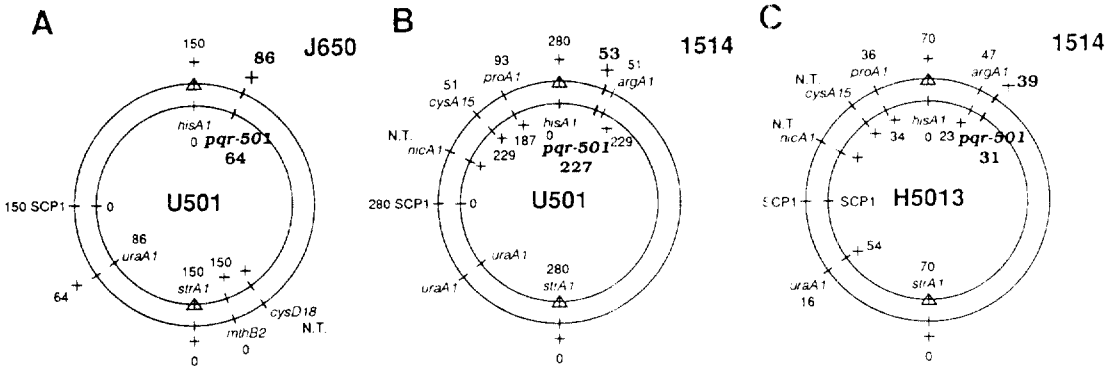
Fig. 5. Phenotypic characterization of paraquat-resistant mutant, U501. Spores of J1501 (A and B) and U501 strains (C and D) were grown on NA plates (A and C), or on NA plates containing 50 μM paraquat (B and D).

markers to be used in genetic mapping (see below), they all turned out to maintain the same genetic background as the parental strain J1501 (Table 1).

Genetic characterization of paraquat-resistant mutants

We crossed paraquat-resistant mutant U501 (in J1501 background) and strain J650, and selected for *his*$^+$ *strA1* recombinants. 150 recombinants were selected and tested for their *ura* and *mth* markers (Fig. 6A). When they were grown on NA plates, two types of colonies were observed; J1501 and J650 type. Colonies of J1501 type grew and differentiated normally, while colonies of J650 type grew poorly and produced no aerial mycelium on NA plates. When recombinants were grown on NA plates containing 50 μM paraquat, colonies of J1501 type segregated again into two phenotypes; wild type (no sporulation) and mutant type (sporulation). Only those recombinants showing normal growth on NA plates as well as on NA-PQ plates were considered to have received the mutated gene. Because the resistance to paraquat segregated independently with respect to the *uraA* locus, the mutant phenotype could be roughly located clockwise of the *hisA* locus (Fig. 6A), and this locus was named as *pqr* (paraquat-resistance).

For more definitive mapping, U501 was further crossed with strain 1514 which has the *argA* marker around 1 o'clock position. 280 *his*$^+$ *strA1* recombinants were selected and tested for their *pro*, *cys*, and *arg* markers. Colonies of 1514 type were indistinguishable from J1501 type on NA plates. But they were more sensitive to



	ura$^+$	*uraA1*	*mth*$^+$	*mthB2*
pqr$^+$	36	50	86	0
pqr-501	28	36	64	0
	$p > 0.995$			

	pro$^+$	*proA1*	*arg*$^+$	*argA1*
pqr$^+$	30	23	5	48
pqr-501	157	70	224	3
	$p > 0.1$		$p < 0.005$	

	pro$^+$	*proA1*	*arg*$^+$	*argA1*
pqr$^+$	12	27	0	39
pqr-501	22	9	23	8
	$p > 0.01$		$p < 0.005$	

Fig. 6. Mapping of *pqr*-501. (A) Strain U501 (inner circle) was crossed with strain J650 (outer circle). (B) Strain U501 (inner circle) was crossed with strain 1514 (outer circle). (C) Strain H5013 (inner circle) was crossed with strain 1514 (outer circle). Selections for *his*$^+$ *strA1* (triangles) excluded parental genomes. Numbers around the circles indicate allele frequencies among the recombinants. Segregations of *pqr-501* with respect to other markers are tabulated.

paraquat and could be differentiated as much smaller colonies on NA-PQ plates. When recombinants were grown on NA-PQ plates, only two types of colonies appeared; 1514 type (no sporulation) and mutant type (sporulation). So, the mutant phenotype could be segregated more clearly than in mating with J650 strain and this mutation was regarded to have occurred at a single locus. The frequency at which paraquat-resistant recombinants appeared suggested two possible positions on the genetic map; around 1 o'clock near *argA* or around 10 o'clock near *cysA15*. Based on higher cosegregation frequency between *pqr* and *argA*, the *pqr* locus was more likely to be located near *argA* (Fig. 6B).

Because the mutant U501 was SCP1$^-$, an SCP1$^+$ (NF) derivative of U501 was isolated from its cross with J650 strain to confirm the result obtained above. An NF and *ura*$^+$ derivative of U501 (H5013) was selected to use the *uraA* marker of 1514 strain in further crossing. In the cross between H5013 and 1514 strain, 70 *his*$^+$ *strA1* recombinants were selected and tested for their *pro, arg,* and *ura* markers donated by both NF parents. The gradient of allele frequencies was less steep than in SCP1$^-$ ×NF cross, and gave a clearer result. The *pqr* phenotype tightly cosegregated with the *argA* phenotype again (Fig. 6C).

Other paraquat-resistant mutants U605 and U607 were also crossed with J650 or 1514 strain and their recombinant progeny were analyzed in the same way. In every cross of U605 and U607, we obtained data that suggested the location of the *pqr* locus near the *argA* locus (data not shown). However, the precise location of the *pqr* locus against the *argA* locus was ambiguous. In crosses between U501 and 1514, H6054 (an NF and *ura*$^-$ derivative of U605) and 1514, and U607 and 1514, the *pqr* locus seemed counterclockwise of *argA*, while in crosses between H5013 and 1514, U605 and 1514, and H6071 (an NF and *ura*$^-$ derivative of U607) and 1514, it seemed clockwise of *argA*. So, we compared the difference in the number of recombinants showing the *pqr* phenotype and the *arg*$^+$ phenotype. When *pqr* was assumed to be counterclockwise of *argA*, the relative distance between *pqr* and *argA* was calculated to be shorter than 1% (for example 2/280 in the cross between U501 and 1514). On the other hand, when *pqr* was assumed to be clockwise of *argA*, it was greater than 10% (for example 8/70 in the cross between H5013 and 1514). Therefore, we tentatively assigned the *pqr* locus clockwise of the *argA* locus. This ambiguity might probably have resulted from the very close distance between *pqr* and *argA*, and the exact location of the *pqr* locus need be determined by physical mapping of the cloned *pqr* gene.

The paraquat-resistant phenotype of these three mutants was unmistakably clear when compared with wild type cells on NA plates upon paraquat treatment or on NA-PQ plates. A single gene or an operon (or at most a cluster of a few operons) is thought to be responsible for this phenotype from three aspects. (1) the paraquat-resistant mutants showed no other difference from wild type in their morphology when grown without paraquat (2) in crosses with 1514 strain, only two types of colonies appeared on NA-PQ plates. (3) in crosses with 1514 strain, *pqr* phenotypes of U501, U605, and U607 were all assigned to about 1 o'clock with tight linkage with the *argA* locus. This result implies that three *pqr* mutations are different alleles of a single locus. To find out the identity of the *pqr* locus and its function in the differentiation of *S. coelicolor,* characterization of the cloned *pqr* gene is necessary.

Acknowledgements

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References

1. **Champness, W.C.,** 1988. New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J. Bacteriol.* **170**, 1168-1174.
2. **Champness, W.C., P. Riggle, T. Adamidis, and P. Vandervere,** 1992. Identification of *Streptomyces coelicolor* genes involved in regulation of antibiotic synthesis. *Gene.* **115**, 55-60.
3. **Chater, K.F.,** 1993. Genetics of differentiation in *Streptomyces. Ann. Rev. Microbiol.* **47**, 685-713.
4. **Farr, S.B. and T. Kogoma,** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium. Microbiol. Rev.* **55**, 561-585.
5. **Fernandez-Moreno, M.A., J.L. Caballero, D.A. Hopwood, and F. Malpartida,** 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* transfer RNA gene of *Streptomyces. Cell.* **66**, 769-780.
6. **Gramajo, H.C., E. Takano, and M.J. Bibb,** 1993. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. *Mol. Microbiol.* **7**, 837-845.
7. **Halliwell, B. and J.M.C. Gutteridge,** 1989. Free radicals in biology and medicine. 2nd ed. Oxford University Press, New York.
8. **Hassan, H.M. and I. Fridovich,** 1979. Intracellular production of superoxide radical and of hydrogen peroxide

by redox active compounds. *Arch. Biochem. Biophys.* **196**, 385-395.

9. **Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward, and H. Schrempf,** 1985. Genetic manipulation of *Streptomyces:* A laboratory manual. The John Innes Foundation, Norwich.
10. **Imlay, J.A. and S. Linn,** 1988. DNA damage and oxygen radical toxicity. *Science.* **240**, 1302-1309.
11. **Kieser, H.M., T. Kieser, and D.A. Hopwood,** 1992. A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome. *J. Bacteriol.* **174**, 5496-5507.
12. **Kim, H.-P., J.-S. Lee, Y.C. Hah, and J.-H. Roe,** 1994. Characterization of the major catalase from *Streptomyces coelicolor* ATCC 10147. *Microbiol.* **140**, 3391-3397.
13. **Kullik, I. and G. Storz,** 1994. Transcriptional regulators of the oxidative stress response in prokaryotes and eukaryotes. *Redox Report.* **1**, 23-29.
14. **Lee, J.-S., Y.C. Hah, and J.-H. Roe,** 1993. The induction of oxidative enzymes in *Streptomyces coelicolor* upon hydrogen peroxide treatment. *J. Gen. Microbiol.* **139**, 1013-1018.
15. **Merrick, M.,** 1976. A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor. J. Gen. Microbiol.* **96**, 299-315.
16. **Toledo, I., A.A. Noronha-Dutra, and W. Hansberg,** 1991. Loss of NAD(P)-reducing power and glutathione disulfide excretion at the start of induction of aerial growth in *Neurospora crassa. J. Bacteriol.* **173**, 3243-3249.
17. **Williams, S.T., M. Goodfellow, G. Alderson, E.M.H. Wellington, P.H.A. Sneath, and M.J. Sackin,** 1983. Numerical classification of *Streptomyces* and related genera. *J. Gen. Microbiol.* **129**, 1748-1813.
18. **Youn, H.-D.,** 1995. Ph. D. Thesis. Characterization of enzymes and differentiation related with oxygen metabolism in *Streptomyces* sp. IMSNU-1. Seoul National University, Seoul.
19. **Youn, H.-D., Y.-I. Yim, K. Kim, Y. C. Hah, and S.-O. Kang,** 1995. Spectral characterization and chemical modification of catalase-peroxidase from *Streptomyces* sp.. *J. Biol. Chem.* **270**, 13740-13747.