Jour. Microbiol. December 1995 p. 339-343 Vol. 33, No. 4

Pleiotrophic Effect of a Gene Fragment Conferring H_2O_2-resistance in *Streptomyces coelicolor*

Tae-Han Um, Chung-Hun Oh, Jong-Soo Lee[1], Yong-Doo Park[1], Jung-Hye Roe[1], and Jae-Heon Kim*

Department of Microbiology, College of Natural Sciences, Dankook University, Cheonan 330-714, Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea
[1]Department of Micrbiology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

(Received November 13, 1995/Accepted December 4, 1995)

We isolated a 10 kb *Bam*HI fragment originated from the chromosome of a H_2O_2-resistant mutant strain of *Streptomyces coelicolor*, which confer H_2O_2-resistance to *S. lividans* upon transformation. Among various subclones of 10 kb *Bam*HI fragment tested for their H_2O_2-resistant phenotype in *S. lividans*, a subclone containing 5.2 kb *Bam*HI-*Bgl*II fragment was found to be responsible for H_2O_2-resistance. The plasmid containing this 5.2 kb fragment was then transformed into *S. coelicolor* A3(2) and tested for their phenotype of H_2O_2-resistance and the change in various enzymes whose activity can be stained in the gel. We found out that the 5.2 kb insert DNA conferred H_2O_2-resistance in *S. coelicolor* A3(2) at early phase of cell growth. The presence of this DNA also resulted in higher level of peroxidase compared with the wild type cell containing parental vector (pIJ702) only. Esterase activity was also higher in this clone. However, alcohol dehydrogenase activity decreased compared with the wild type. These results suggest that the presence of a gene in 5.2 kb *Bam*HI-*Bgl*II DNA fragment causes multiple changes in *S. coelicolor* related to its response against hydrogen peroxide. The result also implies that not only peroxidase but also esterase may function in the defense mechanism against H_2O_2.

Key words: *S. coelicolor*, H_2O_2-resistance gene, peroxidase, esterase, alcohol dehydrogenase, activity staining

Reactive oxygen species are produced endogenously in all aerobically growing cells. They can oxidize membrane fatty acids and proteins, and can damage DNA (5). The responses of bacterial cells to these oxidative stresses have been studied mostly in *Escherichia coli* and *Salmonella typhimurium* (4, 13). In *Bacillus subtilis*, several proteins are induced by H_2O_2 (10), and the sensitivity of *B. subtilis* to H_2O_2 is growth-cycle-dependent. Increased sensitivity in certain sporulation mutants has also been reported (1, 2).

It has been reported that *S. coelicolor* contains an inducible response system against oxidative stress (9). They also reported of H_2O_2-resistant mutants, which contain elevated levels of oxidoreductases, such as catalase, peroxidase, glucose-6-phosphate dehydrogenase. Among these resistant mutants, strain N7 was further analyzed to find the gene responsible for the H_2O_2-resistance as well as elevated levels of oxidative defense enzymes. Lee (8) has constructed a chromosomal library of strain N7 in low copy number plasmid pIJ922, and has screened for H_2O_2-resistant transformants following transformation of the plasmid library into *S. lividans*. Among such transformants, clone B6 containing 10 kb BamHI fragment of N7 chromosomal DNA exhibited the most clear phenotype of H_2O_2-resistance and elevated levels of oxidative defense enzymes (8).

In this report we present the result of further analysis of the clone by narrowing down the region conferring H_2O_2-resistance in 10 kb fragment, and examining the effect of the cloned gene in *S. coelicolor* A3(2) strain for its viability against H_2O_2 and the levels of various enzymes.

* To whom correspondence should be addressed.

Materials and Methods

Strains and plasmids

Clone B6 is a *S. lividans* transformant which contains 10 kb *Bam*HI fragment of the chromosomal DNA of H_2O_2-resistant mutant N7 of *S. coelicolor* (Muller) on low copy number plasmid pIJ922 (8). Clone B6′ is a derivative of B6 and contains 5.2 kb *Bam*HI-*Bgl*II fragment. The 5.2 kb insert DNA from B6′ was further subcloned into pIJ702, a multicopy plasmid and transformed into a wild type *S. coelicolor* A3(2) strain M145 (*S. coelicolor* B6′).

Media

The strains were preserved on YEME agar medium (yeast extract-malt extract) from International Streptomyces Project which consisted of 2 g dextrose, 2 g yeast extract, 5 g malt extract and 1 l distilled water. For selecting transformants, thiostreptone was added into the medium to the final concentration of 50 μg/ml. For shaking cultures, modified YEME was used which contained 10 g dextrose, 2 g yeast extract, 5 g malt extract, 20 mM MOPS (pH 7.5) and 1 l distilled water. Minimal media was prepared as described by Hopwood *et al.* (7).

Inoculum and culture conditions

Spore solutions were obtained by scratching the YEME agar culture with sterile 0.1% Tween 80 and filtered through cotton plug to remove the mycelial fragments. The spore solutions were stored at −20℃ and used as the inoculum. To test the H_2O_2-resistance, spores were diluted and spread onto the YEME agar surface to give approximately 200 colonies per plate and incubated for 12, 24, and 36 h at 30℃. 10 ml of H_2O_2 solution of different concentrations (0.2~5 mM) was poured onto the agar surface at different culture time and incubated for 1 h at room temperature, during which only the H_2O_2-resistant colonies survived. Then, the H_2O_2 solution was removed from the plates and incubated further until surviving colonies were observed. The survival rate was estimated by counting the number of colonies. To obtain mycelia for the enzyme assays, 250 ml Erlenmeyer flasks containing 50 ml modified YEME solution were inoculated with 100 μl spore solution (10^7~10^8 spores/ml) and incubated with shaking at 120 rpm at 30℃. During the incubation, various concentrations of H_2O_2 was added into the flasks at different time.

Activity staining of enzymes on non-denaturing gel electrophoresis

Mycelia grown in shaking culture were harvested by filtration using filter paper (Whatman No. 1), washed twice with distilled water and resuspended in 0.1 M phosphate buffer (pH 7.2). Mycelia were then disrupted by ultrasonication for 3 min with cooling intervals and centrifuged for 30 min at 12,000 rpm at 4℃. The supernatant was used as crude extracts for the enzyme activity staining on non-denaturing gels prepared according to the methods described by Hames and Rickwood (6). Depending on the enzymes to be assayed the gel concentrations were adjusted from 7.5% to 10%. About 10 μg of crude enzyme extract was loaded per well and 200 V electric power was applied until the tracking dye had moved to the bottom of the gel. The gels were soaked in the reaction mixtures for the staining of enzyme activities. The preparation of reaction mixtures and methods for the activity staining were performed as described by Pasteur *et al.* (11). Peroxidase was stained in 30 ml of 0.1 M phosphate buffer (pH 7.2) containing 0.5 g L-dihydroxyphenylalanine and 0.5 ml of 30% hydrogen peroxide. The gels were incubated in the dark until the black brown bands appeared at 37℃. Esterase was stained in the reaction mixture which was prepared by mixing 50 ml of 0.1 M phosphate buffer (pH 7.2) and 1 ml of β-naphthyl acetate (3% in acetone). After the incubation of the gels for 30 min at 37℃ 20 mg of *o*-dianisidine was added to the reaction mixture and incubated further until the red bands appeared. Alcohol dehydrogenase was stained in the reaction mixture prepared by mixing 40 ml of 0.2 M Tris-HCl (pH 8.0) containing 16 mg EDTA, 0.2 ml of 0.5 M $MgCl_2$, 3 ml absolute ethanol, 2 ml NAD (1% in water), 1 ml NBT (1% in water), 0.3 ml MTT (1% in water) and 0.5 ml PMS (1% in water). The gels were incubated in the dark until the blue bands appeared.

Results and Discussion

Functional mapping of 10 kb *Bam*HI fragment conferring H_2O_2-resistance in *S. lividans*

The 10 kb *Bam*HI fragment from *S. coelicolor* responsible for H_2O_2-resistance in clone B6 was further subcloned into pIJ702, a multicopy plasmid, and transformed into *S. lividans* (8). Transformants containing various restriction fragments of 10 kb DNA were tested for the H_2O_2-resistant phenotype compared with the wild type *S. lividans* with and without plasmid pIJ702. H_2O_2-resistance was tested in two separate ways. The growth rate was estimated by measuring A_{640} after treating cells with 5 mM H_2O_2 of cells grown to A_{640} of 0.05 to 0.1 in minimal medium. As a separate measurement, colonies grown for 30~36 h at 30℃ after inoculation of spores on minimal agar medium were treated with 3 mM H_2O_2

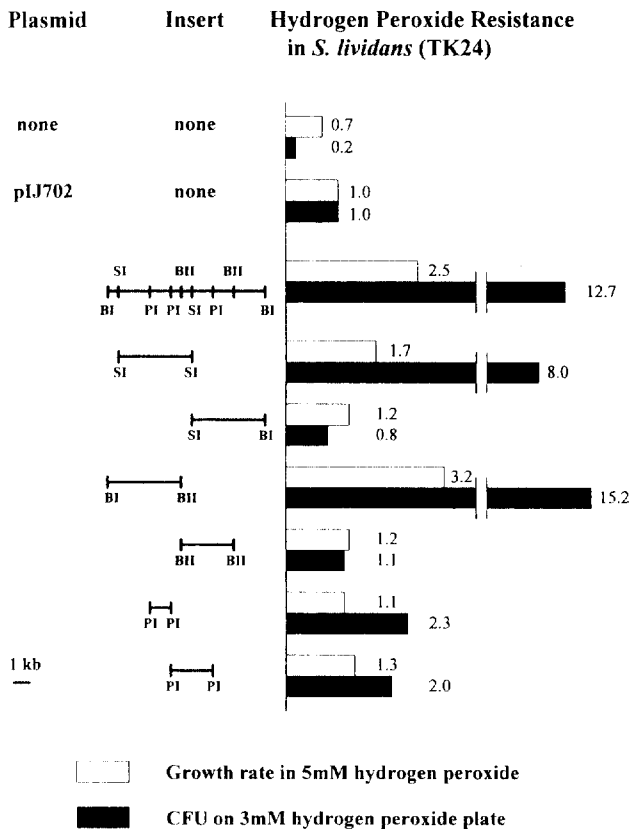

Fig. 1. Functional mapping of 10 kb insert from H_2O_2-resistant clone B6. Various restriction fragments of 10 kb *Bam*HI fragment were subcloned in pIJ702 and transformed into *S. lividans*. The transformed cells were tested for H_2O_2-resistance by two ways; 1) cells grown to A_{640} of 0.05~0.1 in minimal medium were treated with hydrogen peroxide to a concentration of 5 mM and then growth rate was estimated by measuring A_{640}, 2) as a separate measurement, colonies grown for 30~36 h at 30°C after inoculation of spores were treated with 3 mM hydrogen peroxide for 1 h. Surviving colonies (CFU) were counted. The growth rate or CFU of *S. lividans* containing pIJ plasmid only were regarded as 1.0. Restriction sites used were; BI, *Bam*HI; SI, *Sal*I; BII, *Bgl*II; PI, *Pst*I.

for 1 h and the surviving colonies were counted. In both measurements, the values obtained with the wild type *S. lividans* containing pIJ702 plasmid without any foreign DNA insert were regarded as 1.0 and the relative values for each subclones were estimated and presented in Fig. 1. We confirmed that the presence of 10 kb *Bam*HI fragment caused significant increase in H_2O_2-resistance in *S. lividans* when present in multicopy plasmid. The growth rate in the presence of H_2O_2 in liquid medium increased 2.5 fold, whereas the number of surviving colonies on minimal agar plate increased 12.7 fold due to 10 kb *Bam*HI fragment. Among various subclones of *Bam*HI fragment, both *Sal*I fragment and *Bam*HI-*Bgl*II fragment (5.2 kb) conferred significant increase in H_2O_2-resistance comparable to the clone containing the full sized 10 kb insert. This suggest that the gene(s) responsible for H_2O_2-resistance lies within the boundary of these restriction fragments. We observed that the *Bam*HI-*Bgl*II fragment caused more resistance than *Sal*I fragment. We named the H_2O_2-resistant *S. lividans* transformant containing this 5.2 kb *Bam*HI-*Bgl*II fragment as B6′ and used this clone for further analysis.

Effect of 5.2 kb *Bam*HI-*Bgl*II fragment on H_2O_2-resistance in *S. coelicolor*

We next transferred this recombinant plasmid from B6′ into *S. coelicolor* A3(2) and tested for the extent of H_2O_2-resistance as described in Materials and Methods. Colonies were grown for either 12, 24, or 36 h following inoculation of spores on YEME agar medium, and treated with various concentrations of H_2O_2 for 1 h. As shown in Fig. 2, the survival of *S. coelicolor* strains was dependent on the incubation time as well as the concentration of hydrogen peroxide added. As for the incubation time, the 12 h grown cultures were most sensitive against all concentrations of hydrogen peroxide, only about 0.02% of wild type *S. coelicolor* survived even at the lowest concentration of 0.2 mM H_2O_2 (Fig. 2A), whereas the 24 h or 36 h grown cultures exhibited significantly higher survival rates, nearly 100% at 0.2 mM H_2O_2 (Fig. 2B and C). In light of the fact that 0.2 mM hydrogen peroxide was known to be non-lethal to *S. coelicolor* (Muller) (9), the lethal effect should be dependent on the growth phase. The importance of a 12 h incubation was noticed by other several papers which reported that sensitivity to the various stimulants of secondary metabolite production (14) or metabolic state of streptomycetes was changed at around 12 h (3). At the same time the toxic effect of hydrogen peroxide increased very clearly at above 0.8 mM, where no survivors were detected. This lethal concentration was approximately the same as those reported in the previous report (9) and for other bacteria as well (4). *S. coelicolor* A3(2) strain harboring recombinant plasmid from H_2O_2-resistant *S. lividans* clone B6′ (*S. coelicolor* B6′) exhibited its resistance only when hydrogen peroxide was treated at 12 h growth. Three percent of the total colonies survived, in contrast to the wild type strain or the strain with only pIJ702. Meanwhile, *S. coelicolor* B6′ was not resistant to the hydrogen peroxide treatment when grown for 24 or 36 h. These results showed that there is a hydrogen peroxide sensitive phase in the growth of *S. coelicolor* A3(2), and the resistance gene originated from *S. coelicolor* (Muller) could confer the resistance to *S. coelicolor* A3(2) in this sensitive phase.

Changes in the level of various enzymes in *S. coelicolor* B6′ containing 5.2 kb insert DNA

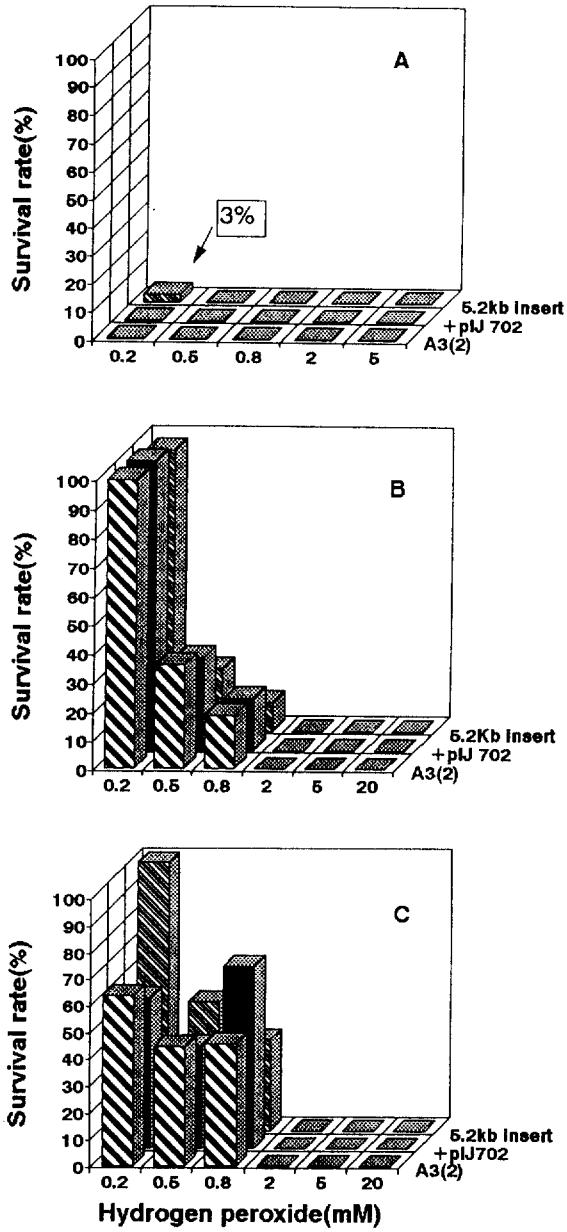

Fig. 2. The survival rate of *S. coelicolor* B6′ against various concentrations of H_2O_2. *S. coelicolor* B6′ cells containing 5.2 kb *Bam*HI-*Bgl*II insert DNA were treated with H_2O_2 for 1 h and measured for the survival rate as described in Materials and Methods. Wild type *S. coelicolor* A(3) with or without pIJ702 plasmid were treated in the same way. The times of H_2O_2 treatment were 12 h (A), 24 h (B), or 36 h (C) after inoculation.

We next proceeded to test the change in the level of various enzymes in *S. coelicolor* B6′ using activity staining. We modified the method for the activity staining on the gel that has been used in the population genetics of bacteria (12). Polyacrylamide gels of various percentage were used to ensure better resolution of activity bands. We tested 13 different enzymes (peroxidase, esterase, alcohol dehydrogenase, isocitrate dehydrogenase,

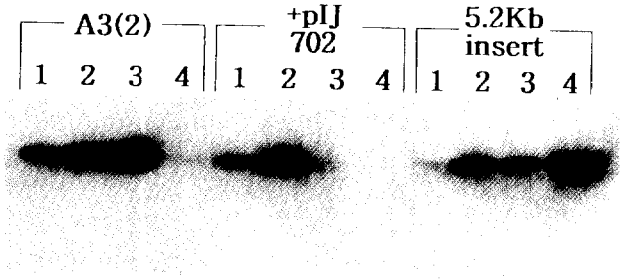

Fig. 3. Peroxidase activity staining of *S. coelicolor* cell extracts. Wild type *S. coelicolor* A3(2) with or without pIJ702 and *S. coelicolor* B6′ strain were grown for 12 h after inoculation and treated with H_2O_2 for 12 h at concentrations of 0.2 (lane 2), 0.5 (lane 3), or 0.8 mM (lane 4). H_2O_2-untreated cells were grown for 24 h (lane 1). Cells were harvested and prepared for activity staining as described in the text.

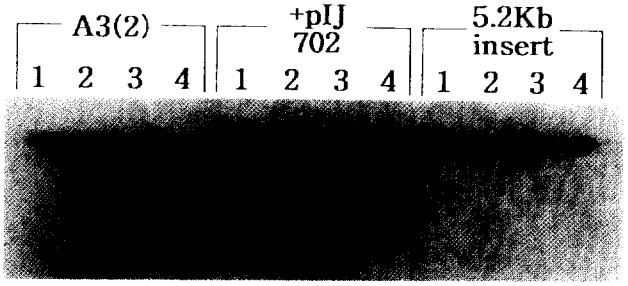

Fig. 4. Esterase activity staining. Cell extracts were prepared as described in the legend to Fig. 3.

malate dehydrogenase, aconitase, glutamate dehydrogenase, hexokinase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, glyceraldehyde phosphate dehydrogenase and hydroxybutyrate dehydrogenase). Among these enzymes, the level of peroxidase and esterase was maintained at significantly higher level in *S. coelicolor* B6′, compared with the wild type with and without pIJ702 plasmid. As shown in Fig. 3, peroxidase activity bands were maintained when *S. coelicolor* culture was treated with H_2O_2 at concentrations up to 0.5 mM. However, upon treatment with 0.8 mM H_2O_2 the peroxidase activity disappeared in cell extracts of wild type *S. coelicolor* and *S. coelicolor* with only pIJ702 plasmid. However, in B6′ clone containing 5.2 kb *Bam*HI-*Bgl*II insert DNA, the level of peroxidase was maintained at the level even higher than the amount present after treatment with 0.2 or 0.5 mM H_2O_2. Therefore, the most likely explanation for this observation is that 5.2 kb insert DNA contains a gene responsible for overproduction of peroxidase. Esterase was also maintained at higher level in *S. coelicolor* B6′ clone compared with the wild type with and without pIJ702 plasmid (Fig. 4). Treating the culture with 0.8 mM H_2O_2 significantly reduced the level of esterase in cell extracts of the wild type *S. coelicolor* with and without pIJ702. Among the

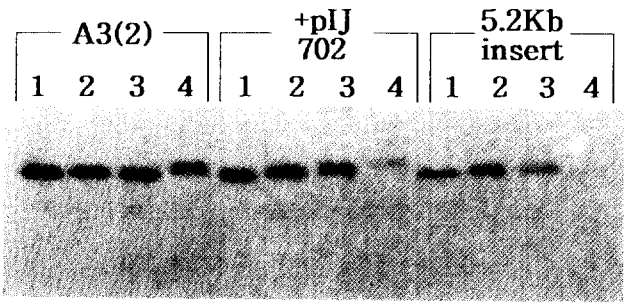

Fig. 5. Alcohol dehydrogenase activity staining. Cell extracts were prepared as described in the legend to Fig. 3.

enzymes tested, alcohol dehydrogenase activity was also affected by the cloned 5.2 kb *Bam*HI-*Bgl*II DNA fragment (Fig. 5). Different from peroxidase and esterase, this enzyme was significantly reduced in *S. coelicolor* B6′ compared with the control strains upon treatment with 0.8 mM H_2O_2. Treatment with lower concentrations of H_2O_2 also decreased alcohol dehydrogenase activity more in strain B6′ than with the control strains.

From the above results, we can conclude that the 5.2 kb DNA fragment from H_2O_2-resistant *S. coelicolor* mutant contains a gene, whose gene product confers H_2O_2-resistance to the cell and regulates the level of at least 3 enzymes. The overproduction of peroxidase is consistent with the increase in H_2O_2-resistance. However, we cannot explain how the increase in esterase or the decrease in alcohol dehydrogenase is related with H_2O_2-resistance. For further analysis, we need to identify the gene product(s) from this gene fragment and to obtain disruption mutants of this gene in *S. coelicolor* to confirm its function. At this point, we can only speculate that the gene product from this 5.2 kb DNA may act either as a regulator of transcription of genes for peroxidase, esterase, and alcohol dehydrogenase or as either a direct or indirect modifier of enzyme activities.

Acknowledgements

This work was supported by a KOSEF research grant for SRC (Research Center for Molecular Microbiology, Seoul National University).

References

1. **Bol, K.D. and R.E. Yasbin,** 1990. Characterization of an inducible oxidative stress system in *Bacillus subtilis*. *J. Bact.* **172**, 3503-3506.
2. **Dowds, B.C.A., P. Murphy, D.J. McConnel, and K.M. Devine,** 1987. Relationship among oxidative stress, growth cycle, and sporulation in *Bacillus subtilis*. *J. Bact.* **169**, 5771-5775.
3. **Ensign, J.C.,** 1988. Physiological regulation of sporulation of *Streptomyces griseus*, p. 309-315. *In* Y. Okami, T. Beppu, and H. Ogawara (eds.), Biology of actinomycetes '88. Japan Scientific Society Press, Tokyo, Japan.
4. **Farr, B.S. and T. Kogoma,** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **55**, 561-585.
5. **Halliwell, B. and J.M.C. Gutteridge,** 1989. Free radicals in biology and medicine. Clarendon Press, Oxford, U.K.
6. **Hames,B.D. and D. Rickwood,** 1990. Gel electrophoresis of proteins. IRL Press, Oxford, U.K.
7. **Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, D.J. Lydiate, C.P. Smith, J.M. Ward, and H. Schrempf,** 1985. Genetic manipulation of *Streptomyces*, a Laboratory manual. John Innes Institute, Norwich, U.K.
8. **Lee, J.S.,** 1994. The response of *Streptomyces coelicolor* (Muller) against hydrogen peroxide stress. Ph.D. Thesis, Seoul National University, Seoul, Korea.
9. **Lee, J.S., Y.C. Hah, and J.H. Roe,** 1993. The induction of oxidative enzymes in *Sterpomyces coelicolor* upon hydrogen peroxide treatment. *J. Gen. Micro.* **139**, 1013-1018.
10. **Murphy, P., B.C.A. Dowds, D.J. McConnel, and K.M. Devine,** 1987. Oxidative stress and growth temperature in *Bacillus subtilis*. *J. Bact.* **169**, 5766-5770.
11. **Pasteur, N., G. Pasteur, F. Bonhomme, J. Catalan, and J. Britton-Davidian,** 1988. Practical isozyme genetics. Ellis Horwood Limited, Chichester, U.K.
12. **Selander, R.K., D.A. Caugant, H. Ochman, J.M. Musser, M.N. Gilmour, and T.S. Whittam,** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51**, 873-884.
13. **Storz, G., L.A. Tartaglia, S.B. Farr, and B.N. Ames,** 1990. Bacterial defenses against oxidative stress. *Trends Genet.* **6**, 363-368.
14. **Vanek, Z., J. Novak, and V. Jechova,** 1988. Primary and secondary metabolism, p. 389-394. *In* Y. Okami, T. Beppu, and H. Ogawara (eds.), Biology of actinomycetes '88. Japan Scientific Society Press, Tokyo, Japan.