Mycoscience 40: 81–85, 1999

Short Communication

Characteristics of stress response in a mushroom-pathogenic bacterium, *Pseudomonas tolaasii*, during the interaction with *Pleurotus ostreatus* and carbon/nitrogen starvation in vitro

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Accepted for publication 25 December 1998

A brown blotch bacterium, *Pseudomonas tolaasii* strain PT814, expresses a high degree of cross-protection against generalized stress imposed by physical/chemical treatment, H_2O_2, UV, high temperature, ethanol and NaCl during the interaction with *Pleurotus ostreatus*. Stress resistance was also noted in the bacterium in vitro under limited carbon and nitrogen sources. In addition, changes in cell morphology from a "metabolically active" rod to an "energy-saving" spherical shape were detected during starvation and the interaction. All the changes under stress were reversible. A homologue of *rpoS* (σ^S), a regulator that controls such physiological status during starvation in other bacteria, was identified in *P. tolaasii* strain PT814. Data suggest that the bacterium is able to withstand a complex stress environment for its survival through changes in its metabolic pattern.

Key Words——*Pleurotus ostreatus*; *Pseudomonas tolaasii*; *rpoS*; stress-response; survival.

Bacteria have evolved mechanisms to adapt themselves to a natural habitat where they are frequently exposed to nutrient limitation as well as physical/chemical stresses. Such adaptive mechanisms have been studied from the angle of stress physiology mostly in enterobacteria, *Escherichia coli* (Migula) Castellani & Chalmers, marine *Vibrio* spp., and *Salmonella typhimurium* (Loeffler) Castellani & Chalmers (Hengge-Aronis, 1993; Nystrom et al., 1991; Spector and Cubitt, 1992). Under such an adverse environment, bacteria become resistant to generalized or physical/chemical stresses imposed by oxidation, UV-radiation, heat, solvents and osmolytes, switching their metabolic status to limit the use of energy and changing their morphology from a "metabolically active" rod to an "energy-saving" spherical shape (Kolter et al., 1993). Genetic analysis revealed that all these events are attributable to the expression of various genes that are concurrently controlled by an alternative σ factor (*rpoS*) of RNA polymerase present in the bacteria (Hengge-Aronis, 1993). Recently, physiological characteristics similar to those reported in enterobacteria have been detected in plant-growth-promoting pseudomonads, *Pseudomonas fluorescens* (Trevisan) Migula and *P. putida* (Trevisan) Migula, and phytopathogenic *P. syringae* van Hall, in which the mechanisms involved play a critical role in their ecology (Givskov et al., 1994; Klotz, 1993; Sarniguet et al., 1995).

Pseudomonas tolaasii Paine strain PT814 is the causal agent of brown blotch disease in a cultivated mushroom, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Suyama and Fujii, 1993). The bacterium is known to persist not only in the host but also in water, spawn, and other materials in the cultivation facility where nutrients are very scarce as compared with their optimum such as under in vitro culture (Suyama, 1994; Suyama and Fujii, 1993). Elucidation of the mechanisms involved in the survival of bacteria may give a clue to the control of the bacterial reservoir for the prevention of disease development in the cultivation of *P. ostreatus* and other edible fungi. In the present study, the ability of *P. tolaasii* strain PT814 to cross-protect itself from generalized stress in response to changes in growth conditions was examined. Along with physiological data, genetic evidence showing the presence of a stress-responding regulator, *rpoS*, in *P. tolaasii* strain PT814 provides information about the physiological phenomena involved in survival of the mushroom pathogenic bacterium.

Stress response during starvation and host-parasite interactions *Pseudomonas tolaasii* strain PT814 was grown in *P. ostreatus* fruiting bodies at 14 and 24°C, and periodically harvested as described by Tsukamoto et al. (1998; Fig. 1A). The harvested bacterial cells were washed with the STV medium, collected by centrifuga-

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tion ($4,000 \times g$ for 5 min), resuspended in the STV medium, and exposed to various components of generalized stress, e.g., H_2O_2 (0.3, 0.6, and 1.2 M/30 min), UV (150, 300, and 600 erg), 15% ethanol (10, 20, and 40 min), 42°C (10, 20, and 40 min) and osmotic stress with 2.5 M NaCl (5, 10, and 20 min). Composition of the STV medium was the same as that of minimal salts-glucose medium described by Murata et al. (1991) except for the addition of 0.02% glucose instead of 0.4% and 0.015 mM of ammonium sulfate instead of 7.6 mM. In a parallel experiment in vitro, the bacterium was grown in the minimal salts-glucose broth medium at 24°C, and a portion of the culture was harvested during the exponential growth phase for physical/chemical stress treatments, while another portion was placed in the STV medium for further culture (Fig. 1A). Samples in the STV medium were harvested 2 and 4 h after the bacterial growth stopped, and cross-protection against stresses was tested (Fig. 1A). Viable bacterial cell numbers were determined based on colony-forming units on Pseudomonas agar F (Difco) after appropriate dilutions.

Bacterial cells grown in *P. ostreatus* fruiting bodies were more resistant to all the stresses examined than those in the exponential growth phase in minimal salts-glucose medium regardless of the growth temperature used (Figs. 1B–F). Resistance to H_2O_2, UV-radiation, and temperature at 42°C was remarkable in that the viable cell counts were more or less the same as in the control without treatment (Figs. 1B–D). It was also noted that the bacterium grew as well on *P. ostreatus* fruiting bodies at 14°C as it did at 24°C, the optimum temperature in the minimal salts-glucose medium, though at a rate far below that of optimum growth in vitro. Cross-protection against these stresses was noted in bacterial cells on the fruiting bodies at various growth stages such as 2 and 4 d after the inoculation in *P. ostreatus*.

Bacterial cells collected from cultures in the STV medium showed a significant level of cross-protection against treatment with H_2O_2, 42°C, NaCl, and ethanol (Fig. 1). They were, however, as sensitive to the UV-radiation as those from the exponential growth (Fig. 1C). The stress resistance was enhanced by a prolonged starvation period, e.g., cells subjected to starvation for 4 h

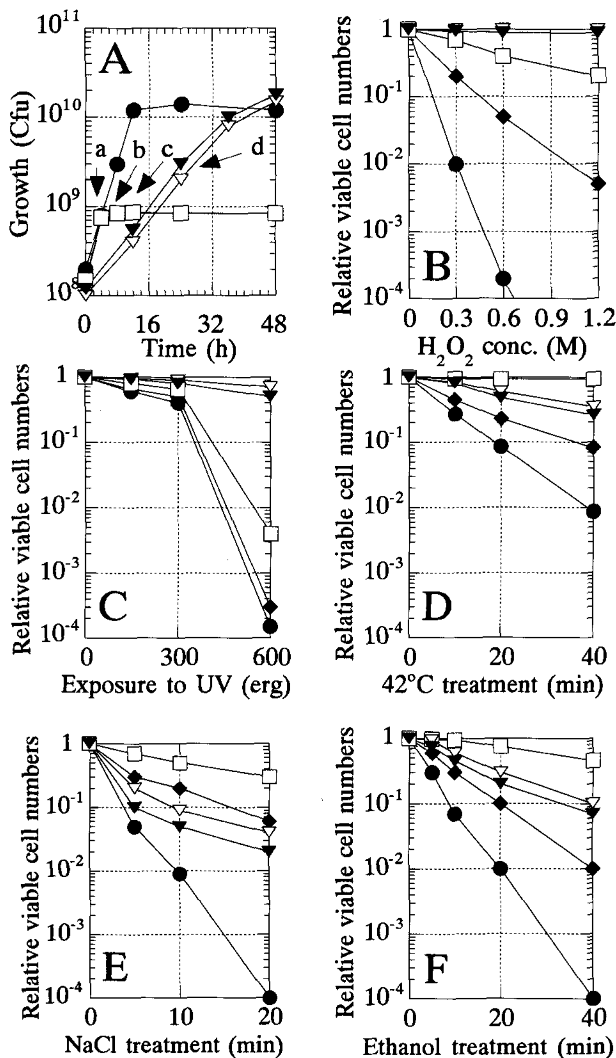

Fig 1. Survival of *P. tolaasii* strain PT814 exposed to generalized stress.

A. Growth curve of *P. tolaasii* strain PT814. Approximately 1×10^8 cells of the bacterium were precultured in minimal salts-glucose medium to the early log phase, then reinoculated in minimal salts-glucose medium (closed circle) or STV medium (open box) and cultured further at 24°C, or reinoculated on fruiting bodies of *P. ostreatus* and incubated further at 14°C (open triangle) or 24°C (closed triangle). Samples were harvested at the growth phase indicated by arrows: (a) exponential phase in vitro, (b) starvation for 2 h in vitro, (c) starvation for 4 h in vitro and (d) culture on *P. ostreatus* for 24 h. Bacterial growth is expressed in colony-forming units/ml (CFU).

B–F. Survival of *P. tolaasii* strain PT814 subjected to various pre-culture treatments under various stresses. Bacterial cells collected from the pre-culture by centrifugation ($4,000 \times g$ for 5 min) were washed with the STV medium, collected again by centrifugation and resuspended in the media (basic medium: STV) for stress treatments. Viable cell numbers were determined based on colony-forming units on *Pseudomonas* agar F after washing with sterile water and appropriate dilution. The term "relative viable cell numbers" stands for viable cell numbers after exposure to stress/viable cell numbers without treatment. Symbols: closed circle, exponential phase cultures in vitro; closed box, starvation for 2 h in vitro; open box, starvation for 4 h in vitro; closed triangle, culture on *P. ostreatus* at 24°C; open triangle, culture on *P. ostreatus* at 14°C.

B. Survival of *P. tolaasii* strain PT814 in the presence of hydrogen peroxide. Bacterial cells resuspended in the STV medium were exposed to 0.3, 0.6, and 1.2 M H_2O_2 for 30 min.

C. Survival of *P. tolaasii* strain PT814 under various doses of UV.

D. Survival of *P. tolaasii* strain PT814 under 42°C treatment.

E. Survival of *P. tolaasii* strain PT814 under high osmotic pressure with 2.5 M NaCl.

F. Survival of *P. tolaasii* strain PT814 in the presence of 15% ethanol.

better protected themselves than those exposed to the stress for 2 h (Fig. 1). Water extracts of *P. ostreatus* fruiting bodies and trehalose, which were previously reported to activate tolaasin production in *P. tolaasii* strain PT814 (Murata and Magae, 1996; Murata et al., 1998), did not affect the stress resistance when incorporated into the minimal salts medium.

Among the stresses, exposure to UV acted differently on the physiology of *P. tolaasii* strain PT814 under starvation and on the host. It is most likely that at least two types of stress resistance mechanisms are involved: (i) the starvation-inducible general stress resistance controlled by *rpoS* (Hengge-Aronis, 1993) and (ii) the SOS system controlled by *recA* that operates in response to DNA damage caused by UV, mytomycin C, and nalidixic acid (Walker, 1987). It is assumed that the bacterium expresses strong resistance to UV during the host-parasite interaction as a result of its response to DNA-damaging agents from the host, *P. ostreatus*, which operates in addition to the response to nutrient limitation.

The changes in cell morphology from a "metabolically active" rod to an "energy saving" spherical shape were detected during both starvation and the interaction with *P. ostreatus* fruiting bodies (Fig. 2). It is interesting to note, however, that although bacterial cells associated with the fruiting bodies were as small as those under starvation in vitro, they showed a septum between attached cells indicating the occurrence of cell division (Fig. 2). In contrast, no sign of cell division was found in the starvation culture (Fig. 2). The spherical cells returned to the rod shape and lost the resistance to stresses when returned to nutrient-rich in vitro culture. The acquisition of the ability to tolerate generalized stress during starvation by *P. tolaasii* strain PT814 and during the interaction with *P. ostreatus* suggests the presence of a system for survival controlled by an alternative sigma factor, σ^S, reported in other bacteria during starvation, which may also operate during the host-parasite interaction.

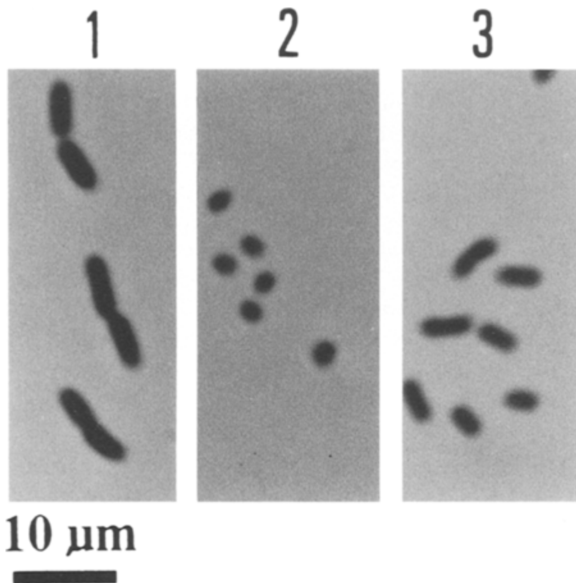

Fig. 2. Cell morphology of *P. tolaasii* strain PT814 during the exponential growth phase in minimal salts-glucose medium (1), starvation in the STV medium (2), and after the interaction with *P. ostreatus* fruiting bodies (3).

Bacteria were collected, stained with 2% crystal violet-0.8% ammonium oxalate-20% ethanol, and observed under a light microscope (×1,000).

Molecular cloning of *rpoS* in *P. tolaasii* strain PT814 To determine whether *P. tolaasii* strain PT814 is equipped with a system involved in stress resistance in response to starvation, a search was conducted for an *rpoS* homologue in a genomic library of *P. tolaasii* strain PT814 constructed in a cosmid vector pLAFR5 (Keen et al., 1988; Murata et al., 1998). Colony hybridization was conducted in the library by using the [α-P^{32}]dCTP-labeled *Eco*RI fragment of pJEL5649 that contains *rpoS* of *P. fluorescens* strain Pf-5 (Sarniguet et al., 1995). We obtained four plasmids showing strong hybridization signals. Since restriction analysis revealed that the four cosmids consisted of the same DNA segment, we further investigated one representative plasmid, pHHM121, in detail.

A 3.1 kb *Eco*RI fragment of pHHM121 was found to carry the *rpoS* homologue by Southern hybridization analysis using the same probe as that employed for colony hybridization. The fragment was cloned in a vector, pBluescript SK$^+$ (Stratagene, La Jolla, CA), from which various subclones and deletion derivatives were further constructed (Fig. 3). Nucleotide sequence analysis conducted on pHHM121 derivatives by using Dye primer FS core reaction kit and ABI prism 377 sequencer (Perkin Elmer Japan, Urayasu) revealed the presence of an open reading frame (ORF) of 1,008 bp, sufficient to encode

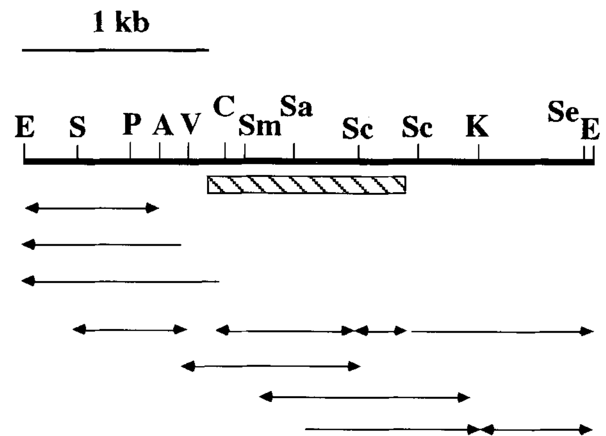

Fig. 3. Restriction endonuclease map of the 3.1 kb *Eco*RI DNA segment of *P. tolaasii* strain PT814 containing the *rpoS* homologue, and schematic representation of further subcloning for nucleotide sequencing.

The cloned *Eco*RI fragment is indicated by a solid bold line. Arrows below the line depict the insert DNA of the subclones used for nucleotide sequencing and the direction of sequence analysis. The hatched bar indicates the ORF predicted to encode *rpoS*. Symbols: A, *Apa*I; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sc, *Sac*II; Se, *Spe*I; Sm, *Sma*I; V, *Eco*RV.

Fig. 4. Comparison of amino acid alignment of predicted RpoS of *P. tolaasii* strain PT814 (Pt) with RpoS of *P. fluorescens* Pf-5 (Pf; Sarniguet et al., 1995) and *E. coli* (Ec; Mulvey and Loewen, 1989).

Regions conserved among sigma factors (Lonetto et al., 1992) are boxed in. Based on information currently available (Lonetto et al., 1992), region 2.1 operates for DNA strand opening and binding to the core polymerase, region 2.3 for DNA strand opening, region 2.4 for recognition of the −10 promoter region, region 3.1 for DNA binding, region 3.2 for binding the core polymerase, and regions 4.1 and 4.2 for recognition of the −35 promoter region. Roles of regions 1.2 and 2.2 have not yet been clarified. Sequences carrying a helix-turn-helix motif are indicated. Asterisks denote amino acid residues that are identical in all three sequences.

336 amino acid residues (Figs. 3, 4; GenBank/EMBL/DDBJ accession number AB006073). This ORF is preceded by a putative Shine-Dalgarno sequence (AAGG; Shine and Dalgarno, 1974) located 9 bp upstream of the putative ATG start codon. The probability that a coding region is present in this ORF gives a score of 1.31 in the TESTCODE indicator based on computation by the method of Fickett (1982). This value satisfies the requirement for the standard of coding sequences (>0.95) and is the highest probability in the sequenced 3,120 bp DNA.

The putative gene product encoded in the ORF has an amino acid sequence 82% identical with the sequence of RpoS reported in *P. fluorescens* and *E. coli* (Fig. 4). It contains four regions conserved within the family of RNA polymerase σ factors of which regions 2 and 4 are highly conserved as compared with regions 1 and 3, which is consistent with the observation reported in other members (Fig. 4; Lonetto et al., 1992). The most conserved sequence was found in region 2, which participates in the binding of the core polymerase (region 2.1), opening of DNA strand (regions 2.1 and 2.3), and sequence-specific contacts in the -10 promoter region (region 2.4) (Fig. 4). Regions 3 and 4 of the predicted product of ORF also contain a typical helix-turn-helix motif, confirming their role in DNA-binding (Fig. 4; Lonetto et al., 1992). The data strongly suggest that the cloned ORF of *P. tolaasii* strain PT814 encodes the starvation-induced stress response regulator, RpoS.

Concluding remarks The present study demonstrated that a devastating bacterial pathogen of cultivated mushrooms, *P. tolaasii*, activates a stress-resistant system in response to starvation as well as host interactions. With respect to the host-parasite interactions, only a few cases have been reported of the activation of cross-protection against generalized stress during both starvation and the interactions in enterobacteria (Kowarz et al., 1994) and, so far, none in plant- or fungus-interacting pseudomonads. While the expression of stress resistance confers on bacteria the means of survival, regulators involved in the cross-protection have been reported to control the production of secondary metabolites that play a role in bacterial ecology. For instance, *rpoS* has been reported to control the production of systemic virulence factors in *S. typhimurium* (Kowarz et al., 1994) and antifungal agents in a plant-growth-promoting bacterium, *P. fluorescens* (Sarniguet et al., 1995). *recA*, the regulator controlling the DNA-repair-system in response to DNA damage, is required for pectin lyase production in a plant pathogenic bacterium, *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey, Harrison, Breed, Hammer & Huntoon (McEvoy et al., 1992).

The fact that tolaasin production is strongly activated during the stationary growth phase suggests that the production of tolaasins, the toxins primarily responsible for brown blotch disease, in *P. tolaasii* strain PT814 could be regulated by a starvation-regulatory system controlled by *rpoS* (Murata and Magae, 1996; Murata et al., 1998). Whether host components that activate the stress resistance in *P. tolaasii* strain PT814 also stimulate pathogenic factor production in the bacterium has to be clarified by further studies at the molecular level. Cloned *rpoS* will be useful in such an approach in order to construct a *P. tolaasii* strain carrying inactivated *rpoS* in the genome and to analyze at the molecular level the role of regulators in the control of pathogenicity under the influence of environmental stimuli. Further investigations of host factors and bacterial regulatory elements may allow elucidation of the mechanism of mushroom-pathogen interactions and offer a clue to new means of control.

Acknowledgements——The author is grateful to Dr. J. E. Loper, U.S. Department of Agriculture, ARS-HCRL, for providing the plasmid pJEL5649. The author thanks Mr. H. Yamada for the cultivation of *P. ostreatus* and Mrs. R. Ohno for her help throughout the experiment.

Literature cited

Fickett, J. W. 1982. Recognition of protein coding regions in DNA sequences. Nucleic Acid Res. **10**: 5303–5318.

Givskov, M., Eberl, L., Moller, S., Poulsen, L. K. and Molin, S.

1994. Response to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. **176**: 7–14.

Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell **72**: 165–168.

Keen, N. T., Tamaki, S., Kobayashi, D. and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70**: 191–197.

Klotz, M. G. 1993. The importance of bacterial growth phase for plant virulence and pathogenicity testing: coordinated stress response regulation in fluorescent pseudomonads? Can. J. Microbiol. **39**: 948–957.

Kolter, R., Siegele, D. A. and Tormo, A. 1993. The stationary phase of the bacterial life cycle. Ann. Rev. Microbiol. **47**: 855–874.

Kowarz, L., Coynault, C., Robbe-Saule, V. and Norel, F. 1994. The *Salmonella typhimurium katF* (*rpoS*) gene: cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. J. Bacteriol. **176**: 6852–6860.

Lonetto, M., Gribskov, M. and Gross, C. A. 1992. The σ^{70} family: sequence conservation and evolutionary relationships. J. Bacteriol. **174**: 3843–3849.

McEvoy, J. L., Murata, H. and Chatterjee, A. K. 1992. Genetic evidence for an activator required for induction of pectin lyase in *Erwinia carotovora* subsp. *carotovora* by DNA-damaging agents. J. Bacteriol. **174**: 5471–5474.

Mulvey, M. R. and Loewen, P. C. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. Nucleic Acid Res. **23**: 9979–9991.

Murata, H. and Magae, Y. 1996. Toxin production in a mushroom pathogenic bacterium, *Pseudomonas tolaasii* strain PT814 is activated by signals present in a host, *Pleurotus ostreatus*, and those accumulating in the medium in the course of bacterial growth. In: Mushroom biology and mushroom products, (ed. Royse, D. J.), pp. 483–494. Pennsylvania State Univ. Press, College Park, Penn., USA.

Murata, H., McEvoy, J. L., Chatterjee, A., Collmer, A. and Chatterjee, A. K. 1991. Molecular cloning of an *aepA* gene that activates production of extracellular pectolytic, cellulolytic, and proteolytic enzymes in *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. **4**: 239–246.

Murata, H., Tsukamoto, T. and Shirata, S. 1998. *rtpA*, a gene encoding a bacterial two-component sensor kinase, determines pathogenic traits of *Pseudomonas tolaasii*, the causal agent of brown blotch disease of a cultivated mushroom, *Pleurotus ostreatus*. Mycoscience **39**: 261–271.

Nystrom, T., Albertson, N. H., Flardh, K. and Kjelleberg, S. 1991. Physiology and molecular adaptation to starvation and recovery from starvation by the marine *Vibrio* sp. S14. FEMS Microbiol. Ecol. **74**: 129–140.

Sarniguet, A., Kraus, J., Henkels, M. D., Muehlchen, A. M. and Loper, J. 1995. The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. Proc. Natl. Acad. Sci. USA **92**: 12255–12259.

Shine, J. and Dalgarno, L. 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**: 1342–1346.

Spector, M. P. and Cubitt, C. L. 1992. Starvation-inducible loci of *Salmonella typhimurium*: regulation and roles in starvation survival. Mol. Microbiol. **6**: 1467–1476.

Suyama, K. 1994. Bacterial mushroom disease caused by *Pseudomonas tolaasii*. Proc. 10th Sym. for Kinoko Gijutsu Shudankai, pp. 12–15. (In Japanese.)

Suyama, K. and Fujii, H. 1993. Bacterial disease occurred on cultivated mushroom in Japan. J. Agric. Sci. (Tokyo Univ. Agric.) **38**: 35–50. (In Japanese.)

Tsukamoto, T., Shirata, A. and Murata, H. 1998. Isolation of a Gram-positive bacterium effective in suppression of brown blotch disease of cultivated mushrooms, *Pleurotus ostreatus* and *Agaricus bisporus*, caused by *Pseudomonas tolaasii*. Mycoscience **39**: 273–278.

Walker, G. C. 1987. The SOS response of *Escherichia coli*. In: *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2, (eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. and Umbarger, H. E.), pp. 1346–1357, Amer. Soc. Microbiol., Wash. DC, USA.