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Effect of Superoxide Dismutase Gene Inactivation on Virulence of *Pseudomonas aeruginosa* PAO1 toward the Silkworm, *Bombyx mori*▽

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To investigate the role of superoxide dismutase (SOD) in virulence against the silkworm, *Bombyx mori*, mutants of *Pseudomonas aeruginosa* PAO1 lacking manganese-SOD (PAO1*sodM*), iron-SOD (PAO1*sodB*), or both (PAO1*sodMB*) were generated. The mutants were injected into the hemocoel of *B. mori*. The virulence decreased in the order PAO1 = PAO1*sodM* > PAO1*sodB* > PAO1*sodMB*. In particular, PAO1*sodMB* was avirulent at a dose of 10^5 cells or less. The *sod* double mutant PAO1*sodMB* was then complemented with either pSodM or pSodB in *trans*. In both the complemented strains, the virulence was partially restored. Of the two plasmids, pSodB contributed more to the virulence of *P. aeruginosa* against *B. mori*. The results of growth in *B. mori* hemolymph broth and microscopic analysis suggested that a longer lag phase and superoxide sensitivity correlated with decreased virulence in *sod* mutants. In conclusion, the SODs are required for full virulence of *P. aeruginosa* against *B. mori* and Fe-SOD is more important than Mn-SOD in the infection process.

Pseudomonas aeruginosa is ubiquitous and is found in diverse environments including soil, freshwater, and marine environments. It is also an opportunistic pathogen in three distinct groups of organisms: vertebrates, invertebrates, and plants (17, 18).

Similar to other pathogens, *P. aeruginosa* must overcome the oxidative stress response generated by the host for successful infection. During the process of active infection, the primary source of exogenous oxidative stress for pathogenic bacteria is attack by host phagocytic cells. Phagocytes utilize the cytotoxic effects of many of the reactive oxygen species, such as superoxide, hydrogen peroxide, and the highly toxic hydroxyl radical. These reactive oxygen species can damage the nucleic acids, proteins, and cell membranes of pathogens. On the other hand, pathogens have effective enzymatic pathways of oxidant inactivation, including those catalyzed by superoxide dismutase (SOD), catalase/peroxidase, and glutathione in combination with glutathione peroxidase and glutathione reductase (12, 13). SOD represents the first line of defense against superoxide stress by converting superoxide into hydrogen peroxide and oxygen, thereby protecting cells from the toxic effects of superoxide.

P. aeruginosa possesses both manganese-cofactored SOD (Mn-SOD) and iron-cofactored SOD (Fe-SOD), and the genes encoding these proteins (*sodM*, previously referred to as *sodA*, and *sodB*, respectively) have been cloned and characterized (14, 15, 16). The expression of these SODs is controlled by environmental factors. In the presence of relatively high concentrations of extracellular iron, Fe-SOD is preferentially expressed, whereas the expression of Fe-SOD decreases and Mn-SOD is produced under iron-limited conditions (5, 14). For example, *P. aeruginosa* PAO1 produces mainly Fe-SOD in Luria-Bertani (LB) broth. In contrast Mn-SOD is produced in low-phosphate succinate (LPS) broth (16).

Since superoxide is endogenously produced under aerobic conditions, inactivation of SOD often results in decreased viability. In fact, *sodB* and *sodM sodB* double mutants of PAO1 have been shown to grow very slowly. In addition, the double mutants demonstrated severe auxotrophy (16).

Hassett et al. (16) also reported that a mutation in *sodM* only slightly increased sensitivity to paraquat, a superoxide generator. However, the *sodB* mutant and, to a greater extent, the double mutant demonstrated a marked increase in sensitivity to paraquat (16).

The isolation of Mn-SOD-deficient *P. aeruginosa* from blood indicates that Fe-SOD is more important than Mn-SOD to its virulence in humans (5). However, the interaction of the pathogen with other hosts is not well understood. The silkworm, *Bombyx mori*, is a good insect model with which to study the interaction between insects and opportunistic bacterial pathogens since it is one of the best-characterized insect species, both phenotypically and genotypically (9, 19, 20). In this study, we constructed *sodM*, *sodB*, and double mutants in PAO1, a wild-type strain of *P. aeruginosa*, and then compared the virulence levels of these mutants in *B. mori*.

MATERIALS AND METHODS

Organisms, plasmids, and media. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *P. aeruginosa* PAO1 was a kind gift of J. Kato, Hiroshima University, Hiroshima, Japan. The strain was originally donated by the Anand M. Chakrabaty laboratory, University of Illinois at Chicago.

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TABLE 1. Bacteria, plasmids, and primers used in this study

Bacterium, plasmid, or primer	Description or sequence[a]	Reference or source
Bacteria		
Pseudomonas aeruginosa		
PAO1	Wild type	Hiroshima University, Japan, via J. Kato
PAO1*sodM*	Mn-SOD mutant of PAO1; *sodM*::Ω*aac* Gm^r	This study
PAO1*sodB*	Fe-SOD mutant of PAO1; *sodB*::Ω*Tc* Tc^r	This study
PAO1*sodMB*	SOD double mutant of PAO1; *sodM*::Ω*aac sodB*::Ω*Tc* Gm^r Tc^r	This study
Escherichia coli		
DH5α	F^- *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* λ^- (ϕ80d*lacZ*Δ*M15*)	25
S17-1	*thi pro hsdR recA*; chromosomal RP4; Tra^-; Tp^r Sm/Sp^r	29
Plasmids		
pGEM-T Easy	PCR cloning vector; Amp^r *lacZ* f1 *ori*/*ori*	Promega
pBBR1MCS2	Broad-host-range vector; Km^r	22
pK18*mobsacB*	Allelic-exchange suicide vector; Km^r *sacB oriT* (RP4) *lacZ*	27
pHP45Ω*aac*	Source of gentamicin resistance cassette; Amp^r Gm^r	3
pHP45Ω*Tc*	Source of tetracycline resistance cassette; Amp^r Tc^r	11
pGSodM	3.0-kb *sodM* fragment in pGEM-T Easy	This study
pGSodB	1.1-kb *sodB* fragment in pGEM-T Easy	This study
pSodM	3.0-kb *sodM* fragment in pBBR1MCS2	This study
pSodB	1.1-kb *sodB* fragment in pBBR1MCS2	This study
pGSodM5′	2.5-kb fragment of 5′ *sodM* in pGEM-T Easy	This study
pGSodM3′	0.5-kb fragment of 3′ *sodM* in pGEM-T-Easy	This study
pGSodB5′	0.5-kb fragment of 5′ *sodB* in pGEM-T Easy	This study
pGSodB3′	0.7-kb fragment of 3′ *sodB* in pGEM-T Easy	This study
pGSodMHind	3.0-kb *sodM* fragment containing HindIII site in pGEM-T Easy	This study
pGSodBHind	1.1-kb *sodB* fragment containing HindIII site in pGEM-T Easy	This study
pGSodMΩaac	2.9-kb mutated *sodM* fragment in pGEM-T Easy	This study
pGSodBΩTc	3.3-kb mutated *sodB* fragment in pGEM-T Easy	This study
pKSodM	2.9-kb mutated *sodM* fragment in pK18*mobsacB*	This study
pKSodB	3.3-kb mutated *sodB* fragment in pK18*mobsacB*	This study
Primers		
sodMf(−2350)	TTCAATTGTCCGCCTCTAT	This study
sodMr626	CATCACGGGTAACACTAGG	This study
sodMf133HindIII	GCGAAGCTTGGCACGCCCTATGC	This study
sodMr124HindIII	CTTAAGCTTCGGCGTTCAGGTTGT	This study
sodBf(−346)	CGCGGTTTCTGTCTTC	This study
sodBr760	GGCCTTCCTTGTTCGT	This study
sodBf110HindIII	CCTAAGCTTTGAACCTGAACAACCTGA	This study
sodBr109HindIII	TCAAAGCTTCCACGTAGGTGTTGTGGT	This study

[a] Abbreviations for phenotype: Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Sp^r, spectinomycin resistance; Sm^r, streptomycin resistance; Tp^r, trimethoprim resistance.

Cultures of *Escherichia coli* and *P. aeruginosa* were routinely grown in LB medium at 37°C and 30°C, respectively. For the superoxide dismutase assay and mortality study, *P. aeruginosa* strains were grown in LB and LPS (7) broth. Antibiotics were used at the following concentrations (*E. coli* and *P. aeruginosa*): 50 μg/ml ampicillin, 5 μg/ml streptomycin, 50 μg/ml trimethoprim, 15 and 50 μg/ml gentamicin (Gm), 15 and 50 μg/ml tetracycline (Tc), and 30 and 200 μg/ml kanamycin (Km). In all the experiments, PAO1*sodM*, PAO1*sodB*, and PAO1*sodMB* were cultured in the medium containing Gm, Tc, and both Gm and Tc, respectively. The strains of *P. aeruginosa* carrying pBBR1MCS2 and derivatives were cultured in the media supplemented with Km. Eggs of silkworm (*B. mori*) hybrids of Kinshu × Showa were obtained from Ueda Sanshu (Ueda, Nagano, Japan). Larvae were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) at 25°C. In all the studies, *B. mori* worms at the fourth larval instar were used.

Construction of superoxide mutants. The 3.0-kb *sodM* fragment was amplified by PCR using KOD Plus polymerase (TOYOBO Co., Ltd., Osaka, Japan) and the primers sodMf(−2350) and sodMr626 (95°C for 2 min, 35 cycles of 15 s at 94°C and 30 s at 55°C, and 3 min at 68°C). The A-overhanging *sodM* fragment was cloned into pGEM-T Easy to obtain pGSodM. In addition to the above primers, we used specific primers (sodMf133HindIII and sodMr124HindIII) to create suitable restriction sites in *sodM*. The 2.5-kb 5′ region and 0.5-kb 3′ region of *sodM* were amplified and cloned into pGEM-T Easy to create pGSodM5′ and pGSodM3′, respectively. To construct pGSodMHind, a small EcoRI-HindIII fragment was eliminated from pGSodM5′ and subsequently ligated with the 0.5-kb EcoRI-HindIII fragment of pGSodM3′. Then, the Ω*aac* cassette from pHP45Ω*aac* was inserted into the artificial HindIII site of pGSodMHind, designated pGSodMΩaac. To construct pKSodM, a 2.9-kb BamHI-EcoRI fragment of pGSodMΩaac harboring a disrupted *sodM* gene was ligated into pK18*mobsacB*. To create pSodM, a 3.0-kb EcoRI fragment of pGSodM was cloned into a broad-host-range plasmid, pBBR1MCS2. pKsodM and pSodM were used for the disruption and complementation of *sodM*, respectively. For the disruption and complementation of *sodB*, pKsodB and pSodB were constructed using a similar procedure.

The plasmid pKsodM or pKsodB was mobilized from the *E. coli* strain S17-1 (29) into *P. aeruginosa* PAO1, followed by selection on LB containing 10% sucrose and appropriate antibiotics to obtain *sodM*- or *sodB*-disrupted mutants.

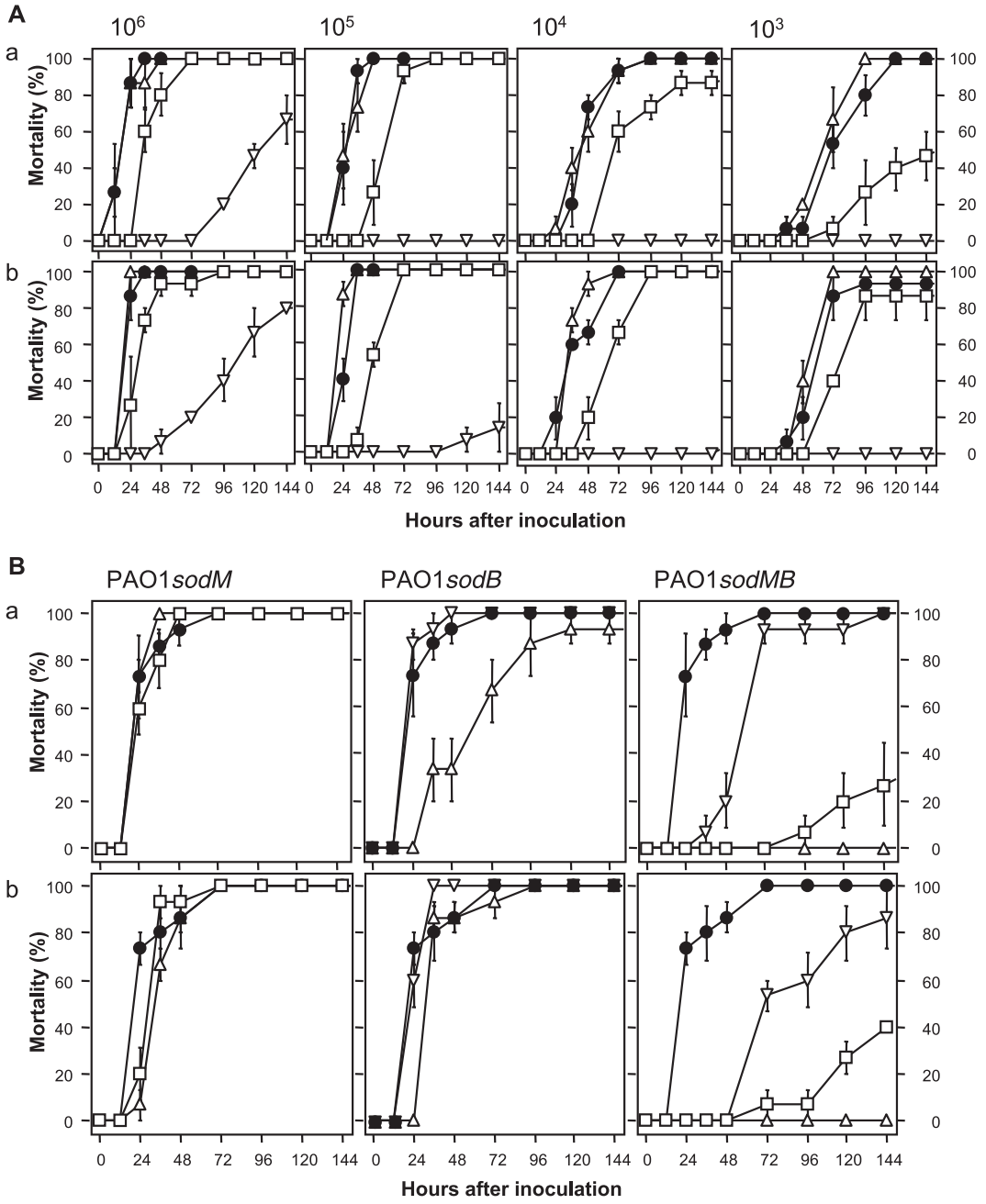

FIG. 1. Mortality assay of the *sod* mutants (A) and the complemented strains (B) of *P. aeruginosa* PAO1 in *B. mori.* Each strain was precultured in LB (a) or LPS (b). *P. aeruginosa* PAO1 (circles), PAO1*sodM* (triangles), PAO1*sodB* (squares), and PAO1*sodMB* (inverted triangles) were injected into the fourth instar of *B. mori* larvae at various doses (A). Similarly, the complemented strain was inoculated (B). PAO1(pBBR1MCS2), the *sod* mutants carrying pBBR1MCS2, the *sod* mutants carrying pSodM, and the *sod* mutant carrying pSodB are represented as circles, triangles, squares, and inverted triangles, respectively. In the inoculation test using the complemented strains, the dose was 10^5 cells. Five larvae were injected per dilution. Each data point is an average of three replicates, and the error bar indicates the standard error of the mean.

Similarly, to create a *sodM sodB* double mutant, the gentamicin-resistant *sodM* mutant was mated with *E. coli* S17-1 containing pKsodB. The *sodM*, *sodB*, and *sodM sodB* double mutant strains were designated PAO1*sodM*, PAO1*sodB*, and PAO1*sodMB*, respectively.

Similarly plasmids pSodM and pSodB were transferred into the *sod* mutants by biparental mating for complementation. Plasmids were extracted from the complemented strains by the alkaline lysis procedure and analyzed.

***B. mori* mortality studies.** Virulence of the *sod* mutants toward *B. mori* was estimated by the method reported by Chieda et al. (6). Briefly, the inocula were cultured in LB and LPS broth with appropriate antibiotics, since *P. aeruginosa* mainly produced Fe-SOD and Mn-SOD in LB and LPS, respectively (5, 16). The cells were harvested at mid-logarithmic phase by centrifugation at 20,630 × *g* for 1 min at 4°C. The pellets were resuspended with sterilized water, and the concentration was adjusted according to the optical density at 660 nm (OD_{660}). The bacterial concentration was adjusted based on the standard curve showing the correlation between OD_{660} and viable cell number determined on LB using PAO1. Prior to inoculation, the bacterial suspension was stained with 10 μg/ml propidium iodide (PI; Dojindo Laboratories, Kumamoto, Japan) to determine the viability of the bacteria. Ten microliters of the bacterial suspension was injected with a syringe into fourth-instar *B. mori* larvae. The mortality of the

inoculated larvae was monitored. Five larvae were injected per dilution, and three replicates per trial were performed.

Bacterial growth in the hemolymph of *B. mori*. The hemolymph was collected from the fourth instar of *B. mori*. To inactivate the host's defense reactions, comprised of a phenol oxidase cascade and phagocytosis, the collected hemolymph was immediately treated at 60°C for 10 min. After centrifugation at 20,630 × *g* for 15 min at 25°C, the supernatant was filter sterilized (0.45 μm) and the filtrate was designated *B. mori* hemolymph (BMH) broth. The strains were grown overnight in LB broth with appropriate antibiotics at 30°C. BMH broth (3 ml) was inoculated with 3 μl of the LB culture and incubated at 25°C until the OD_{660} reached 0.1. At this point, a 30-μl aliquot of preculture was inoculated into 3 ml of fresh BMH broth with appropriate antibiotics and shaken at 70 rpm under aerobic conditions at 25°C to mimic the hemolymph of *B. mori* with the exception of the host's defense reactions. The OD_{660} was automatically recorded every 10 min with an Advantec TVS062CA biophotorecorder (Advantec Toyo Co. Ltd., Tokyo, Japan).

Microscopic analysis. The hemolymph of the *B. mori* worms inoculated with 10^5 cells of various strains of *P. aeruginosa* was collected every 6 h after inoculation. Inoculation was carried out as described above. The hemolymph smears on the slide were stained with Giemsa solution, mounted with Permount (Fisher Scientific, Fair Lawn, NJ), and observed under a light microscope.

Growth of wild-type strain in *B. mori*. PAO1(pBBR1MCS2) was cultured in LB containing Km. *B. mori* worms were inoculated with 10^5 cells of the strain as described above. To monitor the bacterial growth, the hemolymph was collected and diluted and then spread on LB plates supplemented with Km.

RESULTS

Inactivation of *sodM* and *sodB* of *P. aeruginosa*. The *sodM*, *sodB*, and *sodM sodB* double mutants of *P. aeruginosa* PAO1 were created. The insertion of Ω cassettes in the mutants was confirmed by Southern hybridization (data not shown). In the complemented strains, expected plasmids were detected (data not shown).

Mortality assay. In order to study the possible impact of SOD on the virulence of *P. aeruginosa* PAO1 against *B. mori*, various doses of SOD mutants were inoculated into the larvae. Inocula were cultured in LB and LPS broth and collected during the logarithmic growth phase. The results of the PI staining indicated that almost all bacteria were viable at this stage in all strains (data not shown). The inocula were adjusted at 10^8, 10^7, 10^6, and 10^5 CFU/ml based on OD_{660}. Data from three inoculation experiments were analyzed (Fig. 1).

Inoculation with PAO1 and PAO1*sodM* at 10^6, 10^5, 10^4, and 10^3 cells resulted in mortalities of 100, 100, 90, and 50%, respectively, within 72 h, and almost all larvae died within 144 h with no significant differences between these strains (Fig. 1A). On the other hand, PAO1*sodB* displayed a decreased rate of mortality in comparison with the above two strains. The mutant showed a marked decline in virulence when the inoculation dose was low. In contrast, almost all larvae inoculated with PAO1*sodMB* at 10^5 cells or less survived until 144 h. The virulence decreased in the order PAO1 = PAO1*sodM* > PAO1*sodB* > PAO1*sodMB*. Although the type of medium did not influence this order, PAO1*sodB* was more virulent when it was precultured in LPS.

The virulence of complemented strains was also investigated (Fig. 1B). In this assay, PAO1 harboring pBBR1MCS2 was used as a positive control. In the case of *sodM*, no significant difference was observed among PAO1(pBBR1MCS2), PAO1 *sodM*(pBBR1MCS2), and PAO1*sodM*(pSodM). The virulence of PAO1*sodB* was completely restored when the *sodB* gene was reintroduced in the mutant by the plasmid pSodB. The *sod* double mutant, PAO1*sodMB*, was complemented with either pSodM or pSodB. In both of the complemented strains, the virulence was partially restored. Of the two plasmids, pSodB contributed more to the virulence of *P. aeruginosa* against *B. mori*.

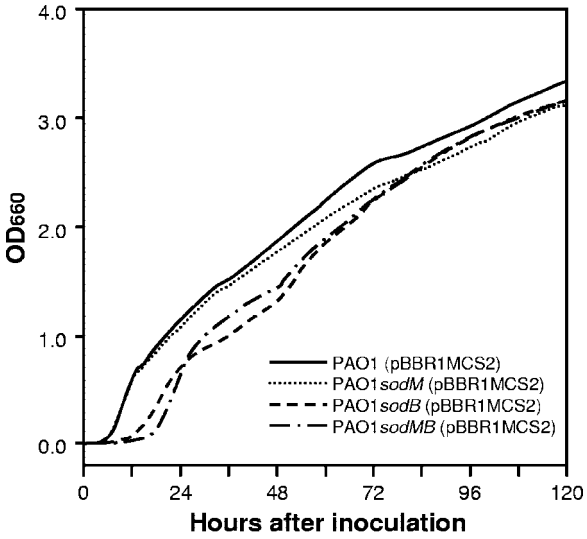

FIG. 2. Growth of *P. aeruginosa* strains in BMH broth. The strains were grown overnight in LB broth at 30°C. Three microliters of the LB culture was subcultured into 3 ml of BMH broth and incubated at 25°C until an OD_{660} of 0.1 was reached. Then, a 30-μl aliquot of preculture was inoculated into 3 ml of fresh BMH broth and shaken at 70 rpm under aerobic conditions at 25°C. The OD_{660} was automatically recorded with an Advantec TVS062CA biophotorecorder (Advantec Toyo Co. Ltd., Tokyo, Japan). Three experiments were repeated to ensure reproducibility and gave equivalent results. Each plot represents the average of triplicates.

Bacterial growth in the extracted hemolymph of *B. mori*. Growth of *P. aeruginosa* strains in BMH broth is shown in Fig. 2. All strains could propagate in the broth, and the OD_{660} reached 3.0 at around 120 h after inoculation. The growth rates during the logarithmic phase were similar among the strains. For example, the required times for the OD_{660} to increase from 0.1 to 2.0 were 46.7, 51.2, 52.8, and 47.3 h for PAO1, PAO1*sodM*, PAO1*sodB*, and PAO1*sodMB*, respectively. However, longer lag phase times were observed in PAO1*sodB* and PAO1*sodMB* than in PAO1 and PAO1*sodM*. The OD_{660} of the cultures of PAO1and PAO1*sodM* reached 0.1 within 6.3 h after inoculation. However, the PAO1*sodB* and PAO1 *sodMB* strains required 13.2 and 17.3 h, respectively, to reach this OD value.

Microscopic analysis. Bacterial propagation in the hemocoel of the *B. mori* worms infected with *P. aeruginosa* strains at a dose of 10^5 cells was investigated using light microscopy (Fig. 3). A few cells of bacteria were observed 6 h after inoculation with PAO1. Although an increase in the bacterial population was achieved by 12 h, a significant decrease occurred 18 h after inoculation. The cell number then increased markedly at 24 h after inoculation. A similar change of cell density was observed in the case of PAO1*sodM*. In contrast, PAO1*sodB* was detected at low density only at 12 h after inoculation. At other time points (6, 18, and 24 h), no bacterial cells were observed. Cells of PAO1*sodMB* were not detected at any observation times.

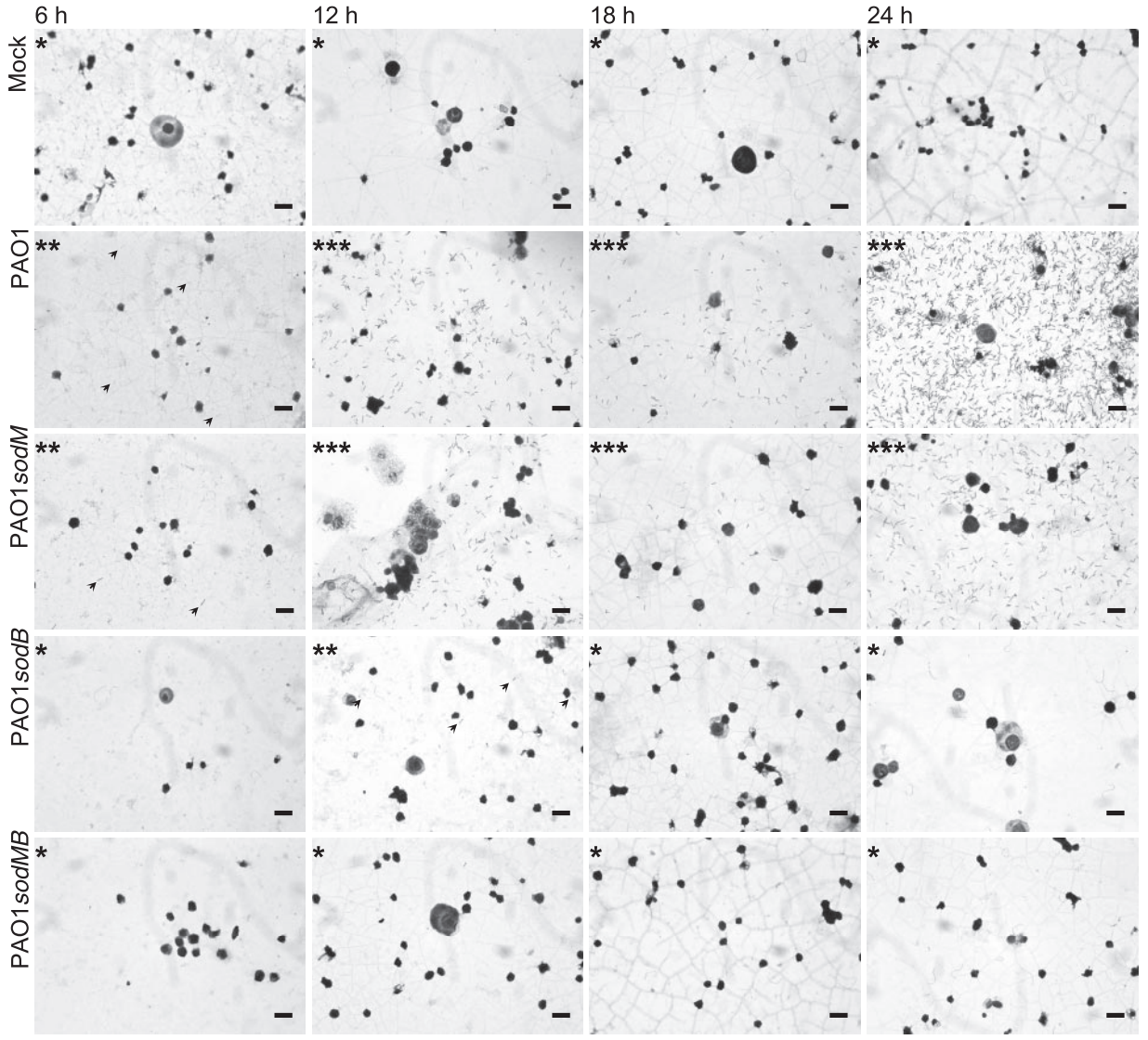

FIG. 3. Light microscopic observation of the hemolymph from *B. mori* inoculated with *P. aeruginosa* strains. From each strain 10^5 cells were injected into the fourth-instar larvae. The hemolymph was collected every 6 h after inoculation. The smear of collected hemolymph was stained with Giemsa solution. A single asterisk means that bacterial cells were not observed. A double asterisk means that the bacterial density was low. A triple asterisk means that the bacterial density was high. On the plate, some of the bacterial cells are shown with arrowheads. Bars, 20 μm.

Bacterial growth in *B. mori*. Growth of PAO1(pBBR1MCS2) in the hemocoel of *B. mori* larvae was monitored (Fig. 4). The bacterial concentrations were 7.8×10^4, 2.1×10^4, and 4.0×10^4 CFU/ml after 3, 6, and 9 h after inoculation, respectively. The concentration gradually decreased until 15 h and then increased. At the 24th hour after inoculation, the population reached approximately 3.9×10^5 CFU/ml.

DISCUSSION

P. aeruginosa opportunistically infects not only vertebrate animals but also invertebrate animals. The contribution of SOD to bacterial virulence has previously been discussed for many animal pathogens, including *Vibrio shiloi* (1), *Haemophilus ducreyi* (4), *Aeromonas salmonicida* subsp. *salmonicida* (8), *Mycobacterium tuberculosis* (10), *Staphylococcus aureus* (21), *Streptococcus agalactiae* (23), *Shigella* spp. (24), *Salmonella enterica* serovar Choleraesuis (26), and *Helicobacter pylori* (28). *P. aeruginosa* PAO1 possesses two SODs, Mn-SOD and Fe-SOD (15). To understand the role of these SODs in the infection process of invertebrates, we constructed *sod* mutants and inoculated these mutants into the silkworm, *B. mori*. Previously it was shown that *P. aeruginosa* PAO1 produced mainly Fe-SOD in LB broth, whereas it produced Mn-SOD in LPS broth (16). This result meant that PAO1*sodB* that had been precultured in LB did not possess SOD in the cell. Similarly, no SOD was produced by PAO1*sodM* when the mutant was cultured in LPS. To avoid the effect of the medium on the virulence of the *sod* mutants, we separately prepared the inocula cultured in LB and LPS broth and then inoculated *B. mori* in this study.

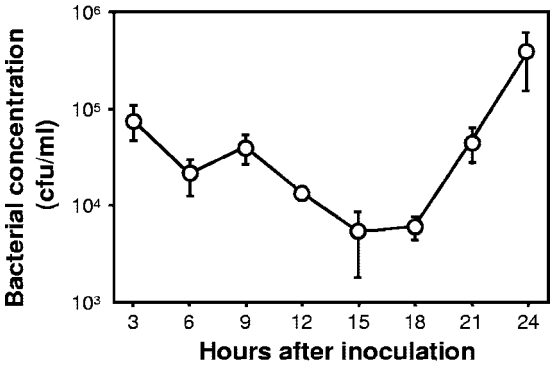

FIG. 4. Growth of *P. aeruginosa* PAO1(pBBR1MCS2) in *B. mori*. Cells (10^5) were injected into the fourth-instar larvae. A hemolymph sample of a single larva was collected every 3 h after inoculation. Three samples were independently obtained from different larvae. The number of viable cells in the hemolymph was estimated by a plate count procedure. Each plotted point represents the average of triplicates. Error bars indicates standard errors.

In comparison with PAO1, PAO1*sodM* was of approximately equal virulence, but the virulence of PAO1*sodB* declined. The double mutant did not have a mortality effect on *B. mori* at doses of $\leq 10^5$ cells. To confirm whether the virulence in the double mutant could be restored by complementation with *sodM* and *sodB*, *B. mori* worms were inoculated with PAO1*sodMB* harboring pBBR1MCS2, pSodM, or pSodB. The virulence of the complemented strain of PAO1*sodMB* was in the following order: PAO1*sodMB*(pSodB) > PAO1*sodMB*(pSodM) > PAO1*sodMB*(pBBR1MCS2). The difference in culture conditions (LB or LPS) had no effect on the order of virulence against *B. mori* (Fig. 1). These results indicate that both Mn-SOD and Fe-SOD are functional but that Fe-SOD is more important than Mn-SOD in the virulence of *P. aeruginosa* against *B. mori*.

Hassett et al. reported that Fe-SOD is the key enzyme in aerobic metabolism and against oxidative stress (16). In their studies, the *sodB* mutant grew much more slowly than the *sodM* mutant or the wild-type strain. The double mutant demonstrated an even lower rate of aerobic growth than the *sodB* mutant. The double mutant was especially incapable of growth in glucose minimal medium, suggesting auxotrophy. This auxotrophy in PAO*sodB* and PAO1*sodMB* constructed in the present study was confirmed, and the cells cultured in the media used in this study also had the same characteristics (data not shown). To ascertain the effect of growth speed and auxotrophy on virulence, the growth in BMH broth under aerobic conditions at 25°C, which mimics in vivo conditions, was monitored. The growth speeds were similar during the logarithmic phase among all the strains in this broth. However, the length of the lag phase was prolonged in PAO1*sodB* and PAO1*sodMB*. This phenotypic change could be one reason for the decreased virulence seen in these strains. However, the reduced virulence could not be completely explained by only the length of the lag phase, because all strains were able to grow under the environmental conditions. The initial concentration in BMH broth was approximately 10^6 CFU/ml. This concentration was equivalent to the bacterial cell density in hemolymph inoculated with 10^5 cells, since the total volume of hemolymph was 100 to 200 μl in fourth-larval-instar silkworms. At this concentration, PAO1*sodMB* was avirulent (Fig. 1).

Microscopic analysis was then performed to clarify the behavior of the bacterial cells injected into hemolymph. A few of the cells of PAO1*sodB* could be detected at 12 h after inoculation, while no cells of the double mutant PAO1*sodMB* were observed. In the *sod* mutants of PAO1, sensitivity to paraquat, a superoxide generator, was increased in the order PAO1 < *sodM* mutant < *sodB* mutant < *sodM sodB* double mutant (16). In the interaction between host and microorganism, superoxide is generated by macrophages and other phagocytic cells. This first defense mechanism is conserved in insects (2). SODs have been reported to enhance bacterial survival by inhibition of the macrophage oxidative burst. Thus, the increase of sensitivity to oxidative stress would be another factor contributing to the reduced virulence of certain strains. More detailed analysis is needed to clarify the interaction between the *sod* mutants and the phagocytic cells in *B. mori*.

The bacterial population decreased 15 to 18 h after inoculation (Fig. 4). The growth curve of the viable bacterial cells in the hemocoel of *B. mori* infected with PAO1(pBBR1MCS2) also supported the decline of the bacterial cells in our microscopic observations. This population decline may have been due to the antibacterial substances produced by the host cells. *B. mori* produces antibacterial substances, including cecropin, attacin, lebocin, and moricin. These compounds are mainly released from the fat body and from hemocytes after bacterial infection (30). Since the population of PAO1*sodB* was low at 12 h after inoculation, almost all of the mutant cells would have been killed by this subsequent defense response induced by the host cell. The sensitivity to these antibacterial substances must be investigated in the *sod* mutants.

Taken together, the results of this study suggest that SODs are required for the full virulence of *P. aeruginosa* against *B. mori* and that Fe-SOD is more important than Mn-SOD in the infection process.

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