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Unusual Growth Phase and Oxygen Tension Regulation of Oxidative Stress Protection Enzymes, Catalase and Superoxide Dismutase, in the Phytopathogen *Xanthomonas oryzae* pv. oryzae

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The enzymes catalase and superoxide dismutase play major roles in protecting phytopathogenic bacteria from oxidative stress. In *Xanthomonas* species, these enzymes are regulated by both growth phase and oxygen tension. The highest enzyme levels were detected within 1 h of growth. Continued growth resulted in a decline of both enzyme activities. High oxygen tension was an inducing signal for both enzyme activities. An 80,000-Da monofunctional catalase and a manganese superoxide dismutase were the major forms of the enzymes detected at different stages of growth. The unusual regulatory patterns are common among several *Xanthomonas* strains tested and may be advantageous to *Xanthomonas* species during the initial stage of plant-microorganism interactions.

A compatible interaction between a pathogen and a particular plant host results in disease development, whereas an incompatible interaction leads to localization and eventual destruction of the microorganism at the infection site. Increased production of reactive oxygen species by the host during the first few hours of infection has been associated with incompatible interaction, suggesting that this plays an important role in an active plant defense (5, 21). Furthermore, oxidative stress is an inevitable consequence of aerobic life, since normal cellular metabolism generates many forms of reactive oxygen species. Thus, in plant-microorganism interactions, the invading pathogens must detoxify reactive oxygen species from the host-generated oxidative stresses as well as from their own metabolism. In bacteria, the most effective protective enzyme systems involve superoxide dismutase and catalase, which catalyze the degradation of superoxide and hydrogen peroxide.

Xanthomonas is an important genus of phytopathogens known to cause severe damage on every economically important crop. *Xanthomonas oryzae* pv. oryzae is the causative agent of the most devastating bacteria diseases of rice (20). Currently, very little is known about the oxidative stress response of *Xanthomonas* spp., whereas extensive studies on the regulation and enzymology of superoxide dismutase (SOD) and catalase (KAT) in other microorganisms have been carried out (6, 7, 9, 15, 16, 18, 22). Multiple KAT and SOD isozymes have been detected, and certain of these appeared to be under both growth and aeration regulation (6, 16). These parameters are important components in host-bacterium interactions. In the work described in this communication, we have demonstrated growth phase and oxygen tension regulation of both enzymes in *Xanthomonas* spp.

X. oryzae pv. oryzae was grown aerobically on a shaker at 28°C in a 125-ml flask containing 20 ml of SB medium (19). The starting inoculum was prepared by subculturing late-log-phase cells (5%) into fresh medium. At the indicated time, the bacteria were collected and clear lysates were prepared by sonication of cell pellets in phosphate buffer (pH 7.8) followed by centrifugation at 8,000 × *g*. KAT and SOD were assayed as previously described (1, 17). The protein concentration was determined by the method of Bradford (3). *X. oryzae* pv. oryzae KAT and SOD activities were highest during the early log phase, i.e., during the first 1 h of growth. Maximum levels were attained as the culture was emerging from the lag phase, and the levels subsequently declined as growth proceeded. Levels of both enzymes were several fold lower when the culture reached early stationary phase (Fig. 1). This pattern of enzyme activity was unexpected, because previous studies of KAT and SOD in other bacteria showed that the highest levels were recorded during early stationary phase with the lowest activities reached during the early log phase of growth (6–8, 11–13, 15, 16, 18). However, a recent report indicates that in *Haemophilus influenzae* the highest level of the monofunctional KAT is found during the log phase (2).

The declines in KAT and SOD levels during the stationary phase could be due to a decrease in the rate of enzyme synthesis or to the appearance of inhibitory factors. The latter possibility was ruled out by mixing of lysates prepared from stationary-phase cells with lysates from early-log-phase cells prior to enzyme assays. The treatment did not have any inhibitory effects on the activity of either enzyme (data not shown). However, the induction of KAT during early log phase was due to de novo enzyme synthesis and can be blocked by either a transcription (rifampin) or a translation (chloramphenicol) inhibitor (Fig. 2). The effectiveness of both inhibitors were evaluated by measuring the decrease in the total protein concentration and the cessation of growth. However, the induction of SOD during the growth phase was not blocked by these inhibitors. This could be a result of the high stability of SOD carrying over from the stationary phase coupled with the decrease in total protein concentrations caused by the inhibitors, which resulted in the increase in SOD specific activity. This notion was supported by observations that the total protein concentration had decreased and there was no increase in

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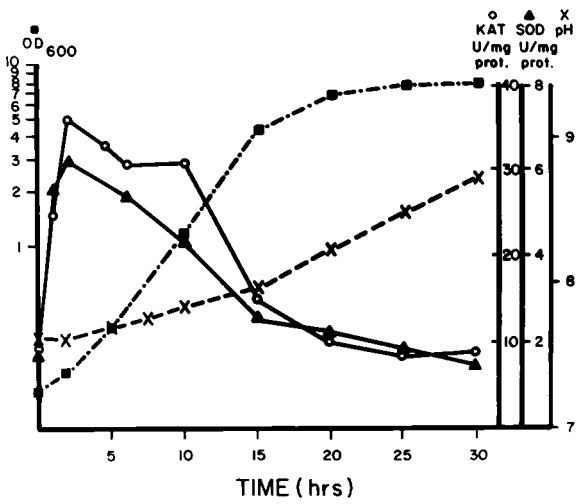

FIG. 1. Growth phase regulation of *X. oryzae* pv. oryzae KAT and SOD. *X. oryzae* pv. oryzae culture was grown aerobically in a shaker shaking at 100 rpm at 28°C in SB medium (19); the starting inoculum was from late-log-phase cells (5%). For KAT and SOD, 1 U of activity is defined as the decomposition of 1 μmol of H_2O_2 per min at pH 7 (2) and the amount required to inhibit the rate of reduction of cytochrome *c* by 50% (17), respectively. The pH of the medium was also determined. Growth was measured spectrophotometrically at 600 nm. The results shown are typical results from three independently performed experiments.

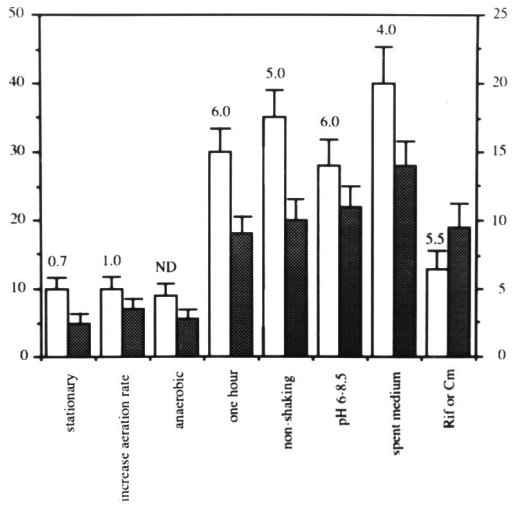

FIG. 2. *X. oryzae* pv. oryzae KAT (□) and SOD (▨) levels (in U per milligram of protein) in response to various growth conditions. Unless otherwise stated, all cultures were inoculated and grown as described in the legend to Fig. 1. "Stationary" indicates a stationary-phase culture. "Increase aeration rate" indicates a stationary-phase culture except that 10 ml of the culture was removed and the shaker speed was increased to 130 rpm to increase the aeration rate. "anaerobic" indicates subculturing of the culture into SB medium which was prepared by keeping fresh medium in an anaerobic jar without shaking for addition 1 h. "One hour" indicates subculture of the culture into fresh medium followed by aerobic growth for 1 h. "Non-shaking" indicates the same treatment as "one hour" except that the culture was kept without shaking. "pH 6–8.5" indicates the same conditions as "one hour" except that the pH of the medium was adjusted to 6, 7, 8, and 8.5. The variations in medium pH gave similar results. Therefore the representative results of enzyme activities are shown. "Spent medium" indicates the same treatment as "one hour" except that the fresh medium was replaced by spent medium prepared by centrifugation and filtration (to remove bacteria) of *X. oryzae* pv. oryzae stationary-phase cultures. The pH of the spent medium was 7.8. "Rif or Cm" indicates the same conditions as "one hour" except that rifampin (50 μg/ml) or chloramphenicol (50 μg/ml) was added. Both treatments gave similar results, and the representative results of enzyme activities were shown. The numbers above of the bars indicate the level of dissolved oxygen (in milligrams per milliliter) (10a). ND, the oxygen tension in the anaerobic culture could not be accurately determined. The vertical bars represent the range of three independently performed experiments.

SOD activity in the presence of inhibitors during early log phase.

The pattern of enzyme regulation in *X. oryzae* pv. oryzae PXO 86 was also seen with three other *X. oryzae* pv. oryzae strains, an *X. campestris* pv. campestris strain, a *X. campestris* pv. vesicatoria strain, and an *X. campestris* pv. malvacaerum strain (Table 1). We do not know the significance of the different enzymes levels observed in different bacterial strains. As expected, the *Escherichia coli* strain showed higher enzyme levels during the stationary phase (Table 1), consistent with previous reports (7, 16, 18).

The unusual pattern of KAT and SOD regulation prompted an examination of the inducing conditions. The production of both enzymes is known to be affected by variations in aeration rate, oxygen tension, medium components, and pH (16, 18). These parameters were investigated in *X. oryzae* pv. oryzae. Alterations in the aeration rate of stationary-phase cultures (both increases [Fig. 2] and decreases [data not shown]) and of early-log-phase cultures (decreases [Fig. 2]) had no effects on the enzyme levels. However, under all conditions tested where the oxygen tension of the medium was high, both KAT and SOD were rapidly induced regardless of the rate of aeration (Fig. 2). The data indicated that high oxygen tension and not the aeration rate was the inducing signal for synthesis of both enzymes. The notion was further

TABLE 1. Induction of KAT and SOD during early log phase in various *Xanthomonas* species

Species	KAT activity (U/mg of protein) at[a]:		Change in KAT activity (1 h/24 h)	SOD activity (U/mg of protein) at[a]:		Change in SOD activity (1 h/24 h)
	1 h	24 h		1 h	24 h	
X. oryzae pv. oryzae strain PXO 61	9.9	4.2	2.4	5.4	3.0	1.8
X. oryzae pv. oryzae strain PXO 79	21.5	8.9	2.4	6.4	3.1	2.1
X. oryzae pv. oryzae strain PXO 86	37.5	9.8	3.8	7.4	3.4	2.2
X. oryzae pv. oryzae strain PXO 99	41.3	9.4	4.4	4.2	2.1	2.0
X. campestris pv. campestris	6.9	3.2	2.2	11.3	6.5	1.7
X. campestris pv. malvacaerum	8.6	4.4	1.9	13.2	6.4	2.1
X. campestris pv. vesicatoria	8.3	4.0	2.1	5.0	2.4	2.0
E. coli	12.0	38.1	0.3	1.1	5.7	0.2

[a] The values for 1 h indicate the subculturing of a stationary-phase culture into fresh medium, and the values for 24 h indicate a stationary-phase culture. Growth conditions were as described in the legend to Fig. 1 for all *Xanthomonas* strains. *E. coli* was grown aerobically in LB medium at 37°C. The experiments were performed in triplicate, and the mean values are shown.

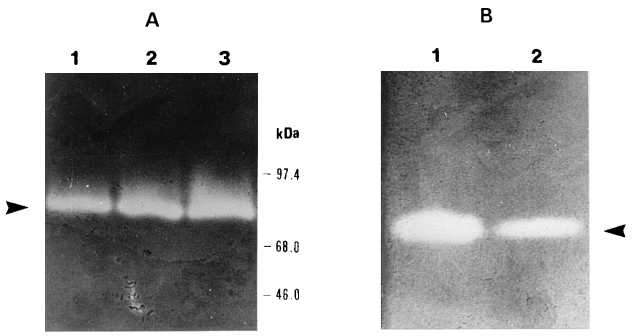

FIG. 3. Visualization of *X. oryzae* pv. oryzae KAT and SOD at different stages of growth. (A) KAT (2 U) was loaded into each lane. SDS-PAGE was carried out as described by Laemmli (14), except that samples were not boiled prior to loading. After electrophoresis, the gel was renatured by the method of Ikeda et al. (10) and stained for KAT activity (8). Lanes: 1, 2-h culture; 2, 8-h culture; 3, 24-h culture. The numbers to the right indicate the molecular masses of protein markers in kilodaltons. (B) SOD (4 U) was loaded into each lane of native polyacrylamide gel. Gel electrophoresis and staining for SOD activity were carried out as previously described (4). Lanes: 1, 2-h culture; 2, 24-h culture. The number of hours indicates the time after the inoculation.

supported by data that anaerobic growth abolished the induction of both enzymes (Fig. 2).

To test the possibility that metabolites produced during stationary-phase growth could influence SOD and KAT synthesis, the enzyme activities were monitored in *X. oryzae* pv. oryzae grown in spent medium (Fig. 2). The levels of KAT and SOD were higher in the spent-medium culture than in the parallel culture grown in fresh medium (Fig. 2). Thus, low enzyme activities at stationary phase were not due to any inhibitory components of the medium on enzyme synthesis. However, the preparation of spent medium resulted in increases in the oxygen tension of the medium and, together with some metabolites in the medium, may result in synergistic effects on enzyme induction. Similar results in which metabolites, especially organic acids, and pH of spent media act as inducers of KAT synthesis have been reported (18). The effects of variation in medium pH on the induction of KAT and SOD were examined by adjusting the pH of fresh medium. The results indicated that different pHs ranging from 6 to 8.5 have no effects on enzyme induction (Fig. 2).

Bacterial KAT enzymes can be divided into two groups, namely, a bifunctional peroxidase/KAT and a monofunctional KAT, on the basis of their properties (16). The induction of specific forms of KAT isozymes at different stages of growth has been reported (16, 18). We were interested in analyzing *X. oryzae* pv. oryzae KAT isozymes at different stages of growth with the KAT activity gel. The native KAT activity gel did not give a clear separation of *X. oryzae* pv. oryzae KAT. Thus, modified Laemmli (14), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an additional renaturation step (10) followed by KAT activity staining (4, 8) was used. One large disperse band of *X. oryzae* pv. oryzae KAT with a molecular mass of 80,000 Da was detected at different stages of growth (Fig. 3A). This band of positively staining KAT activity did not have peroxidase activity, indicating that it was a monofunctional KAT enzyme (23). The analysis of SOD isozymes from different stages of growth was carried out with a native PAGE gel and SOD activity staining (4). The results showed only one major form of SOD detected at both early-log and stationary phases (Fig. 3B). The SOD inhibition study to differentiate different SOD isozymes indicated that the major *X. oryzae* pv. oryzae SOD activity was a Mn SOD (the SOD activity was unaffected by KCN and H_2O_2 treatment [reference 4 and data not shown]). The data supported the notion that the increase in KAT and SOD activities during early log phase was due to increased synthesis of a monofunctional KAT isozyme and a Mn SOD.

In conclusion, the regulation of KAT and SOD in *Xanthomonas* species is different from that in other bacteria. The unusual enzyme regulation may have beneficial effects for *Xanthomonas* species, especially in the plant-microorganism interactions. Increased production of reactive oxygen species is associated with the initial phase of the active defense response by the plant (5, 19). At this stage of infection, the bacteria are most susceptible to plant defense responses. However, at this stage the small number of bacteria and the high oxygen tension induce high-level expression in *Xanthomonas* spp. of the enzymes KAT and SOD, which protect against oxidative stress and hence may protect the bacteria from the initial defense reactions by the plant.

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