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Cloning and Characterization of *katA*, Encoding the Major Monofunctional Catalase from *Xanthomonas campestris* pv. phaseoli and Characterization of the Encoded Catalase KatA

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Abstract. The first cloning and characterization of the gene *katA*, encoding the major catalase (KatA), from *Xanthomonas* is reported. A reverse genetic approach using a synthesized *katA*-specific DNA probe to screen a *X. campestris* pv. phaseoli genomic library was employed. A positively hybridizing clone designated pKat29 that contained a full-length *katA* was isolated. Analysis of the nucleotide sequence revealed an open reading frame of 1,521 bp encoding a 507-amino acid protein with a theoretical molecular mass of 56 kDa. The deduced amino acid sequence of KatA revealed 84% and 78% identity to CatF of *Pseudomonas syringae* and KatB of *P. aeruginosa*, respectively. Phylogenetic analysis places *Xanthomonas katA* in the clade I group of bacterial catalases. Unexpectedly, expression of *katA* in a heterologous *Escherichia coli* host resulted in a temperature-sensitive expression. The KatA enzyme was purified from an overproducing mutant of *X. campestris* and was characterized. It has apparent K_m and V_{max} values of 75 mM [H_2O_2] and 2.55×10^5 μmol H_2O_2 μmol heme^{-1} s^{-1}, respectively. The enzyme is highly sensitive to 3-amino-1,2,4-triazole and NaN_3, has a narrower optimal pH range than other catalases, and is more sensitive to heat inactivation.

Xanthomonas spp. is an important group of bacterial phytopathogens. During plant– microbe interactions, bacteria are exposed to plant-generated reactive oxygen species (ROS) including superoxide, H_2O_2, and organic peroxide [1]. ROS function as bacteriocidal agents and as secondary signal molecules to further activate plant defense responses [5]. To survive and proliferate, bacterial pathogens must overcome reactive oxygen species. Catalases (E.C. 1.11.1.6) have an important protective role against H_2O_2, because they convert H_2O_2 to oxygen and water. There are two major types of catalases: a bifunctional catalase-peroxidase and a monofunctional catalase coded by different genes [10, 13]. In most bacteria, catalases are the principal protection against H_2O_2 toxicity, and inactivation of the genes encoding catalases leads to increased sensitivity to H_2O_2. Moreover, novel roles of catalase on protection against electrophiles have also been reported in bacteria [15].

X. campestris pv. phaseoli (*Xp*) produces at least two monofunctional catalase isozymes denoted KatA and KatE encoded by *katA* and *katE*, respectively [14]. KatA is the major catalase, accounting for over 90% of the total activity present in all phases of growth. KatE could be detected only as cells entered the stationary phase of growth. Previously, *katX* encoding a monofunctional catalase from *X. oryzae* pv. oryzae and *katE* from *Xp* have been cloned and characterized [8, 14]. Here, we report the cloning of *katA* and the first purification and biochemical characterization of the major catalase KatA from *Xp*. Heterologous expression of *katA* in an *Escherichia coli katG katE* mutant was also performed.

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Materials and Methods

Growth conditions and media. All *Xanthomonas* strains were grown aerobically at 28°C in Silva-Buddenhagen (SB) medium containing

appropriate antibiotics [8]. All *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium.

Purification of the *Xp* KatA. All manipulations were carried out at 4°C. The crude extract was prepared by using a French press followed by high-speed centrifugation. To the clear lysate, 2.5% streptomycin sulfate was added, and the precipitate was removed by centrifugation. The supernatant was fractionated with 30, 40, 50, and 60% of saturation ammonium sulfate precipitation. At each step, the solution was stirred gently to ensure complete dissolution and precipitation. The precipitate from 60% ammonium sulfate containing the highest catalase-specific activity was dissolved and dialyzed overnight at 4°C against 4 L of 50 mM potassium phosphate buffer (pH 7.0). The protein was fractionated on a 2 × 10 cm hydroxylapatite column equilibrated in the same buffer eluted with a linear gradient of NaCl (0–0.5 M). The catalase-containing fractions were pooled, concentrated by ultrafiltration (Amicon), dialyzed overnight in the same buffer, and stored at −70°C.

Partial amino acid determination. Purified KatA was run on the SDS-PAGE after pretreatment with BrCN and blotted onto PVDF filter. The protein bands were cut from the filter and sent to the Tripartite Microanalytical Center at the University of Victoria (Victoria, Canada) to determine their partial amino acid sequences.

DNA isolation, cloning, PCR, and nucleotide sequencing. *Xanthomonas* genomic DNA was purified as previously described [14]. Primers for amplification of *Xp katA*, designed based on the partial amino acid sequence of KatA protein (NGVHAYKL) and the conserved region of monofunctional catalases (DNFFQETEQ), were BT50 [5′ AAC GG(G/C) GT(G/C) CA(C/T) GC(G/C) TA(C/T) AA(A/G) CT(G/C) GT(G/C) AA(C/T) GC(A/G) CA(A/G)GG 3′] and BT54 [5′ TG(C/T) TC(A/G/C) GT(C/T) TC(C/T) TG (A/G) AA(A/G) AA(A/G) TT(A/G) TC 3′], respectively. The two primers, *Xp* genomic DNA, the PCR reaction mix, and two units of Taq polymerase were mixed and used to amplify a portion of the *katA* gene through 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The resulting 344-bp PCR product was cloned into pGEM-T Easy vector (Promega) and used as a probe for screening the *Xp* genomic library constructed in a λZipLox vector (Bethesda Research Laboratories). Nucleotide sequencing was performed with the fluorescence dye terminator ABI prism kits and analyzed on an automated DNA sequencer ABI 377. The *katA* sequence has been deposited in the GenBank under the accession number AF461425.

Biochemical characterization of KatA. Catalase activity was estimated by the method of Rorth and Jensen [11] in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase in defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 in 1 min at 37°C. The optimum pH range of the enzyme was determined in 50 mM potassium phosphate buffer adjusted to the appropriate pH values. For values beyond the buffering capacity, the pH was adjusted by the addition of HCl or NaOH directly before the assay was performed. To estimate thermal stability, the purified enzyme was incubated at different temperatures for 10 min before the residual activity was measured at 37°C. The inhibition study was done by incubating KatA with indicated concentrations of various inhibitors for 1 min prior to assay for catalase activity.

Cell lysate preparation for catalase activity gels. Cell lysate preparation and catalase activity gel staining were performed as previously described [14]. Bacterial cells were lysed in 50 mM sodium phosphate buffer pH 7.0 by brief sonication, followed by centrifugation at 10,000 *g* for 10 min. Supernatants were used for catalase activity gels. Catalase isozymes were visualized on native PAGE gels performed as previously described [14]. Catalase activity appears as colorless bands against a dark brown background.

Results and Discussion

Cloning and characterization of *Xp katA*. The *katA* gene from *Xp* was cloned by a reverse genetic approach. The partial amino acid sequence of KatA, NGVHAYKL, was determined from trypsin digest fragments. The KatA amino acid sequence and another sequence derived from a highly conserved region of bacterial monofunctional catalases were used to design two degenerate oligonucleotide PCR primers which yielded a 344-bp PCR product that was cloned into pGEM-T-easy vector, giving pKat111. The nucleotide sequence of the fragment showed significant similarity to other monofunctional catalases. The 344-bp DNA fragment was then used as a probe to screen for the full-length *katA* gene from an *Xp* genomic library [14]. A positive clone named pKat29 containing *katA* was isolated and sequenced. An open reading frame of 1521 bp encoding a 507-amino acid protein with a predicted molecular mass of 56 kDa was identified. The encoded protein showed a high degree of amino acid sequence identity with other monofunctional catalases, including 84% and 78% identity with CatF of *P. syringae* and KatB of *P. aeruginosa*, respectively. The gene was therefore designated *katA*. A phylogenetic analysis of a limited group of catalase sequences was carried out, revealing that *katA* is a clade I catalase [10] more closely related to plant than to mammalian catalases (Fig. 1).

Xp katA was sub-cloned from pKat29 into a pGEM-5Zf vector giving pGemkatA for transformation into the catalase-deficient *E. coli* strain UM255 (*katG2, katE12::Tn10, recA*) [9]. Cell lysates prepared from UM255 harboring pGemkatA and from *Xp* were compared on non-denaturing gels stained for catalase activity, revealing a similar band of catalase in both extracts (Fig. 2B). Expression of KatA in UM255 harboring pGemkatA was more efficient at 28°C (955 units mg protein^{-1}) as compared with 37°C (119 units mg protein^{-1}). This effect may be the result of poorer folding of KatA in the heterologous *E. coli* host at 37°C and to the enhanced temperature sensitivity noted above.

Purification of KatA from *Xp*HR. Catalase activity gels have shown that *Xp* produces two isozymes of monofunctional catalases coded by distinct genes [14]. Because many catalase genes have been isolated from bacteria, but very few enzymes have been purified and characterized, the purification and characterization of KatA, the major catalase in *Xp*, was initiated. *Xp* has a low total catalase activity, which made purification of the enzyme difficult [14], but a multiple peroxide-resistant

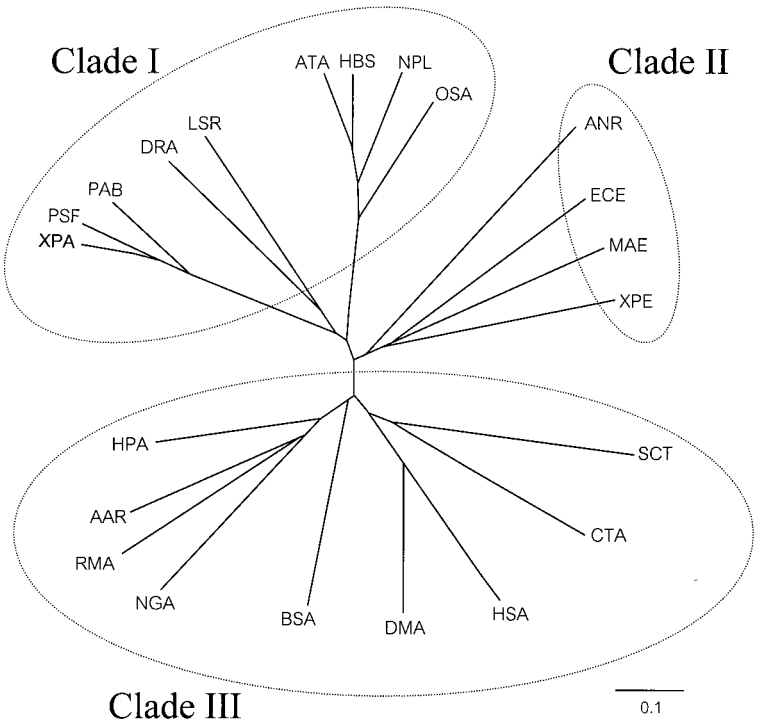

Fig. 1. A phylogenetic tree of monofunctional catalases. A phylogenetic tree was constructed by the neighbor-joining method by using the Tree program for the phylogenetic analysis of Clustal W [12]. The results were drawn with the program PHYLODENDRON (Version 0.8 d 1994; Department of Biology, University of Indiana [http://iubio.bio.indiana.edu]). XPA, *X. campestris* pv. phaseoli KatA (AF461425); PSF, *P. syringae* CatF (U03465); PAB, *P. aeruginosa* KatB (U34896); DRA, *Deinococus radiodurans* KatA (D63898); LSR, *Listeria seeligeri* Kat (M75944); ATA, *Arabidopsis thaliana* CatA (X64271); HBS, *Hevea brasiliensis* (AF151368); NPL, *Nicotiana plumbaginifolia* Cat2 (Z36976); OSA, *Oryza sativa* CatA (X61626); ANR, *Aspergillus niger* CatR (Z23138); ECE, *E. coli* KatE (M55161); MAE, *Mycobacterium avium* KatE (L41246); XPE, *X. campestris* pv. phaseoli KatE (AF170449); SCT, *Saccharomyces cerevisiae* CatT (X04625); CTA, *Candida tropicalis* CatA (M18832); HAS, *Homo sapiens* Catalase (P04040); DMA, *Drosophila melanogaster* CatA (X52286); BSA, *B. subtilis* KatA (M80796); NGA, *Neisseria gonorrhoeae* KatA (U35457); RMA, *Rhizobium meliloti* KatA (U59271); AAR, *Actinobacillus actinomyceterium* (AF162654); HPA, *Helicobacter pylori* KatA (U67458). Bar, 0.1 change per site.

mutant designated *Xp*HR that produces 200- to 300-fold higher levels of catalase has been isolated [3]. This mutant was used as the source for KatA, the major catalase of *Xp* (Fig. 2). The purified catalase has a specific activity of 150,000 U mg protein^{-1} (at 60 mM H_2O_2), somewhat higher than some other bacterial catalases such as *E. coli* KatE and *P. aeruginosa* catalases [2, 6]. SDS-PAGE analysis of the purified enzyme showed that the preparation was greater than 90% pure, and the major protein band had a molecular weight of 56 kDa (Fig. 2A) similar to other small subunit monofunctional catalases [4].

Biochemical characterization of KatA. The apparent K_m and V_{max} values of the KatA for H_2O_2 at pH 7 and 37°C were determined to be 75 mM of H_2O_2 and 2.55×10^5 μmol H_2O_2 μmol heme^{-1} s^{-1}, respectively. The k_{cat} is 2.55×10^5 s^{-1} (Fig. 3A,B). Catalases generally have low substrate affinities with apparent K_m values for H_2O_2 of 10.6 and 44.7 mM for KatB and KatA from *P. aeruginosa*, and 60 mM for CatF from *P. syringae* [2, 4], and *Xp* KatA is similar to these.

KatA is very sensitive to the catalase inhibitors, 3-amino-1,2,4-triazole (K_i = 2.0 mM) and NaN_3 (K_i = 1.0 μM), in sharp contrast to *E. coli* KatE, which is highly resistant to both inhibitors but similar to the catalases from *Pseudomonas*. KatA had a broad pH range for optimal activity from 6.0 to 9.0, outside of which activity dropped off rapidly (Fig. 3D). This differs from monofunctional catalases from *E. coli* [6] and *Bacillus subtilis* [7], which have even wider ranges of optimum pH.

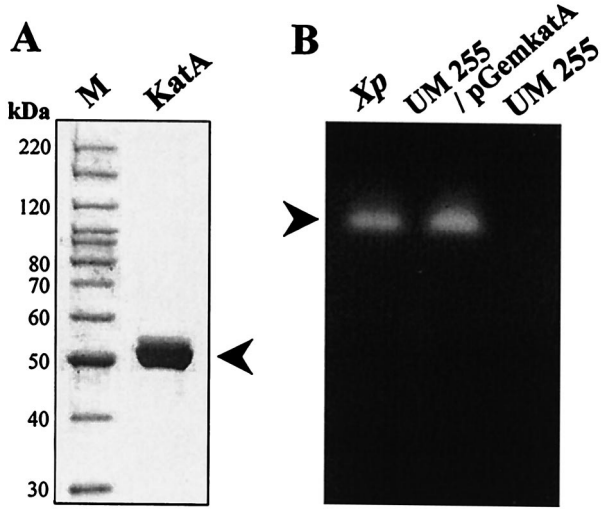

Fig. 2. Analysis of purified KatA and catalase activity gel. In (A), 5 μg of purifed KatA was analyzed on an 8% SDS-PAGE gel stained with Coomassie Blue (KatA). M represented protein molecular weight markers. In (B), catalase activity staining of the protein lysates separated on 7.5% SDS-PAGE to visualize KatA in UM255 (100 μg total protein), UM255 harboring pGemkatA (5 μg total protein), and exponential phase *Xp* (100 μg total protein), respectively. Arrow indicates the position of KatA.

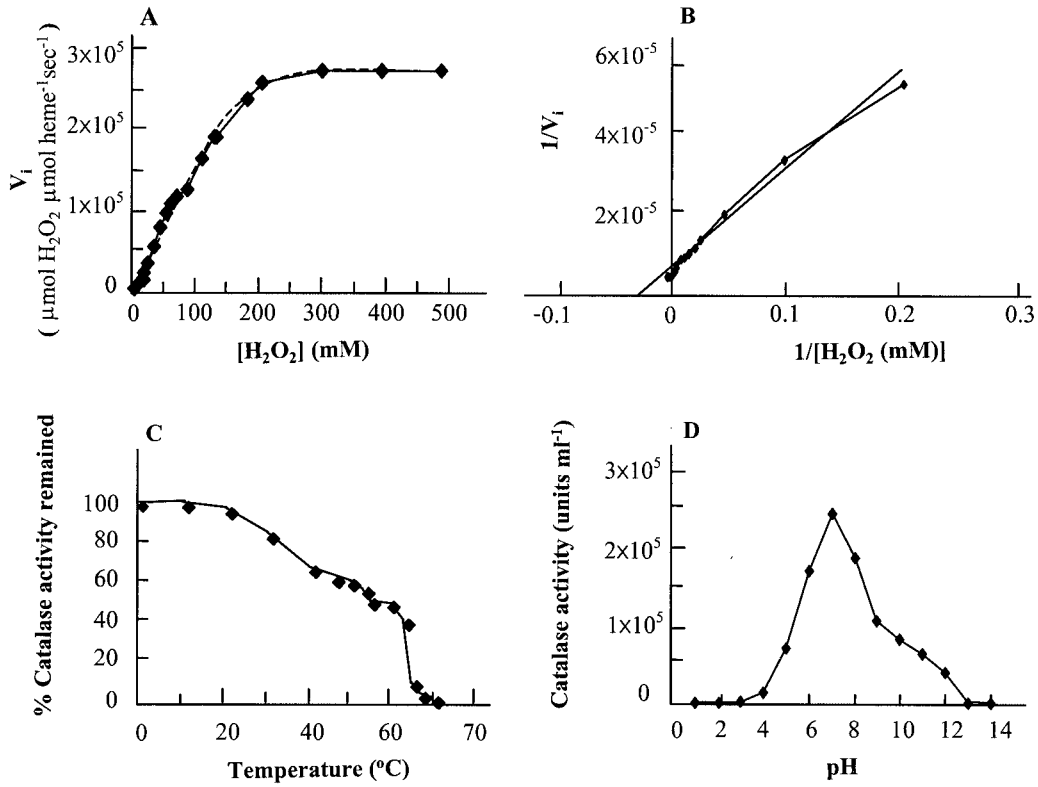

Fig. 3. Biochemical characterization of KatA. The apparent V_{max} (A) and K_m (Lineweaver-Burk plot, B) determinations with different concentrations of H_2O_2 as substrate. The effect of temperature (C) and pH (D) on catalase activity. All experiments were performed as described in Materials and Methods.

Incubation of KatA for 10 min at 30°C resulted in a loss of 20% of the activity, while incubation for 10 min at 55°C resulted in a loss of 50% of the activity (Fig. 3C); thus, KatA was much more sensitive to heat inactivation than many other catalases. In conclusion, KatA from *Xp*HR differs in some properties from and is similar in others to characterized bacterial catalases.

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