A minor catalase/peroxidase from *Burkholderia cenocepacia* is required for normal aconitase activity

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The opportunistic bacterium *Burkholderia cenocepacia* C5424 contains two catalase/peroxidase genes, *katA* and *katB*. To investigate the functions of these genes, *katA* and *katB* mutants were generated by targeted integration of suicide plasmids into the *katA* and *katB* genes. The catalase/peroxidase activity of the *katA* mutant was not affected as compared with that of the parental strain, while no catalase/peroxidase activity was detected in the *katB* mutant. However, the *katA* mutant displayed reduced resistance to hydrogen peroxide under iron limitation, while the *katB* mutant showed hypersensitivity to hydrogen peroxide, and reduced growth under all conditions tested. The *katA* mutant displayed reduced growth only in the presence of carbon sources that are metabolized through the tricarboxylic acid (TCA) cycle, as the growth defect was abrogated in cultures supplemented with glucose or glycerol. This phenotype was also correlated with a marked reduction in aconitase activity. In contrast, aconitase activity was not reduced in the *katB* mutant and parental strains. The authors conclude that the KatA protein is a specialized catalase/peroxidase that has a novel function by contributing to maintain the normal activity of the TCA cycle, while KatB is a classical catalase/peroxidase that plays a global role in cellular protection against oxidative stress.

**INTRODUCTION**

Aerobic organisms produce reactive oxygen species (ROS), including the superoxide anion, hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals, as endogenous by-products of the cellular metabolism in the presence of oxygen (Cline, 1975; Fridovich, 1978; Storz & Imlay, 1999). Pathogenic microorganisms may also encounter ROS upon interaction with host cells during infection (Brown *et al.*, 1995; Cline, 1975). Accumulation of ROS can be bactericidal due to damage of cellular proteins, membranes and nucleic acids (Cline, 1975; Fridovich, 1978; Imlay & Linn, 1988). Many organisms produce specialized enzymes that neutralize ROS. Catalase, an enzyme found in both prokaryotes and eukaryotes, detoxifies H$_2$O$_2$ into water and oxygen (Zamocky & Koller, 1999), and it can be grouped into three distinct enzyme families: mono-functional catalases (HPII class), bifunctional catalase/peroxidases (HPI class), and manganese-dependent catalases (Switala & Loewen, 2002; Zamocky & Koller, 1999; Zamocky *et al.*, 2000). Bifunctional catalase/peroxidases are haem co-factorized enzymes that have been described in simple eukaryotes and prokaryotes, and are evolutionarily linked to plant peroxidases (Zamocky & Koller, 1999; Zamocky *et al.*, 2000). This class of enzymes has been associated with virulence in various bacterial pathogens, including *Legionella pneumophila* and *Agrobacterium tumefaciens* (Bandypadhyay & Steinman, 1998, 2000; Bandypadhyay *et al.*, 2003; Xu & Pan, 2000).

*Burkholderia cepacia* is an aerobic, Gram-negative, catalase-positive bacterium found in soil and water environments (Coenye & Vandamme, 2003). Strains identified as *B. cepacia* belong to a group of at least nine closely related species or genomovars, collectively referred to as the *B. cepacia* complex (Coenye & Vandamme, 2003). *B. cepacia* complex strains have become medically important multidrug-resistant opportunistic pathogens, particularly in patients with cystic fibrosis and chronic granulomatous disease (Govan *et al.*, 1996; Govan & Deretic, 1996; Speert *et al.*, 1994). Infections in cystic fibrosis patients by *B. cepacia* complex bacteria are often associated with increased morbidity and mortality. Some infected patients also succumb to a rapidly progressive necrotizing pneumonia, termed the cepacia syndrome (Bals *et al.*, 1999; Govan & Vandamme, 1998; Govan & Deretic, 1996; Tablan *et al.*, 1985; Tummler & Kiewitz, 1999). Chronic inflammation of the lungs and airways in cystic fibrosis presumably contributes to the release of ROS, resulting in damage to lung tissue (Bals *et al.*, 1999). The survival and persistence...
of B. cepacia in this highly oxidative environment sharply contrasts with the observation that B. cepacia complex isolates are killed by oxidative mechanisms of neutrophils (Speert et al., 1994).

Previously, we have shown that B. cepacia complex strains can survive intracellularly in both macrophages and amoebae (Lamothe et al., 2004; Marolda et al., 1999; Saini et al., 1999). In addition, B. cepacia complex strains can survive within a murine macrophage cell line in the presence of an oxidative burst, and reduced nitric oxide production (Saini et al., 1999). These observations suggest that bacterial resistance or adaptation to oxidative damage may play a role in the infectivity and persistence of B. cepacia complex strains within the airways of patients with cystic fibrosis (Valvano et al., 2005). In fact, the degree of survival of several B. cepacia complex isolates in the presence of exogenous H2O2 can be directly correlated to the level of catalase activity (Lefebre & Valvano, 2001). On average, Burkholderia cenocepacia strains produced the highest levels of catalase activity, and displayed an increased survival upon challenge with H2O2 (Lefebre & Valvano, 2001).

More than 80% of the B. cepacia complex strains that are isolated in Canada from patients with cystic fibrosis are identified as B. cenocepacia, and they all belong to a single lineage, ET12, that has been shown to be transmissible among patients (Speert et al., 2002). All B. cenocepacia isolates examined in our study produce a bifunctional catalase/peroxidase (Lefebre & Valvano, 2001). In this work, we identified two genes from one of these isolates (strain C5424) that encode the functional catalase/peroxidase enzymes KatA and KatB. We demonstrate that a katA-deficient mutant exhibits a carbon-source-dependent growth defect, and increased sensitivity to H2O2 under iron limitation. A katB-deficient mutant, in contrast, showed reduced growth and hypersensitivity to H2O2 under all conditions tested. We provide evidence demonstrating that KatB is the major catalase/peroxidase enzyme in C5424, while KatA is a specialized catalase/peroxidase that plays a novel functional role to protect critical tricarboxylic acid (TCA) cycle enzymes.

**METHODS**

**Bacterial strains, plasmids and culture media.** Bacterial strains used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) broth, or Tris minimal (TM) medium consisting of 100 mM NaCl, 40 mM KCl, 20 mM NH4Cl, 1 mM Na2SO4, 2 mM KH2PO4, 100 mM Tris base, 0-2% Casamino acids, 2 μg thiamine ml-1, 0-1 mM CaCl2, 0-1 mM MgSO4 and 20 μg tryptophan ml-1, pH 7-4. TM was supplemented with various carbon sources, such as glucose, glycerol, pyruvate, 2-oxoglutarate, fumarate, succinate and citrate at final concentrations of 0-2% (w/v). For growth of Escherichia coli, media were supplemented with 20 μg tetracycline ml-1, 50 μg trimethoprim ml-1, 30 μg gentamicin ml-1, 30 μg chloramphenicol ml-1, 40 μg kanamycin ml-1 and 100 μg ampicillin ml-1, as required. For growth of B. cenocepacia, 100 μg trimethoprim ml-1 and 100 μg chloramphenicol ml-1 were used. L-Arabinose was added to final concentrations ranging from 0-02 to 1-0% (w/v), as required. For some experiments, cultures were supplemented either with the iron chelator x, x’-dipiridyl (final concentrations ranging from 50 to 400 μM, as required), or with ferrous ammonium sulphate [Fe(NH4)2(SO4)2] at a final concentration of 100 μM.

**General molecular techniques.** Molecular techniques used for DNA manipulations were conducted by standard procedures, as described by Sambrook et al., (1990). Restriction enzymes were purchased from Roche Diagnostics, and used as recommended by the manufacturer. T4 DNA polymerase (Roche), mung bean nuclelease (Pharmacia), poly nucleotide kinase (Fermentas) and T4 DNA ligase (Roche) treatments were also conducted using the conditions recommended by the manufacturers. DNA transformations in E. coli were carried out using a calcium chloride protocol, as described elsewhere (Cohen et al., 1972). Transfer of plasmids into B. cepacia complex strains was conducted by tri-parental mating (Craig et al., 1989) using an E. coli helper strain carrying the plasmid pRK2013 (Table 1). DNA sequencing was done in the Mobix Sequencing Facility at the University of McMaster, Hamilton, Ontario, and the DNA Sequencing Facility at York University, Toronto, Ontario. The Basic Local Alignment Sequence Tool (BLAST) was used to analyse the sequences obtained in this work and the B. cenocepacia J2315 genome.

**PCR amplifications.** PCR amplifications were performed in a PTC-0200 DNA engine (MJ Research) with either Pwo polymerase (Roche) or Taq polymerase (Qiagen), using the supplied Q solution for G + C-rich templates, and B. cenocepacia chromosome as a template. The DNA sequences of the oligonucleotide primers are indicated in Table 2. The specific conditions for PCR amplification were optimized for each primer pair, and they are available from the authors upon request. PCR amplification products were separated in 0-7 or 1-2% agarose gels, and purified using the QiAgel quick gel extraction system (Qiagen).

**Identification of a katA gene fragment by degenerate PCR amplification.** We designed two degenerate oligonucleotide primers, katdeg-NT and katdeg-CT (Table 2), from comparisons of sequence alignments of genes encoding bifunctional catalase/peroxidases in other bacteria, especially from those micro-organisms with a high mol% G + C. PCR amplifications with B. cenocepacia C5424 genomic DNA, using the conditions indicated in Table 2, resulted in a 473 bp product that was subsequently treated with T4 polymerase and T4 polynucleotide kinase to facilitate its cloning into HindIII-cleaved pUC19. The 473 bp DNA insert of the resulting plasmid, pML10, was sequenced using M13 forward and M13 reverse primers, and BLAST analysis of the DNA sequence confirmed that the cloned 473 bp amplicon encoded an internal fragment of a bifunctional catalase/peroxidase, which we designated KatA.

**Southern blot analysis.** The 473 bp amplicon (katA probe) was labelled directly with DIG-11-UTP using the kat-NT and kat-CT primers (Table 2) and a PCR labelling kit (Roche), as recommended by the manufacturer. B. cenocepacia genomic DNA was isolated, and individually digested with Sall, NsiI and BamHI. Southern blot analysis of genomic DNA was conducted as described elsewhere. Briefly, DNA was separated on a 0-5% agarose gel, and transferred to a nitrocellulose membrane by capillary action. The membrane was incubated with the katA probe under high-stringency conditions (50%, v/v, formamide at 42 °C). Hybridization signals were detected by chemiluminescence with disodium 3-(4-methoxyxipiro [1,2-dioxetano-3,2’-(5’-chloro)tricyclo[3.3.1.1^9]decan-4-yl]phenyl phosphate (CSPD) as recommended by the manufacturer (Roche).

**Cloning of katA and katB genes.** Sall- and NsiI-digested DNA fragments from B. cenocepacia C5424 were purified from an agarose gel, and used to construct a genomic library by ligation to Sall- and
NsiI-cleaved pKS-Bluescript, respectively. Ligation mixes were transformed into *E. coli* DH5α, and transformants were plated on LB agar plates with ampicillin and 0.2% (w/v) X-Gal and 2 mM IPTG. Colonies with a white colour phenotype were pooled into groups of 50. Plasmid DNA was extracted from each pool, and screened by Southern blot hybridization using the katA probe, as described above. Pools yielding positive signals were further split into smaller groups, and rescreened until plasmids from individual colonies hybridizing to the probe were isolated. These experiments resulted in the isolation of plasmids pML25, pML26 and pML30, which span the *katA* gene and its flanking sequences (Fig. 1, Table 1). The DNA inserts in these plasmids were fully sequenced, and the sequence that carries the *katA* gene encoding trimethoprim resistance, was constructed for the complementation of the C5424 _katA*-defective mutant (see below) by digesting pMLBAD-*katA* with EcoRI and PstI, and ligating the resulting 2.3 kb fragment carrying the complete _katA_ gene into pKMBAD.

The coding region of _katB_ was amplified by PCR using the Qiagen Taq polymerase system, and the primers katB-NT and katB-CT (Table 2). The 2.2 kb amplicon was digested with _MfeI_ and _PstI_, and ligated into _EcoRV/PstI_-digested pKMBAD. The ligation mixtures were transformed into _E. coli_ DH5α, and the plasmids from trimethoprim/chloramphenicol-resistant transformants were screened by restriction digestion to identify pKMBAD-*katB*.

**Functional complementation of the *E. coli* catalase-deficient strain UM2.** Catalase activity in strain UM2 alone, or harbouring pMLBAD-*katA*, pKMBAD-*katA* or pKMBAD-*katB*, was assessed on agar plates by visualizing bubbles upon the addition of 3% H₂O₂ solution to the edge of colonies.

### Construction of *katA* and *katB* mutants.

pSUP202-Tp, a modified version of the suicide plasmid pSUP202 (Simon _et al._, 1983) that carries the _dfr_ gene encoding trimethoprim resistance, was used to disrupt _katA_ in C5424 by a single cross-over event. An internal fragment of _katA_ was amplified by PCR using primers Amut-NT and Amut-CT (Table 2). The product was ligated into the EcoRV site of pSUP202-Tp, which disrupts the tetracycline-resistance gene in the plasmid. Transformants carrying plasmids with the internal _katA_ fragment were selected for resistance to trimethoprim and sensitivity to tetracycline, and screened by restriction digest to identify pML31. pGP704, a derivative of pGP704 that carries the Pir-dependent R6K origin of replication and the _dfr_ gene flanked by terminator sequences, was used to disrupt _katB_. An internal fragment of _katB_ was amplified with primers KatB-NT and KatB-CT (Table 2). Pools yielding positive signals were further split into smaller groups, and rescreened until plasmids from individual colonies hybridizing to the probe were isolated. These experiments resulted in the isolation of plasmids pML25, pML26 and pML30, which span the _katA_ gene and its flanking sequences (Fig. 1, Table 1). The DNA inserts in these plasmids were fully sequenced, and the sequence that carries the _katA_ gene encoding trimethoprim resistance, was constructed for the complementation of the C5424 _katA*-defective mutant (see below) by digesting pMLBAD-*katA* with EcoRI and PstI, and ligating the resulting 2.3 kb fragment carrying the complete _katA_ gene into pKMBAD.

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**Functional complementation of the *E. coli* catalase-deficient strain UM2.** Catalase activity in strain UM2 alone, or harbouring pMLBAD-*katA*, pKMBAD-*katA* or pKMBAD-*katB*, was assessed on agar plates by visualizing bubbles upon the addition of 3% H₂O₂ solution to the edge of colonies.

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The coding region of _katB_ was amplified by PCR using the Qiagen Taq polymerase system, and the primers katB-NT and katB-CT (Table 2). The 2.2 kb amplicon was digested with _MfeI_ and _PstI_, and ligated into _EcoRV/PstI_-digested pKMBAD. The ligation mixtures were transformed into _E. coli_ DH5α, and the plasmids from trimethoprim/chloramphenicol-resistant transformants were screened by restriction digestion to identify pKMBAD-*katB*.

### Functional complementation of the *E. coli* catalase-deficient strain UM2.** Catalase activity in strain UM2 alone, or harbouring pMLBAD-*katA*, pKMBAD-*katA* or pKMBAD-*katB*, was assessed on agar plates by visualizing bubbles upon the addition of 3% H₂O₂ solution to the edge of colonies.

### Construction of *katA* and *katB* mutants.

pSUP202-Tp, a modified version of the suicide plasmid pSUP202 (Simon _et al._, 1983) that carries the _dfr_ gene encoding trimethoprim resistance, was used to disrupt _katA_ in C5424 by a single cross-over event. An internal fragment of _katA_ was amplified by PCR using primers Amut-NT and Amut-CT (Table 2). The product was ligated into the EcoRV site of pSUP202-Tp, which disrupts the tetracycline-resistance gene in the plasmid. Transformants carrying plasmids with the internal _katA_ fragment were selected for resistance to trimethoprim and sensitivity to tetracycline, and screened by restriction digest to identify pML31. pGP704, a derivative of pGP704 that carries the Pir-dependent R6K origin of replication and the _dfr_ gene flanked by terminator sequences, was used to disrupt _katB_. An internal fragment of _katB_ was amplified with primers KatB-NT and KatB-CT (Table 2). Pools yielding positive signals were further split into smaller groups, and rescreened until plasmids from individual colonies hybridizing to the probe were isolated. These experiments resulted in the isolation of plasmids pML25, pML26 and pML30, which span the _katA_ gene and its flanking sequences (Fig. 1, Table 1). The DNA inserts in these plasmids were fully sequenced, and the sequence that carries the _katA_ gene encoding trimethoprim resistance, was constructed for the complementation of the C5424 _katA*-defective mutant (see below) by digesting pMLBAD-*katA* with EcoRI and PstI, and ligating the resulting 2.3 kb fragment carrying the complete _katA_ gene into pKMBAD.
The product was ligated into the SmaI site of pGPVTp, and transformed into E. coli SY327. Trimethoprim-resistant colonies were screened by restriction digestion and PCR to confirm the presence and orientation of the PCR product to identify pML102. Both pML31 and pML102 (Fig. 1, Table 1) were transferred into B. cenocepacia strain C5424 by tri-parental mating, as described above. Exconjugants containing either pML31 or pML102 that had integrated into the C5424 genome were selected on LB agar supplemented with trimethoprim and gentamicin (to remove E. coli donor and helper strains). The integration of both suicide plasmids was confirmed by Southern blot hybridization using either katA- or katB-specific probes, allowing identification of the katA- and katB-deficient mutant strains MDL1 and MDL2, respectively.

Resistance to H2O2. Bacterial resistance to H2O2 was assessed as described previously (Lefebre & Valvano, 2001). Briefly, cultures were grown to stationary phase in LB broth with and without x,α′-dipyridyl, and approximately 10⁷ cells were incubated with 10 mM H2O2 for 30 min at room temperature. Aliquots were removed, plated on LB agar, and the survival rate was calculated using the following equation: percentage survival = (no. colonies in treated sample/no. colonies at time 0) x 100. Also, H2O2 resistance was assessed by a disk diffusion inhibition assay. Briefly, overnight cultures were diluted to an OD₆₀₀ of 0.4, and inoculated into molten top agar. Sterile disks (8 mm) were treated with 20 μl H₂O₂ dilutions (ranging from 0 to 100 mM), and placed on the agar. Zones of inhibition were measured following 24–36 h incubation at 37°C.

Determination of catalase/peroxidase activity. Crude cell-free lysates of wild-type, katA and katB mutant strains were generated from cultures grown to exponential and stationary phases. Catalase activities within the lysates were measured spectrophotometrically, and also assessed by native PAGE to identify bands with catalase and peroxidase activity, as described by Katsuwon & Anderson (1992) and Lefebre & Valvano (2001).

Table 2. Oligonucleotide primers

Restriction endonuclease sites incorporated in the oligonucleotide sequence are underlined. NA, Not applicable.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>katdeg-NT</td>
<td>TGGTGCGACCGGAATGGAAGATATTCGAGCGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>katdeg-CT</td>
<td>CCATCAATTGATGTCGAACGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>kat-NT</td>
<td>TGGTGCGACCGGAATGGAAGATATTCGAGCGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>kat-CT</td>
<td>CCATCAATTGATGTCGAACGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>katA-NT</td>
<td>GATGAGATGTCGAACGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>katA-CT</td>
<td>GATGAGATGTCGAACGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>Amut-NT</td>
<td>TATGTCGAGCGCAACTCGGAGAGGTGGGACAG</td>
<td>NA</td>
</tr>
<tr>
<td>Amut-CT</td>
<td>TATGTCGAGCGCAACTCGGAGAGGTGGGACAG</td>
<td>NA</td>
</tr>
<tr>
<td>katB-NT</td>
<td>ATTACAATTGATGTCGAACGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>katB-CT</td>
<td>ATTACAATTGATGTCGAACGAAGGGCAGTGCCC</td>
<td>NA</td>
</tr>
<tr>
<td>Bmut-NT</td>
<td>TGGTGGCCGCGGACATCAGCAACAGAGG</td>
<td>NA</td>
</tr>
<tr>
<td>Bmut-CT</td>
<td>TGGTGGCCGCGGACATCAGCAACAGAGG</td>
<td>NA</td>
</tr>
<tr>
<td>PkatA-NT</td>
<td>TATTCTAGACCGCGCGTATGTCGAAGACGAAAGG</td>
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</tr>
<tr>
<td>PkatA-CT</td>
<td>TATTCTAGACCGCGCGTATGTCGAAGACGAAAGG</td>
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<tr>
<td>Pbp-CT</td>
<td>TATTCTAGACCGCGCGTATGTCGAAGACGAAAGG</td>
<td>NA</td>
</tr>
</tbody>
</table>

Fig. 1. Genetic map of the katA (a) and katB (b) loci of B. cenocepacia strains C5424 and J2315. tnp, transposase; orf, conserved ORF of unknown function; merR, regulatory protein; bp, bromoperoxidase; katA, bifunctional catalase/peroxidase; qor, quinine oxidoreductase; chpx, chloroperoxidase; necG, helicase; oxyR, transcriptional regulator; katB, catalase/peroxidase; dpsA, DNA-binding ferritin-like protein; ubiA, prenyltransferase. The relevant restriction endonuclease sites are indicated: S, SalI; N, NsiI; B, BamHI.
Determination of aconitase activity. Aconitase activity was measured from crude cell lysates under aerobic conditions, as described elsewhere (Gruer & Guest, 1994). Briefly, cultures were grown to late stationary phase in LB broth, or LB broth supplemented with 100 μM z,α′-dipyridyl, 50 μM Fe(III) or 1 % (w/v) arabinose, as required, and harvested in late stationary phase. Cells were resuspended in Tris/citrate buffer (20 mM citrate, pH 8), and lysed by sonication disruption. Lysates were cleared by centrifugation, and total protein content was determined using the Bio-Rad system, with BSA as a standard. Aconitase activity was assayed spectrophotometrically at room temperature by monitoring the production of cis-aconitate as an increase of absorbance at 240 nm, following the combination of 0–100 μg total protein with 20 mM isocitrate. Specific aconitase activity was calculated as the change in absorbance per min per mg protein through the linear portion of the curves. For inactivation of AcmB activity, crude cell lysates were incubated with either 1 mM EDTA or 100 μM z,α′-dipyridyl for 60 min prior to the assay. Reconstitution of AcmB activity was conducted by incubating cell lysates with 1 mM DTT and 1 mM Fe(III)SO4 at 0 °C for 30 min prior to the assay.

RESULTS

B. cenocepacia C5424 carries two catalase/peroxidase genes

Following the cloning strategy described in Methods, we identified a gene putatively encoding a bifunctional catalase/peroxidase in B. cenocepacia C5424, which we designated katA (Fig. 1a). Directly upstream from katA, we identified genes encoding proteins with homology to a bromoperoxidase and the transcriptional regulator MerR, respectively (Fig. 1a, bp and merR), while a gene encoding a polypeptide with homology to a quinone oxidoreductase (gor) was found downstream from katA (Fig. 1a). While this work was in progress, the complete genomic sequence of B. cenocepacia strain J2315 became available (http://www.sanger.ac.uk/Projects/B_cenocepacia/). Strains C5424 and J2315 are clonal, and therefore closely related (Mahenthiralingam et al., 2000). Inspection of sequences from the katA region in both strains revealed an identical gene organization to that depicted in Fig. 1(a), which also included two additional ORFs showing homology to a transposase gene (tnp) and a conserved gene of unknown function (orf). This region is located in chromosome 2, and has a lower G+C content (61 mol%) as compared with the mean G+C content of B. cenocepacia (67 mol%), suggesting that it may have been acquired by horizontal transmission.

Another catalase/peroxidase gene, designated katB, was identified in the genome of the related strain J2315 (Fig. 1b). The predicted KatB protein is also homologous to typi-
cal catalase/peroxidase of the HPI class, and shows 82-9 % similarity and 76 % identity to KatA at the amino acid level. A putative oxyR gene is located 621 bp upstream of katB, and is transcribed in the same direction. The organization of these genes resembles that of the katG locus in the related species Burkholderia pseudomallei (Loprasert et al., 2002). Using katBNT and katBCT primers, we confirmed by PCR that katB was also present in the strain C5424 (data not shown).

To determine whether katA and katB encode functional proteins, pML26 and pKMBADkatB (Fig. 1) were transformed into E. coli strain UM2, which carries mutations in both katE and katG genes (Table 1). Restoration of catalase activity in the transformants was assessed by applying 3% H2O2 to the border of colonies, and inspecting them for bubble formation due to oxygen release. The plasmid pKMBAD-katB complemented the catalase deficiency of the E. coli double-catalase mutant UM2, indicating that KatB is functional. In contrast, initial experiments did not show complementation of the catalase deficiency in UM2 (pML26) cells. Lack of complementation could be due to either poor expression of katA from a B. cenocepacia promoter in E. coli, or the absence of a promoter region in the cloned fragment. Therefore, we cloned the coding region of katA under the control of the arabinose-inducible P araBAD promoter of pMLBAD, generating the plasmid pMLBAD-katA. In the presence of arabinose, the catalase-deficient phenotype of E. coli UM2 (pMLBAD-katA) was restored, indicating that the B. cenocepacia KatA protein is functional in E. coli when appropriately expressed.

A katA-deficient mutant of strain C5424 shows reduced resistance to exogenous H2O2 under conditions of iron limitation

We generated a katA mutant carrying a targeted integration of the suicide plasmid pML31 into the katA gene, which was designated MDL1 (Table 1). The site-specific integration of pML31 in B. cenocepacia MDL1 was independently confirmed by Southern blot hybridization and by PCR analysis of genomic DNA (data not shown). Mutant and parental strains were grown to late stationary phase in LB broth, and LB broth supplemented with either 100 μM FeSO4 (to promote oxidative stress conditions) or 100 μM of the iron chelator z,α′-dipyridyl, and then assessed for sensitivity to exogenous H2O2. Both strains showed no significant differences in the levels of H2O2 resistance when grown in LB broth alone or LB broth supplemented with FeSO4 (Fig. 2, and data not shown). In contrast, MDL1 displayed decreased survival (a decrease of approx. 2 log units compared to the parent) after H2O2 challenge under iron limitation (Fig. 2). The survival defect of MDL1 was corrected by pMLBAD-katA as long as the plasmid-encoded katA gene was induced by adding arabinose to the growth medium (Fig. 2). These experiments demonstrate that the sensitivity of MDL1 to H2O2 challenge under iron limitation is associated with the lack of KatA function. Furthermore, this phenotype was complemented by pMLBAD-katA, indicating that it was due to the disruption of the katA gene, and not caused by a polar effect on downstream genes, or a secondary mutation elsewhere.

The phenotype observed in the katA mutant could not be correlated with reduced catalase activity, since no differences in enzymic activity were detected by a spectrophotometric assay in lysates from wild-type and mutant strains, irrespective of the growth phase (exponential or stationary phase) and the composition of the medium (iron

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Bacterial resistance to exogenously added H₂O₂. Bacterial cultures that were grown to late stationary phase in LB broth, or LB broth plus 100 μM 2,6-dipyridyl (dip), were exposed to H₂O₂ at concentrations of 0, 5 and 10 mM. Data represent the means of three experiments. Bars indicate standard deviations. •, C5424 in LB; □, MDL1 in LB; ■, C5424 in LB plus dip; ○, MDL1 in LB plus dip; △, MDL1(pKMBAD-katA) in LB plus dip without induction of katA expression by arabinose; ○, MDL1(pKMBAD-katA) in LB plus dip, but in the presence of 1% arabinose to induce katA expression.

deprecated versus iron replete) as assessed. Also, no differences in catalase bands by specific activity staining were found by native PAGE analysis (Fig. 3, lanes 1 and 2). Thus, we concluded that KatB, rather than KatA, most likely accounted for the majority of the enzymic activity detected in MDL1.

B. cenocepacia C5424 KatB is a general catalase/peroxidase

To investigate the role of katB, we generated a second insertion mutant, which was designated MDL2. This mutant was obtained by homologous recombination using the suicide plasmid pML102 (Table 1), as described in Methods. Site-specific integration of pML102 in B. cenocepacia MDL2 was confirmed by PCR and Southern blot hybridization of genomic DNA (data not shown). Addition of 3% H₂O₂ near the edge of the bacterial growth of the mutant strain on agar plates did not produce bubbling, and the lack of detectable bands of catalase activity in native PAGE (Fig. 3, lane 3) confirmed the absence of KatB in this mutant.

MDL2 did not form isolated colonies on agar plates, and the growth was mainly restricted to zones of high cell density, such as the initial points of streaking (data not shown). Since it was difficult to assess H₂O₂ sensitivity in MDL2 by enumerating c.f.u. as with MDL1, we used instead a disk diffusion inhibition assay. Zones of inhibition in top agar lawns containing C5424, MDL1 and MDL2 were measured after exposure to sterile filter disks impregnated with 20 μl H₂O₂ solution (at concentrations ranging from 0 to 100 mM). C5424 and MDL1 showed comparable levels of inhibition, with zones of 8 and 9 mm, respectively, after challenge with 100 mM H₂O₂, but no inhibition zones were detected at lower H₂O₂ concentrations. In contrast, MDL2 showed hypersensitivity to H₂O₂, with zones of growth inhibition of 10 mm diameter appearing at concentrations as low as 2.5 mM. MDL2 also showed reduced growth in LB broth, as the strain required roughly 60 h to reach stationary phase (OD600 ~ 2.0), in contrast to 24–30 h for either C5424 (parental) or MDL1 (katA mutant). However, the growth defect in MDL2 was not related to iron availability, as it was not affected by the addition of exogenous iron or the presence of 2,6-dipyridyl (data not shown).

Complementation experiments to restore catalase/peroxidase function in MDL2 were conducted using pKMBAD-katA and pKMBAD-katB. In both cases, catalase/peroxidase activity, as determined by native PAGE, was restored in the presence of arabinose. MDL2(pKMBAD-katB) exhibited a single band of catalase activity that co-migrated with the activity seen in the parental C5424, indicating that KatB is the major catalase/peroxidase in the strain (Fig. 3, lane 6). In contrast, MDL2(pKMBAD-katA) showed three distinct bands of weak catalase activity, one of which co-migrated with the band of activity associated with KatB (Fig. 3, lane 4). All bands were a result of KatA expression, as they were absent from MDL2, and from MDL2-pKMBAD-katA grown in the presence of glucose (Fig. 3, lanes 3 and 5). Analysis of the peroxidase activity by native PAGE revealed identical banding profiles to those seen with the catalase-specific stain (data not shown). Based on the phenotypic characterization of the katB mutant MDL2, we conclude that KatB is the major catalase/peroxidase in C5424, which is required for full resistance to H₂O₂ exposure, while KatA is a minor catalase/peroxidase, which is only required under iron limitation.

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MDL1 shows reduced growth in the presence of some substrates of the TCA cycle

Despite the reduced viability of the MDL1 katA mutant under iron limitation in the presence of H₂O₂, its growth...
rate in LB under iron limitation, but in the absence of exogenously added H₂O₂, was comparable with that of the parental C5424 (data not shown). Further experiments using a defined minimal medium supplemented with various carbon sources showed that the growth rate of MDL1 was reduced in the presence of succinate, but not affected in the presence of glucose or glycerol, irrespective of the α,α′-dipyridyl concentration (Fig. 4, and data not shown). Since succinate is catabolized via the TCA cycle, we hypothesized that detoxification of the ROS generated by the biochemical reactions of this cycle (Messner & Imlay, 1999) could require the function of KatA. In the presence of glucose or glycerol, enough ATP for bacterial growth would be generated through the terminal steps of glycolysis (Oexle et al., 1999), effectively bypassing TCA cycle reactions and the requirement for KatA. Thus, we examined the growth rates of C5424 and MDL1 in TM medium supplemented with various carboxylic acids that are catabolized through the TCA cycle. The results show that the growth of the mutant was compromised in cultures supplemented with citrate, 2-oxoglutarate, succinate or pyruvate (Fig. 4). The growth differences observed with MDL1 relative to the parental strain were less obvious for cultures in the presence of fumarate. Furthermore, addition of exogenous iron to the TM medium alleviated the growth deficiency, while the MDL1 grew very poorly in the presence of 25 μM α,α′-dipyridyl (Fig. 4, and data not shown), confirming that the growth defect was linked to both the availability of cellular iron and the particular carbon source supplied.

**The katA mutant strain MDL1 shows reduced aconitase activity**

The results in the previous section suggested that in the absence of KatA, ROS generated through metabolic reactions of the TCA cycle might compromise the stability of an essential enzyme or enzymes that require iron for their function. In other bacteria, aconitase and fumarate reductase are known to possess ROS-sensitive iron–sulphur clusters (Oexle et al., 1999; Varghese et al., 2003). Thus, we determined the aconitase activity in MDL1 and MDL2 mutants. Table 3 shows an almost fourfold reduction in the specific aconitase activity detected in the cell-free lysate from MDL1 [0.41 units (mg protein)⁻¹] relative to the activity in the lysates from C5424 and MDL2 [1.63 and 1.55 units (mg protein)⁻¹, respectively]. To demonstrate an association between aconitase levels and the KatA protein, lysates from C5424, and MDL1 containing pKMBAD (vector control), pKMBAD-κatA or pKMBAD-κatB, were assayed for aconitase activity in the presence of iron limitation (to induce the katA phenotype) and arabinose (to activate the expression of the cloned katA and katB genes under the

![Fig. 4](http://mic.sgmjournals.org/1981)

**Fig. 4.** Growth rate of *B. cenocepacia* strains C5424 (●) and MDL1 (○) in minimal medium. Bacteria were grown in TM medium supplemented with various carbon sources, as indicated in each panel, all at a final concentration of 0.2% (w/v). Data represent the means of three experiments. Bars indicate standard deviations.
control of the arabinose inducible promoter). Table 3 shows that the aconitase activities of cultures grown in the presence of arabinose were reduced by approximately 50% compared with those of cultures grown without the sugar. The overall reduction of aconitase activity in the presence of arabinose is consistent with previous observations in other bacteria demonstrating that one type of aconitase, AcnA, is regulated by both carbon source and iron availability (Cunningham et al., 1997). Despite the overall reduction in aconitase activity in the presence of arabinose, the activity in the lysate from MDL1(pKMBAD) showed a further twofold reduction not observed in lysates from MDL1(katA) and MDL1(katB) (Table 3). We therefore conclude that the reduced aconitase activity in MDL1 is associated with the loss of KatA expression. Furthermore, expression of KatB in MDL1 from a multicopy plasmid pKMBAD-katB restored aconitase activity to levels comparable with the parental and pKMBAD-katA-complemented strains (Table 3). This observation suggests that KatB can compensate for the KatA deficiency only when overexpressed, since the normal production of KatB mediated by its chromosomal gene is not sufficient to prevent the reduction in aconitase activity in MDL1.

In other bacteria, such as E. coli, the aconitase activity can be contributed by two enzymes: (i) AcnB, which is iron- and oxidative-stress-sensitive, and contributes the majority of the aconitase activity in growing cells, and (ii) AcnA, which is predominantly expressed and stable under conditions of oxidative stress, and in stationary phase (Varghese et al., 2003). To determine whether biochemically distinct forms of aconitase also exist in B. cenocepacia, cell-free lysates were pre-incubated with ferrous sulphate and DTT. This experiment resulted in a proportionally similar increase in the aconitase activities of both C5424 and MDL1 lysates (Table 3), indicating that there is an equivalent pool of an aconitase form that is inactivated under aerobic conditions, but can be reactivated in the presence of exogenous iron and reducing conditions. These results suggest that there are at least two biochemically distinct aconitase forms in B. cenocepacia. Furthermore, we conclude that the form of the enzyme contributing to the aconitase activity measured in our assay, and affected in the katA mutant, is resistant to aerobic inactivation.

**DISCUSSION**

We demonstrate in this study that B. cenocepacia C5424 carries two genes, katA and katB, encoding proteins that belong to the HPI class of bi-functional catalase/peroxidases. To determine the function of these proteins, we constructed and characterized the phenotypes of katA and katB insertion mutants. Two lines of evidence support the conclusion that katB encodes the major catalase/peroxidase. First, the katB gene is located next to an oxyR homologue in a similar genetic organization as that of the B. pseudomallei katG, the only catalase/peroxidase found in this bacterium (Loprasert et al., 2002). Second, the katB mutant displayed a dramatic reduction in catalase activity and peroxidase activities, hypersensitivity to H$_2$O$_2$, and also poor growth under all conditions tested. All these phenotypes were complemented by providing the intact katB in trans. Furthermore, preliminary evidence from promoter fusions to a promoterless lacZ reporter gene suggests that the katB gene is highly expressed in late stationary phase and under oxidative stress (M. D. Lefebre & M. A. Valvano, unpublished), two characteristics common to general catalase/peroxidases (Bandypadhyay & Steinman, 1998, 2000; Rava et al., 1999; Steinman et al., 1997; Zheng & Storz, 2000).

We also demonstrate that although the cloned katA encodes a functional catalase/peroxidase, the katA insertion

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Table 3. Aconitase activity determinations

All cultures were grown to stationary phase under the conditions indicated, prior to sonic disruption. Post-lysis treatments of crude cell extracts are indicated in parentheses. dip, 2,6'-dipyridyl, 100 μM; + Fe, 1 mM Fe(NH$_4$SO$_4$)$_2$; DTT, 1 mM; Ara, arabinose, 1%. Values represent the mean of between three and six repeats (±SD).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth condition</th>
<th>Activity [U (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5424</td>
<td>LB</td>
<td>1.63 ± 0.06</td>
</tr>
<tr>
<td>MDL1</td>
<td>LB</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>MDL2</td>
<td>LB</td>
<td>1.55 ± 0.16</td>
</tr>
<tr>
<td>C5424</td>
<td>LB, dip</td>
<td>1.79 ± 0.04</td>
</tr>
<tr>
<td>MDL1</td>
<td>LB, dip (+ Fe, DTT)</td>
<td>2.62 ± 0.03</td>
</tr>
<tr>
<td>MDL1</td>
<td>LB, dip (+ Fe, DTT)</td>
<td>0.69 ± 0.002</td>
</tr>
<tr>
<td>C5424</td>
<td>LB, dip, Ara</td>
<td>1.48 ± 0.03</td>
</tr>
<tr>
<td>MDL1 (pKMBAD)</td>
<td>LB, dip, Ara</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td>MDL1 (pKMBAD-katA)</td>
<td>LB, dip, Ara</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>MDL1 (pKMBAD-katB)</td>
<td>LB, dip, Ara</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>MDL1 (pKMBAD-katB)</td>
<td>LB, dip, Ara</td>
<td>0.57 ± 0.05</td>
</tr>
</tbody>
</table>
mutant did not show any detectable reduction in the enzyme activity under the conditions tested, and displayed a growth defect only in the presence of carbon sources that are metabolized through the TCA cycle. The \textit{katA} mutant was also sensitive to \textit{H}_{2}\text{O}_{2}, but only when bacterial cells were grown under iron limitation. The phenotypes of the \textit{katA} mutant were corrected by complementation with the intact \textit{katA} gene, confirming that they were due to the absence of the KatA protein. The \textit{katA} gene is located on the second-largest chromosome of the clonally related strain J2315, and is part of a putative three-gene operon that also includes a bromoperoxidase and quinone oxidoreductase genes. The location of the \textit{katA} gene, and its association with genes encoding enzymes involved in redox reactions, suggest a specialized role for KatA.

The iron chelator \textit{x}-\textit{x}'-dipyridyl can enter the bacterial cytoplasm, and, under these conditions, some enzymes of the TCA cycle, like AcnB, can be rapidly demetallated (Varghese et al., 2003). We reasoned that a similar situation could occur in \textit{B. cenocepacia} C5424, such that KatA could be required to counteract the effects of iron depletion on one or more TCA cycle enzymes. Consistent with this notion, we observed reduced growth of the \textit{katA} mutant in low-iron-containing minimal medium supplemented with various carbon sources that are metabolized through the TCA cycle, especially with citrate and succinate, while no growth defect was found with glucose or glycerol. Our results demonstrating that the aconitase activity is significantly reduced in the \textit{katA} mutant, but not affected in the parental strains or in the \textit{katB} mutant, suggest that KatA could be involved, directly or indirectly, in stabilizing this enzyme under conditions of iron limitation and TCA substrates in the growth medium.

The reduction in aconitase activity associated with MDL1 was clearly linked with the loss of KatA expression, since complementation of the KatA defect could restore aconitase activity to wild-type levels. Therefore, we propose that in \textit{B. cenocepacia} C5424, and possibly other \textit{B. cepacia} complex strains, KatA may contribute to maintain the function or stability of aconitase under iron limitation. An inspection of the genome sequence of the related \textit{B. cenocepacia} strain J2315 revealed the presence of genes encoding very good homologues of the AcnA and AcnB proteins from \textit{E. coli}, suggesting they may have similar properties. Determination of the aconitase activity in \textit{B. cenocepacia} C5424 and the \textit{katA} mutant in the presence of excess iron and reducing conditions (Table 3) revealed an aconitase form that has similar biochemical properties to AcnB, and its activity does not appear to be affected in the \textit{katA} mutant. Thus, it is possible that KatA acts on another form of aconitase, which may resemble the \textit{E. coli} AcnA. In \textit{E. coli}, AcnB is rapidly inactivated when cellular iron pools are low or under oxidative stress (Varghese et al., 2003). In contrast, AcnA synthesis is induced by oxidative stress and iron limitation, but it appears to require an unidentified cellular component for stability (Varghese et al., 2003). Insertional inactivation of the \textit{B. cenocepacia} \textit{acnA} and \textit{acnB} candidate genes will permit us to directly address this possibility.

The catalase activity of the KatA protein is negligible, since it could only be detected in the \textit{katB} mutant when the \textit{katA} gene was overexpressed under a regulatable strong promoter, and also by complementation of the \textit{E. coli} double-catalase mutant UM2. Our working model predicts that the KatA protein contributes to the stability of an AcnA-like protein itself, or that of another factor involved in maintaining the normal function of the TCA cycle enzymes in the presence of iron limitation. However, it is puzzling why KatB, which is presumably present in large quantities, cannot compensate for the KatA defect. One possible explanation is that KatA carries out its protective role in a fashion that is independent of enzymic function. If this is the case, KatA must presumably possess specific structural motifs, absent or modified in KatB, that are required for this role. Experiments to directly test this possibility, and to elucidate the differential regulation of \textit{katA} and \textit{katB} expression, are under way in our laboratory.

In summary, we have determined that \textit{B. cenocepacia} C5424 carries two catalase/peroxidases that share substantial similarity in their amino acid sequences, but play different functional roles. Future studies are required to further characterize the relationship between KatA and the function of aconitase and possibly other enzymes associated with cellular metabolism, as well as to determine whether both KatA and KatB activities are required for bacterial survival \textit{in vivo}.

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