In vitro resistance of Burkholderia cepacia complex isolates to reactive oxygen species in relation to catalase and superoxide dismutase production

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The Burkholderia cepacia complex comprises groups of genomovars (genotypically distinct strains with very similar phenotypes) that have emerged as important opportunistic pathogens in cystic fibrosis (CF) patients. The inflammatory response against bacteria in the airways of CF individuals is dominated by polymorphonuclear cells and involves the generation of oxidative stress, which leads to further inflammation and tissue damage. Bacterial catalase, catalase-peroxidase and superoxide dismutase activities may contribute to the survival of B. cepacia following exposure to reactive oxygen metabolites generated by host cells in response to infection. In the present study the authors investigated the production of catalase, peroxidase and SOD by isolates belonging to various genomovars of the B. cepacia complex. Production of both catalase and SOD was maximal during late stationary phase in almost all isolates examined. Native PAGE identified 13 catalase electrophoretotypes and two SOD electrophoretotypes (corresponding to an Fe-SOD class) in strains belonging to the six genomovars of the B. cepacia complex. Seven out of 11 strains displaying high-level survival after H₂O₂ treatment in vitro had a bifunctional catalase/peroxidase, and included all the genomovar III strains examined. These isolates represent most of the epidemic isolates that are often associated with the cepacia syndrome. The majority of the isolates from all the genomovars were resistant to extracellular O₂⁻, while resistance to intracellularly generated O₂⁻ was highly variable and could not be correlated with the detected levels of SOD activity. Altogether the results suggest that resistance to toxic oxygen metabolites from extracellular sources may be a factor involved in the persistence of B. cepacia in the airways of CF individuals.

Keywords: cystic fibrosis, chronic granulomatous disease, oxidative stress, hydrogen peroxide, superoxide anion

INTRODUCTION

Metabolism of oxygen in aerobic organisms generates toxic reactive species such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH⁻) (Cline, 1975; Fridovich, 1978; Imlay & Linn, 1988). Accumulation of these byproducts can cause lethal damage to cellular proteins, membranes and nucleic acids. Many organisms produce specialized enzymes that neutralize toxic oxygen metabolites (Cline, 1975; Fridovich, 1978; Imlay & Linn, 1988). Superoxide dismutase (SOD), catalase and catalase-peroxidase are well-known examples of antioxidant enzymes utilized by prokaryotes. SOD detoxifies the O₂⁻ anion by a dismutation reaction that generates H₂O₂ and O₂, while catalase converts toxic H₂O₂ into H₂O and O₂ (Fridovich, 1978). In addition to generation of O₂⁻ and H₂O₂ by the endogenous cellular metabolism, pathogenic micro-

Abbreviations: CF, cystic fibrosis; CGD, chronic granulomatous disease; SOD, superoxide dismutase.
organisms may encounter extracellular reactive oxygen species from the respiratory burst activity of phagocytic cells (Brown et al., 1995; Cline, 1975). Therefore, bacterial SOD and catalase may play an important role in bacterial survival within the host. SODs of *Shigella flexneri* (Franzon et al., 1990), *Salmonella typhimurium* (Fang et al., 1999), *Listeria monocytogenes* (Welch et al., 1979), *Aeromonas salmonicida* (Barnes et al., 1996) and *Photobacterium damselae* (Barnes et al., 1999) have been reported to increase the resistance to killing by oxygen radicals. Catalase activity is thought to protect *Staphylococcus aureus* (Manca et al., 1990), and to a limited extent *Shigella flexneri* (Franzon et al., 1990), *Salmonella typhimurium* (Tsolis et al., 1995), *Mycobacterium tuberculosis* (Manca et al., 1999) and *Agrobacterium tumefaciens* (Xiu & Pan, 2000).

*Burkholderia cepacia* is an aerobic, Gram-negative micro-organism that was originally described as the cause of soft rot in onions (Burkholder, 1950). Over the past 20 years the bacterium has emerged as an important opportunistic pathogen, primarily in chronic granulomatous disease (CGD) and cystic fibrosis (CF) patients (Burkholder, 1950; Govan & Deretic, 1996; Govan & Vandamme, 1998). CGD is a rare genetic disorder (X-linked or autosomal recessive) resulting in a defect in the oxidative killing mechanism of phagocytic cells, thus making them unable to generate the toxic oxygen metabolites normally involved in inactivating engulfed bacteria (Cline, 1973; Speert et al., 1994). These cells still retain their non-oxidative bactericidal activities, mainly through the functions of defensins as well as other bactericidal cationic peptides and proteins (Odell & Segal, 1991).

CF is an autosomal recessive genetic disorder that affects approximately 1 in 2500 people in the Caucasian population. The disorder compromises the transport of chloride ions through apical epithelial cell membranes and leads to pancreatic insufficiency and abnormally thick mucous secretion in the lungs and other organs (Govan et al., 1996; Govan & Deretic, 1996; Govan & Vandamme, 1998). The disease is characterized by bacterial colonization and chronic airway infection and persistent inflammation that progressively compromises the lung function (Bals et al., 1999). Ultimately, chronic inflammation and tissue damage leads to a reduction in the rate of gas exchange at the cellular level and can be fatal. The most common pathogen responsible for the morbidity and mortality in CF patients is *Pseudomonas aeruginosa* (Koch & Holby, 1993). However, in recent years *B. cepacia* has increasingly been isolated from the airways of CF patients, and is often associated with a rapid decay of the lung function accompanied with a sepsis-like syndrome. This condition, referred to as the ‘cepacia syndrome’, occurs in roughly 20% of infected patients (Govan & Deretic, 1996; Govan & Vandamme, 1998; Tablan et al., 1985). The severity of *B. cepacia* infections is amplified by the broad resistance of these micro-organisms to most clinically useful antibiotics, and the fact that they can be effectively transmitted from patient to patient (Govan & Deretic, 1996; LiPuma et al., 1990). Recent taxonomic studies have shown that strains formerly identified as *B. cepacia* can be grouped into six genomovars, each containing phenotypically similar yet genotypically distinct organisms (Vandamme et al., 1997). Collectively, these genomovars are referred to as the *Burkholderia cepacia* complex. Strains belonging to genomovar III have been more commonly linked to fatal infections and constitute most of the epidemic isolates (Vandamme et al., 1997).

Recent investigations in our laboratory have shown that *B. cepacia* can survive intracellularly in macrophages and amoebae (Marolda et al., 1999; Saini et al., 1999). Survival within professional phagocytes suggests that *B. cepacia* may avoid the bactericidal mechanisms employed by these cells. *B. cepacia* strains demonstrate resistance to non-oxidative killing pathways, as they are able to survive in the presence of neutrophils from CGD patients while the micro-organisms are killed by neutrophils of normal individuals (Speert et al., 1994). During colonization and infection of the airways in CF patients, there is a pronounced inflammatory response that results in the release of toxic oxygen and toxic nitrogen compounds (Bals et al., 1999). Survival of *B. cepacia* in a macrophage cell line, in the presence of cell activation and an oxidative burst (Saini et al., 1999), suggests that bacterial resistance to oxidative damage may play a role in the infectivity and persistence of this opportunistic pathogen. *B. cepacia* isolates are known to produce catalase (Palleroni, 1992) but the presence of SOD has not been investigated. However, it is likely that *B. cepacia* strains produce SOD since this family of enzymes is found in essentially all aerobic forms of life (Weisiger & Fridovich, 1973).

We hypothesize that SOD and catalase protect *B. cepacia* from oxidative damage and may contribute to bacterial survival and persistence in infected CF lung and airways. To our knowledge, these enzymes have not been systematically characterized in *B. cepacia* genomovars. As a step towards examining the role of these enzymes in infection, we have investigated SOD and catalase activities of cell-free cell extracts of isolates representing each genomovar. Native PAGE analysis of cell-free extracts was performed to probe for the presence of multiple isozymes at various growth stages. We also compared the survival rate of *B. cepacia* strains following challenge with both extracellular and intracellular O$_2^-$ and H$_2$O$_2$ at concentrations equal or higher than those normally found *in vivo*, as a consequence of the oxidative burst in phagocytic cells. Our data show that isolates of genomovar III display a uniformly high level of survival *in vitro* following oxidative stress mediated by extracellular reactive species.

**METHODS**

**Strains, media and chemicals.** Clinical strains of *B. cepacia* belonging to various genomovars were obtained from the *B. cepacia* Canadian Repository at the Research Institute for Children’s and Women’s Health, Vancouver, British Columbia, Canada, and are shown in Table 1. These isolates...
were genetically differentiated into the various genomovars by P. Vandamme, Laboratory of Microbiology, University of Gent, Belgium, and by E. Mahenthiralingam, Research Institute for Children’s and Women’s Health, Vancouver, British Columbia, Canada (Table 1). Genotypic and phenotypic screening indicated that all strains used in this study represented unique isolates rather than clonal variants. Cultures were grown on Luria–Bertani (LB) medium at 37 °C. Preliminary experiments with liquid cultures were done at 25 °C (Katsuwon & Anderson, 1992). One unit of catalase will decompose 1 μmol H₂O₂ (mg protein)⁻¹ min⁻¹ at 25 °C (Katsuwon & Anderson, 1989). The specific peroxidase activity was determined spectrophotometrically by following the oxidation of o-dianisidine at 460 nm in a reaction buffer containing 50 mM potassium phosphate and 1 mM H₂O₂ under these conditions both SOD and catalase activities could be preserved for several months. The protein concentration of cell-free extracts was measured according to the Bio-Rad protein assay using bovine serum albumin as a standard.

Enzymic activity assays. The specific catalase activity was determined spectrophotometrically by following the disappearance of H₂O₂ over time at 240 nm, upon the addition of cell-free extract (Katsuwon & Anderson, 1992). One unit of catalase will decompose 1 μmol H₂O₂ (mg protein)⁻¹ min⁻¹ at 25 °C (Katsuwon & Anderson, 1989). The specific peroxidase activity was determined spectrophotometrically by following the oxidation of o-dianisidine at 460 nm in a reaction buffer containing 50 mM potassium phosphate and 1 mM H₂O₂ upon the addition of cell-free extract. One unit of peroxidase activity equals 1 μmol H₂O₂ reduced min⁻¹, with ε₉₀ = 11.3 × 10⁴ M⁻¹ cm⁻¹ (Schnell & Steinman, 1995). The specific SOD activity was determined spectrophotometrically by measuring the inhibition of the initial rate of auto-oxidation of 6-hydroxydopamine at 490 nm following the addition of cell-free extract (Heikikila & Cabbat, 1976). A standard curve using known units of SOD activity was generated in order to determine the units of activity in each sample. One unit of SOD activity corresponded to 50% inhibition of the initial activity equals 1 μmol H₂O₂ reduced min⁻¹, with ε₉₀ = 11.3 × 10⁴ M⁻¹ cm⁻¹ (Schnell & Steinman, 1995). The specific SOD activity was determined spectrophotometrically by measuring the inhibition of the initial rate of auto-oxidation of 6-hydroxydopamine at 490 nm following the addition of cell-free extract (Heikikila & Cabbat, 1976). A standard curve using known units of SOD activity was generated in order to determine the units of activity in each sample. One unit of SOD activity corresponded to 50% inhibition of the initial reaction.

Preparation of cell-free extracts. Thirty-millilitre cultures were centrifuged at 7000 g for 10 min. Bacterial pellets were washed with ice-cold 0.05 M sodium phosphate buffer (pH 7.0) and finally resuspended in 3 ml 0.05 M sodium phosphate buffer, supplemented with 0.1% dithiothreitol and stored on ice. Cells were lysed by sonication with a sonifier cell disrupter model W-350 (Branson Ultrasonics) for a total of 3 min in three 60 s pulses on ice. Lysates were centrifuged at 12000 g for 5 min to sediment cellular debris and unlysed cells. Supernatants were stored at -20 °C in 50% (v/v) glycerol. Under these conditions both SOD and catalase activities could be preserved for several months.
rate. Bovine liver catalase and both iron- and manganese-containing SOD enzymes (Fe-SOD and Mn-SOD) from *Escherichia coli* were used as positive controls in the respective assays.

**Native PAGE for identification of catalase, peroxidase and SOD.** We used native PAGE to identify bands of catalase (Katsuwon & Anderson, 1992). Lanes were loaded with 10–20 µg total protein and the electrophoresis was carried out using 10 % Novex gels. Gels were washed with distilled water for 30 min to remove any traces of running buffer. To identify bands of catalase activity the gel was soaked in 200 ml of a 3 mM solution of H$_2$O$_2$ for 10 min and then rinsed in distilled H$_2$O. Following the addition of a 1 % potassium ferricyanide/1 % ferric chloride (w/v) solution for 10 min in darkness, the gel stained dark blue except at sites showing catalase activity, which appeared clear. Bovine liver catalase was used as a positive control. Bands of peroxidase activity were visualized using the method described by Wayne & Diaz (1986). Briefly, following electrophoresis as described above, gels were washed for 30 min in phosphate-buffered saline (PBS). 5 % to 10 % w/v dimethyl sulfoxide (DMSO) was added to the gels to identify bands of peroxidase activity. The gels were soaked in a 200 ml PBS solution containing 10 mM H$_2$O$_2$ and 2.0 mM 3,3'-diaminobenzidine tetrahydrochloride for 30 min. Sites of peroxidase activity appeared brown on a clear background. Type II horseradish peroxidase was used as a positive control. In order to determine whether bands showing peroxidase activity were due to bifunctional catalase-peroxidase enzymes, gels were washed in distilled water and counter-stained for catalase activity as described above. Heat inactivation was performed by incubating samples at 65 °C for 1 min immediately prior to loading (Wayne & Diaz, 1986). Chemical inactivation of catalase activity was performed by incubating extracts with 3-amino-1,2,4-triazole for 20 min. Bands of SOD activity were identified by the native PAGE method described by Beauchamp & Fridovich (1971). Lanes were routinely loaded with 20–40 µg total protein. After electrophoresis, under the conditions as described above, gels were washed for 30 min in distilled water, and incubated with shaking in the dark for 30 min in a solution of 250 µM nitro blue tetrazolium (NBT) dissolved in 200 ml distilled water. Gels were then incubated in a developing solution containing 50 mM potassium phosphate, pH 7.8, 1 mM EDTA, pH 8.0, 20 mM N,N,N',N'-tetramethylethylenediamine (Bio-Rad) and 30 µM riboflavin in the dark with shaking for 20 min. Bands of SOD activity were visualized by exposing the gels to light for 10 min or until sufficient contrast with the background developed. Sites with SOD activity appeared clear on a purple background. Both Fe-SOD and Mn-SOD purified from *E. coli* were used as positive controls in this assay.

**RESULTS**

**Catalase production by strains of the *B. cepacia* complex**

We examined catalase production in a set of 19 isolates representing the six known genomovars of the *B. cepacia* complex (Table 1). These included clinical isolates as well as two isolates from environmental sources. The catalase activity in cell-free extracts was determined spectrophotometrically by following the degradation of H$_2$O$_2$ at 240 nm. An extract from the *E. coli* K-12 catalase-deficient mutant strain UM2, lacking functional *katE* and *katG* genes, was used as a negative control for the assay, while bovine catalase served as a positive control. In general, there was a wide range in the amount of catalase activity produced by individual isolates of genomovars I, II and III (Table 2). This variability was not due to experimental error since similar enzyme units for a given isolate were detected in repeated experiments. No significant differences were observed in the average catalase production among the various genomovar isolates. However, the catalase activity significantly increased in extracts prepared from isolates grown to late stationary phase, ranging from fivefold to several thousandfold with respect to the levels measured in extracts obtained from cells in the exponential phase.

Native PAGE analysis of cell-free extracts, derived from cultures at various growth stages, yielded 13 distinct catalase electrophoretotypes (Fig. 1a). Extracts with enzymic activities below 10$^{-6}$ units (mg protein)$^{-1}$ (Table 2) did not produce detectable bands under the staining conditions used. It should be noted that the band designation simply reflects the mobility of the enzyme in the gel but does not imply that the enzymes with similar mobility are indeed identical (see below).
Late stationary phase
positive control while the this case, type II horseradish peroxidase served as a
by following the oxidation of
ditional peroxidase activity. Thus, peroxidase activity in
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Catalases can exist as monofunctional or bifunctional
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in each strain at the various growth stages are sum-
small variations in amino acid composition that may
affect dramatically the electrophoretic migration into
the gel or may correspond to protein aggregates that
small variations in amino acid composition that may
affect dramatically the electrophoretic migration into
the gel or may correspond to protein aggregates that
cannot be resolved by native PAGE. The bands detected
in each strain at the various growth stages are sum-
arized in Table 3 using the band designations from
Fig. 1(a). Based on the analysis of this limited set of
strains, it appeared that some bands were consistently
associated with particular genomovars. Band 13 was
present in the genomovar V strain CEP040, the geno-
movar VI strain L6, and all genomovar III strains, except
for strain CEP024. Band 11 appeared in all strains
belonging to genomovars II and VI.

Catalases can exist as monofunctional or bifunctional
enzymes. Bifunctional catalases usually carry an ad-
ditional peroxidase activity. Thus, peroxidase activity in
cell-free lysates was determined spectrophotometrically
by following the oxidation of o-dianisidine at 460 nm. In
this case, type II horseradish peroxidase served as a
positive control while the E. coli K-12 catalase-deficient
mutant strain UM2 was used as a negative control for
the assay. Extracts from all isolates belonging to
 genomovars III, IV and VI, but only one from a
genomovar I strain, were positive for peroxidase activity
(Table 2). Strains belonging to genomovar II showed no
detectable peroxidase activity. As with catalase activity,
the level of peroxidase activity measured was maximal
during stationary phase for all strains except for
CEP511. To determine whether the detected peroxidase
and catalase activities were associated with the same
enzyme we conducted native PAGE analysis combining
the specific staining for peroxidase activity with that for
catalase (Fig. 1c). In all peroxidase-positive strains, the
band with peroxidase activity co-migrated with the
band of catalase activity (Fig. 1c, lane 1). No bands of
peroxidase activity, independent of catalase activity,
were detected in any of the extracts investigated. These
observations confirm the presence of bifunctional
catalase/peroxidase enzymes. Furthermore, all bands
showing both enzymic activities disappeared when
samples were incubated at 65 °C for 1 min prior to
loading the gel, while bands corresponding to mono-
functional enzymes were heat-resistant (Fig. 1c, Table
2). The heat sensitivity of the bifunctional enzymes
observed in the native PAGE analysis was corroborated
by similar observations on overall levels of enzyme
activity in heat-treated cell-free extracts (Table 3 and
data not shown). Heat sensitivity is a typical feature

<table>
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<th>Strain</th>
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<th>Late stationary</th>
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<td>Catalase activity</td>
<td>Peroxidase activity</td>
<td>Catalase activity</td>
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<td>FC124</td>
<td>I</td>
<td>1·6</td>
<td>—</td>
</tr>
<tr>
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<td>I</td>
<td>1·5</td>
<td>6·5 × 10⁻⁴</td>
</tr>
<tr>
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<td>—</td>
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<td>II</td>
<td>0·2</td>
<td>—</td>
</tr>
<tr>
<td>C5274</td>
<td>II</td>
<td>2·3 × 10⁻⁷</td>
<td>—</td>
</tr>
<tr>
<td>C7062</td>
<td>II</td>
<td>3·2 × 10⁻⁷</td>
<td>—</td>
</tr>
<tr>
<td>ATCC 17616</td>
<td>II</td>
<td>2 × 10⁻⁸</td>
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</tr>
<tr>
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</tr>
<tr>
<td>L6</td>
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</tr>
<tr>
<td>UM2</td>
<td>E. coli</td>
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</table>
of bifunctional catalase/peroxidases; monofunctional catalases are typically heat resistant (Wayne & Diaz, 1986). Detection of both catalase and peroxidase activities in association with specific electrophoretotypes also allowed us to distinguish between the monofunctional catalase present in genomovar II strains and the bifunctional catalase/peroxidase present in genomovar VI strains, both of which had apparently the same electrophoretotype (band 11). Also, the band 13 present in genomovar III strains corresponded to a bifunctional catalase/peroxidase while the band with similar mobility found in the genomovar V strain CEP040 corresponded to a monofunctional catalase (Table 3). The results indicate that these bands, although identical in gel mobility, clearly corresponded to two different classes of catalases. Furthermore, in the case of the genomovar I strain CEP509, band 1 corresponded to a monofunctional catalase while the bifunctional catalase/peroxidase activity was associated with band 10. Bands 2 and 6, seen in the genomovar III strains CEP024 and C4455, respectively (Table 3), also disappeared after heating, and it is quite possible that these bands corresponded to aggregates of bands 12 and 13. In the genomovar II strains, we observed that the slow-migrating bands 3, 4 and 5 appeared and/or disappeared from the same strain at different growth stages (Table 3). All of these bands were absent in heat-treated extracts, while the corresponding lower band 11 was always detected. Since the genomovar II strains do not produce a detectable heat-sensitive bifunctional catalase/peroxidase we concluded that the slow-migrating bands also corresponded to protein aggregates of a single monofunctional catalase represented by band 11. Therefore we propose that of the 13 electrophoretotypes observed, only bands 1, 7, 8, 9, 10, 11, 12 and 13 represent distinct catalase activities while the remaining bands (2, 3, 4, 5, 6) correspond to multimeric aggregates.

**SOD production by strains of the B. cepacia complex**

Cell-free extracts were also used to determine the specific SOD activity by measuring the inhibition of the initial rate of auto-oxidation of 6-hydroxydopamine at 490 nm. This assay was chosen because it does not suffer any interference from other components in the crude cell-free lysates (Bannister & Calabrese, 1987). The *E. coli* K-12 *sodA sodB* mutant QC779 was used as a negative control for the assay. Units of SOD activity were calculated by generating a standard curve with Fe-SOD from *E. coli* as a control, where 1 unit represented 50% inhibition of auto-oxidation of the substrate. Table 4 shows the SOD activities for each strain at the early exponential and late stationary growth stages. As with catalase activities, the level of SOD activity measured...
Increased dramatically in the extracts from cultures at stationary phase. Likewise, genomovar III strains also appeared to possess, on average, higher levels of SOD activity than the strains belonging to the other genomovars (Table 4). However, following statistical analysis, the levels of SOD activity observed for strains from the various genomovars were not significantly different.

Native PAGE analysis of cell-free cell extracts identified two distinct SOD electrophoreotypes (Fig. 2a, b). In all cases, the SOD activity was maximal at the late stationary stage of growth based on band intensity (Fig. 2c). While the fastest-migrating species (band 2, Fig. 2a) was present in all the strains, the other species (band 1, Fig. 2a) was not equally distributed (Table 4). Based on the banding pattern generated by each strain at the various growth stages, we concluded that the majority of SOD activity was associated with band 2 in all strains except for CEP511 and FC124, which produced a more intense band 1. In all cases, the more intense band was detected at all stages of growth while the other band became apparent only during stationary phase (Table 4). More thorough analysis identified an additional band of activity that was very weak and only became clearly visible when 75 µg total protein was loaded in the gels. This band was exclusively present in the extracts that contained an intense band 2, but disappeared when the extracts were incubated at 65 °C for 1 min. Also, this band of activity was not detected in extracts loaded on gels with 10% acrylamide. Taking into consideration the association of this band with band 2, its heat-sensitive nature (SOD enzymes are typically heat stable), its slow migration and comparatively low amount, we conclude that this band possibly corresponds to a multimeric enzyme aggregate of band 2.

Depending on the metal co-factor, SOD enzymes can be distinguished as Fe-SOD, Mn-SOD and Cu/Zn-SOD. These SOD forms can be separated on the basis of their sensitivities to various inhibitors. Fe-SOD enzymes are inhibited by exposure to H$_2$O$_2$ while the Cu/Zn form is inhibited by potassium cyanide. Mn-SOD enzymes are usually resistant to both inhibitors. Following treatment with the selected inhibitors, bands of activity on native gels are retained or lost depending on the type of SOD present. Initial attempts to classify the SOD enzymes from _B. cepacia_ proved unsuccessful. This was probably due to interactions of the specific inhibitors with other components of the cell-free lysates used for these studies. This was clearly evident with the addition of H$_2$O$_2$ to the samples, as the catalase activity rapidly produced bubbling, a sign of H$_2$O$_2$ degradation. To eliminate

### Table 3. Distribution of catalase electrophoretotypes at various growth stages

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genomovar</th>
<th>Early exponential</th>
<th>Late exponential</th>
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<tr>
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<tr>
<td>CEP103</td>
<td>IV</td>
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<td>N/B</td>
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<tr>
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<td>CEP021</td>
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<td>11</td>
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<td>11</td>
</tr>
<tr>
<td>L6</td>
<td>VI</td>
<td>11</td>
<td>11, 13</td>
<td>11, 13</td>
<td>11, 13</td>
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</table>

Distribution of electrophoretotypes among the isolates of the _B. cepacia_ complex, using the same band designations as shown in Fig. 1(a). Numbers in bold denote bands displaying a bifunctional catalase/peroxidase activity (which also disappeared with heat treatment). Numbers in italics denote bands that correspond to protein aggregates as explained in the text. _N/B_ indicates that a band was not detectable. The _OD$_{400}$ ranges defining the various growth stages are indicated in Methods.
**Table 4.** SOD activity in *B. cepacia* strains according to growth phase

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genomovar</th>
<th>Band profile</th>
<th>SOD activity†</th>
<th>Early exponential‡</th>
<th>Late stationary§</th>
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<tr>
<td>CEP509</td>
<td>I</td>
<td>2</td>
<td>43:1</td>
<td>74:5</td>
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<tr>
<td>ATCC 25416</td>
<td>I</td>
<td>2</td>
<td>52:6</td>
<td>83:5</td>
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<td>FC124</td>
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<td>2</td>
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<td>2</td>
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<td>168:2</td>
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<tr>
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<td>54:7</td>
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<td>2</td>
<td>22:3</td>
<td>209:5</td>
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<td>35:2</td>
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<td>17:3</td>
<td>139</td>
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<tr>
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<td>QC779</td>
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</table>

*Bold numbers indicate the SOD electrophoretotype that appeared with the highest intensity in native PAGE gels; ND, not determined.
†SOD activity was measured spectrophotometrically as described in Methods. Specific activity is indicated as units per mg protein.
‡Early exponential phase cells (OD_{660} = 0.5–0.6).
§Late stationary phase cells (OD_{660} > 2.0).
Catalase and superoxide dismutase in B. cepacia

Fig. 2. Detection of SOD activities in cell-free cell lysates of B. cepacia complex strains using native PAGE followed by staining as described in Methods. (a) Identification of two SOD electrophoretotypes present in strains belonging to genomovars I to VI. Band 1 represents electrophoretotype 1; band 2 represents electrophoretotype 2. Each lane was loaded with 40 µg total protein from extracts generated from late stationary cultures. Lanes: 1, L6; 2, FC124; 3, C5424. (b) Lane overloaded with 75 µg total protein from L6 to visualize a weak band of SOD activity thought to correspond to an aggregate of band 2 (arrow). (c) Stationary-phase induction of SOD activity observed in B. cepacia strain C1484. Each lane was loaded with 20 µg total protein. Visible electrophoretotype corresponds to band 3. EL, early exponential phase; LL, late exponential phase; ES, early stationary phase; LS, late stationary phase, as defined in Methods.

Fig. 3. Determination of the SOD class. Cell-free extracts were pre-treated by heating at 65°C for 1 min to inactivate bifunctional catalase/peroxidase activity and/or with 20 mM 3-amino-1,2,4-triazole for 20 min to inactivate monofunctional catalase activity. Subsequently extracts were treated with 25 mM H2O2 for 20 min at room temperature to inactivate Fe-SOD. Odd-numbered and even-numbered lanes were loaded with untreated and treated extracts, respectively. Lanes: 1 and 2, E. coli Mn-SOD; 3 and 4, E. coli Fe-SOD; 5 and 6, B. cepacia FC124; 7 and 8, B. cepacia C5424.

Therefore, the remaining experiments were conducted using cultures in stationary phase. Strains belonging to genomovar III showed a uniformly high level of resistance to H2O2 at all concentrations tested (Fig. 4). Survival of one strain in this group, C5424, was assayed at higher H2O2 concentrations, showing that it was capable of surviving in up to 100 mM H2O2 (data not shown). In contrast, the susceptibility of strains from the other genomovars showed much more variation among individual isolates, although some strains, notably the two genomovar VI isolates, also displayed high levels of resistance comparable with that observed with genomovar III isolates (Fig. 4). The survival rate could not be directly correlated with levels of catalase activity alone. However, 7 out of 11 strains showing the highest survival rates (above 25% with respect to the initial inoculum) at the highest H2O2 concentrations were those producing a bifunctional catalase/peroxidase. In contrast, only two of the strains producing a bifunctional catalase/peroxidase (FC473 and CEP539) showed poor survival following H2O2 exposure. These results suggest that in most cases a bifunctional catalase/peroxidase may be associated with increased resistance to H2O2 exposure. The genomovar III strain CEP511 exhibited anomalous behaviour in that its percentage survival was not proportional to the amount of H2O2 in the assay medium. This may be related to the presence of other unstable phenotypes (colony morphology, mucoidity and pigment formation) that have been observed with this strain.

The level of resistance to O2⁻ exposure was assessed using the xanthine plus xanthine oxidase (X + XO) system and paraquat, which generate extracellular and intracellular O2⁻, respectively. As membrane bilayers are impermeable to O2⁻ (Steinman, 1993), the results obtained with these assays may vary significantly according to the cellular localization of the SOD enzyme(s). A periplasmic SOD would provide protection against X + XO-generated O2⁻ while a cytoplasmic enzyme would provide protection primarily against paraquat-generated O2⁻ (De Groote et al., 1997; Wilks et al., 1998). The majority of strains analysed except for FC124 and CEP511 showed over 80% survival following exposure to the extracellular O2⁻-generating system for 120 min and there were no significant differences between genomovars II, IV, V and VI in their response to the X + XO challenge (Fig. 5a). Bacterial susceptibility to intracellular O2⁻ was investigated by exposing cells to increasing concentrations of paraquat ranging from 0 to 10 mM. The cells accumulate this compound and its reduction in the cytoplasm causes the formation of O2⁻ anions. In contrast to the uniform survival patterns observed in the X + XO system, the paraquat survival patterns showed a higher level of variability for strains belonging to all genomovars (Fig. 5b). However, statistical analysis did not reveal significant differences among the genomovars. Furthermore, the level of survival following paraquat exposure did not closely follow the pattern of SOD activity in the strains. For instance, the genovar IV strains showed elevated levels of paraquat resistance yet their levels of SOD activity were not significantly different from those of the other strains, while the genomovar III strain, C5424, showed relatively poor paraquat resistance despite showing the highest level of SOD activity. This phenomenon possibly reflects a difference in the level of paraquat accumulated within the various strains. It has been
shown that paraquat toxicity is strongly linked to the cytoplasmic concentration of the drug (Kitzler & Fridovich, 1990). Therefore, different rates of intake and/or efflux of paraquat in the strains could account for our results.

DISCUSSION

The current study focused on the antioxidant enzymes catalase and SOD with the hypothesis that they may play a role in protecting *B. cepacia* from the oxidative killing mechanisms within the host, especially in the airways of CF patients. Strains belonging to the six established genomovars of the *B. cepacia* complex were investigated to identify and characterize their catalase and SOD activities. We observed the presence of 13 distinct electrophoretotypes of catalase based on their differential migration in native PAGE. As this is a non-denaturing system, the distinct migration patterns are not necessarily a consequence of differences in the molecular mass of the enzyme forms. Further analysis demonstrated that many of the slow-migrating electrophoretic forms corresponded to protein aggregates of unique fast-migrating enzymes. Therefore we propose that bands 1, 7, 8, 9, 10, 11, 12 and 13 represent activities associated with distinct catalase enzymes while bands 2, 4, 5 and 6 are thought to be artifacts of protein aggregation. While there appeared to be some association with genomovar assignment and the presence of specific bands, the pool of strains used in this study is not large enough to test this relationship adequately. The overall amount of catalase produced by individual strains increased as the cultures grew from exponential to stationary phase (Table 2). However, only the genomovar VI strain L6 and the genomovar II strain CEP484 showed novel catalase electrophoretotypes in stationary phase as compared to exponential phase (Table 3).

Bifunctional catalase/peroxidase enzymes were detected in all strains examined from genomovars III, IV and VI, but only in one genomovar I isolate, which also
expressed a monofunctional catalase. These enzymes have been found to be associated with virulence in a wide range of lung pathogens such as Mycobacterium tuberculosis (Manca et al., 1999) and Legionella pneumophila (Bandyopadhyay & Steinman 1998). Although in this study a high level of survival to H2O2 exposure in vitro could not be correlated with the amount of catalase activity, 7 out of the 11 strains showing the highest survival rates produced bifunctional catalase/peroxidase and belonged to genomovar I, III and VI. The prevalence of catalase/peroxidase in the genomovar III strains, which are most commonly associated with the cepacia syndrome in CF patients, and their apparent increased resistance to H2O2 exposure provides justification for further study into the role of this enzyme in the pathogenesis of B. cepacia infections. Monofunctional catalases were found in the B. cepacia complex isolates from genomovars I, II and V. Isolates of closely related species such as Pseudomonas aeruginosa, P. putida, P. syringae and Xanthomonas possess multiple catalase isozymes (Brown et al., 1995; Katsuwon & Anderson, 1992; Mongkolsuk et al., 1996). Under our experimental conditions, however, we have found only one enzyme class in most isolates, suggesting either that B. cepacia isolates have fewer catalase forms or, alternatively, that additional catalases may be expressed under conditions of stress not examined in this study.

Electrophoretic analysis also revealed the presence of two electrophoretotypes of SOD in B. cepacia, one of which corresponded to a larger protein aggregate. While SOD is commonly found in three major forms, Fe, Mn and Cu/Zn co-factored enzymes, it is not uncommon for multiple bands of SOD to be detected (Bannister & Calabrese, 1987). Initial attempts to classify the SOD activities using specific inhibitors yielded inconclusive results, possibly due to interference from other components found in cell-free cell lysates. As H2O2 is the standard inhibitor of Fe-SOD enzymes, any catalase activity present in the samples would probably limit the effectiveness of the inhibition. Therefore, following inactivation of the catalase activities we were able to identify that the SOD electrophoretotypes in B. cepacia corresponded to Fe-SODs. Using PCR amplification with degenerate primers, we have recently been able to clone a portion of the B. cepacia SOD gene that shows features of a typical Fe-SOD and that hybridized with all the strains tested (unpublished results). Gene knockout experiments in combination with studies under different growth conditions, currently under way in our laboratory, will permit us to determine whether the Fe-SOD is unique to B. cepacia or other SOD forms are also present in these isolates.

The level of expression of catalase and SOD was associated with the growth stage of the cultures. In all strains the activity of both enzymes increased in the cells entering stationary phase as shown by the band intensities in native gels as well as by the specific activity determinations. This may be important in vivo as it would provide a higher level of protection to those cells able to establish a chronic infection where the bacteria would find themselves under a physiological situation similar to the late stationary phase of growth. Alternatively, other conditions of stress, including tissue inflammation, may stimulate a bacterial response similar to that of stationary phase and induce a high level of expression of these enzymes. The susceptibility of B. cepacia to oxidative killing is suggested by the observation that neutrophils from CGD patients fail to inactivate B. cepacia (Speert et al., 1994). However, this conclusion contrasts with the apparent tolerance of B. cepacia to the highly oxidative environment in the CF lung, where the inflammatory response is dominated by neutrophils. Furthermore, B. cepacia strains isolated from CF patients, especially those of genomovar III, are strong catalase/peroxidase and SOD producers. One possibility to explain this paradox could be that the strains causing infections in CGD individuals are different from those found in CF patients. This may be true, at least in part, since all genomovar III isolates reported by Vandamme et al. (1997) were obtained exclusively from CF patients. In addition, the unique nature of the CF lung and airways may also contribute to the possible escape of B. cepacia from oxidative killing. Studies into the pathology of bacterial infections in the respiratory airways of CF patients have identified chronic inflammation as a major cause of tissue damage and loss of gas-exchange function (Tager et al., 1998). Recruitment of neutrophils to the site of infection is associated with a release of reactive oxygen species and other toxic compounds. Ongoing release of reactive oxygen species may in turn overwhelm the cellular antioxidant defences and lead to the accumulation of toxic levels of these compounds, which may damage the lung cells (Bals et al., 1999; Suttorp & Simon, 1982). Lipopolysaccharide (LPS) has been shown to elicit a powerful inflammatory response in neutrophils (Forehand et al., 1993; Worthen et al., 1988). Interestingly, it has been shown that the LPS of B. cepacia elicits a much greater inflammatory response as measured by O2− release than the LPS of P. aeruginosa, another common CF pathogen (Hughes et al., 1997; Zughaier et al., 1999). These observations are consistent with our own findings indicating that activation of macrophages following phagocytosis of B. cepacia is primarily due to LPS release (Saini et al., 1999). Therefore, it is possible that bacterial antioxidant defences allow B. cepacia to survive and persist despite the oxidative stress in the lung promoted by LPS-mediated stimulation of inflammatory cells. Experimental evidence showing that B. cepacia strains can survive within macrophages in the presence of macrophage cell activation supports these conclusions (Saini et al., 1999). The resistance of B. cepacia strains to toxicity by reactive oxygen species in lung tissue, in association with the ability of these isolates to survive intracellularly (Burns et al., 1996; Marolda et al., 1999; Martin & Mohr, 2000; Saini et al., 1999) may explain, at least in part, their persistence as well as their ability to continually elicit an inflammatory response. Furthermore, a recent study showed that nitric oxide acts synergistically with reactive oxygen species to
kill *B. cepacia* in vitro (Smith et al., 1999) and suggested that persistence of *B. cepacia* in CF patients may be associated with a defect in the inducible nitric oxide synthase activity in these patients (Kelley & Drumm, 1998). Taken together, these factors would explain the high level of tissue damage and lethality commonly associated with *B. cepacia* infection in CF patients, especially with genomovar III isolates. Confirmation of this pathogenic model awaits further experiments using animal models of lung infection as well as the generation of genetically defined catalase- and SOD-deficient mutants.

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**REFERENCES**


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