***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/oxidase, 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 damsela* (Barnes *et al.*, 1999) have been reported to increase the resistance to killing by oxygen radicals. Catalase activity is thought to protect *Staphylococcus aureus* (Mandell, 1975), 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, 1975; 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 & Hoiby, 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 O₂⁻ and H₂O₂ 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

Table 1. Strains used in this study

Strain	Genomovar*	Source†	RAPD type‡	Source or reference§
<i>B. cepacia</i>				
FC124	I	CGD	X	BCCR
CEP509	I	CF	41	BCCR
ATCC 25416	I	Onion	39	ATCC
CEP484	II	CF	47	BCCR
C0514	II	CF	5	BCCR
C5274	II	CF	12	BCCR
C7062	II	CF	20	BCCR
ATCC 17616	II	Soil	30	T. Lessie
CEP024	III	CF	17	BCCR
CEP511	III	CF	40	BCCR
C1484	III	CF	4	BCCR
C4455	III	CF	6	BCCR
C5424	III	CF	2	BCCR
CEP103	IV	Clinical, non-CF	X	BCCR
FC473	IV	CF	16c	BCCR
CEP559	IV	CF	16	BCCR
CEP040	V	CF	8	BCCR
CEP021	VI	CF	10	BCCR
L6	VI	CF	10	BCCR
<i>E. coli</i> K-12				
UM2	NA	GC4468, <i>katE katG</i>	NA	Loewen & Triggs (1984)
QC779	NA	GC4468, <i>sodA sodB</i>	NA	Carlioz & Touati (1986)

* Genomovars II, IV and V of the *B. cepacia* complex are now designated as *B. multivorans* (Vandamme *et al.*, 1997), *B. stabilis* (Vandamme *et al.*, 2000) and *B. vietnamiensis* (Gillis *et al.*, 1995), respectively. NA, Not applicable.

† CGD, chronic granulomatous disease; CF, cystic fibrosis.

‡ RAPD, randomly amplified polymorphic DNA fingerprinting (Mahenthiralingam *et al.*, 1996); X, unassigned profile.

§ BCCR, *B. cepacia* Canadian Repository; T. Lessie, University of Massachusetts.

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 to determine that OD₆₀₀ values (measured with an Ultrospec II spectrophotometer; Pharmacia) of 0.5–0.6, 0.8–0.9, 1.5–2.0 and 2.0 or more corresponded to early exponential phase, late exponential phase, early stationary phase and late stationary phase, respectively. Unless otherwise indicated, chemicals were obtained from Sigma.

Preparation of cell-free cell 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. 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 $\epsilon_M = 11.3 \times 10^4$ 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 (Heikkilä & 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

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_2O_2 for 10 min and then rinsed in distilled H_2O . 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). To identify bands of peroxidase activity the gels were soaked in a 200 ml PBS solution containing 1.0 mM H_2O_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.

In vitro protection assays. The level of protection against H_2O_2 exposure in the strains used in this study was measured as described by Katsuwon & Anderson (1989). Briefly, cultures of *B. cepacia* were grown with shaking at 37 °C in LB broth until late stationary phase. Samples containing 1×10^8 cells ml^{-1} of each strain were treated with various H_2O_2 concentrations and incubated with shaking at 25 °C for 30 min. Control samples received H_2O in place of H_2O_2 treatment. Appropriate serial dilutions were plated in triplicate on LB agar plates. Colonies were counted after 48 h incubation and percentage survival calculated by comparison with colony counts obtained from untreated samples. The level of protection of strains to extracellular O_2^- exposure was measured as described elsewhere (Schnell & Steinman, 1995) with some modifications. Late stationary phase culture samples containing 1×10^8 cells ml^{-1} were incubated with shaking at 37 °C in a mixture containing 250 µM xanthine (X)

and 0.14 units of xanthine oxidase (XO); 100 U catalase ml^{-1} was added to each sample prior to addition of XO to protect cells from the toxicity of any H_2O_2 produced as a consequence of SOD activity. Aliquots were removed at 0, 30, 60 and 120 min and serially diluted in 200 mM phosphate buffer, pH 7.4. Time 0 aliquots were removed before addition of the O_2^- generating components. Appropriate dilutions were plated in triplicate on LB agar plates, incubated for 24–48 h at 37 °C and the colonies counted. Percentage survival was determined using the following equation: % Survival = (No. of colonies, treated sample/No. of colonies at time 0) $\times$ 100. Survival after intracellular O_2^- exposure was measured using the liquid assay. The liquid assay was performed as described by Membrillo-Hernandez *et al.* (1999) with some modifications. Cultures of *B. cepacia* strains were grown to late stationary phase. Samples containing 1×10^8 cells ml^{-1} were incubated with shaking at 37 °C in a mixture containing 0, 2.5, 5 or 10 mM paraquat. Aliquots were removed after 45 min, serially diluted and plated in triplicate on LB agar plates. Following incubation at 37 °C for 24–48 h, colonies were counted and percentage survival was calculated using the equation described above.

Statistical analysis. Data were analysed using the Fisher least significant difference (LSD) method, using 95% confidence intervals.

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_2O_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).

Table 2. Catalase and peroxidase activities in *B. cepacia* strains according to growth phase

See Methods for the OD₆₀₀ ranges defining the growth stages. Specific activity of the enzymes is indicated as units per mg protein.

Strain	Genomovar	Early exponential		Late stationary	
		Catalase activity	Peroxidase activity	Catalase activity	Peroxidase activity
FC124	I	1.6	—	6.6	—
CEP509	I	1.5	6.5×10^{-4}	6.4	3×10^{-3}
ATCC 25416	I	6×10^{-7}	—	0.5	—
CEP484	II	0.1	—	1.3	—
C0514	II	0.2	—	1.1	—
C5274	II	2.3×10^{-7}	—	0.4	—
C7062	II	3.2×10^{-7}	—	11.9	—
ATCC 17616	II	2×10^{-8}	—	0.4	—
CEP024	III	7.2×10^{-2}	$< 10^{-8}$	9.4	5×10^{-3}
CEP511	III	0.2	1×10^{-4}	1.4	6.5×10^{-6}
C1484	III	0.1	8×10^{-5}	0.7	5×10^{-4}
C4455	III	0.6	2.7×10^{-3}	3.9	3×10^{-3}
C5424	III	2.3	9×10^{-3}	29	1×10^{-2}
CEP103	IV	4×10^{-8}	—	0.1	—
FC473	IV	8×10^{-8}	$< 10^{-8}$	1.5	1.8×10^{-3}
CEP559	IV	4×10^{-8}	$< 10^{-8}$	0.9	2.0×10^{-3}
CEP040	V	2×10^{-8}	—	0.4	—
CEP021	VI	0.9	1.5×10^{-4}	2.3	1.6×10^{-4}
L6	VI	0.2	$< 10^{-8}$	12	2.3×10^{-3}
UM2	<i>E. coli</i>	ND	ND	$< 10^{-8}$	$< 10^{-8}$

Likewise, enzymes with different gel mobility could have 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 summarized 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 genomovar 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 additional 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 monofunctional 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

Fig. 1. Detection of catalase activities in cell-free cell lysates from strains belonging to genomovars I through VI of the *B. cepacia* complex. Extracts were analysed by native PAGE followed by staining with potassium ferricyanide. (a) Identification of the 13 catalase electrophoretotypes (lanes 1–13) present in strains belonging to genomovars I–VI of the *B. cepacia* complex. Each lane was loaded with 10–20 µg total protein in order to visualize the relevant electrophoretotypes from the following strains (growth stage indicated in parentheses; see below): lane 1, ATCC 25416 (LS); 2, CEP024 (ES); 3, C0514 (LL); 4, C7062 (ES); 5, CEP484 (ES); 6, C4455 (LS); 7, CEP484 (LL); 8, FC473 (LS); 9, FC124 (LS); 10, CEP509 (LS); 11, CEP021 (LS); 12, CEP024 (LS); 13, CEP511 (LS). Lane 14, extract from *E. coli* UM2 (*katF katG*) used as a negative control. Lane 15, bovine catalase (1 unit) used as a positive control. Additional bands in lanes 2 and 6, corresponding to electrophoretotypes 12 and 13 respectively, are present in the specified samples used. The apparent doublet visible in lane 6 is an artifact of the excess protein loaded to visualize the much weaker band 6. Not all bands associated with the indicated extracts (Table 3) are visible at the protein concentrations loaded in this gel. (b) Stationary-phase induction of catalase activity observed in *B. cepacia* strain C1484. The electrophoretotype corresponds to band 13. EL, early exponential phase; LL, late exponential phase; ES, early stationary phase; LS, late stationary phase. (c) Identification of bifunctional catalase-peroxidase enzymes in strains belonging to the *B. cepacia* complex. Top panel, native PAGE followed by staining with 3,3'-diaminobenzidine tetrahydrochloride for peroxidase activity. Bottom panel, counterstaining using catalase-specific potassium ferricyanide stain. Lanes: 1, C4455; 2, heat-treated C4455; 3, CEP484; 4, heat-treated CEP484.

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

Table 3. Distribution of catalase electrophoretotypes at various growth stages

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 OD₆₀₀ ranges defining the various growth stages are indicated in Methods.

Strain	Genomovar	Early exponential	Late exponential	Early stationary	Late stationary
FC124	I	9	9	9	9
CEP509	I	1, 10	1, 10	1, 10	1, 10
ATCC 25416	I	N/B	N/B	1	1
CEP484	II	7	7	5, 11	5, 11
C0514	II	3, 11	3	N/B	3, 11
C5274	II	N/B	N/B	N/B	7, 11
C7062	II	N/B	N/B	4, 11	5, 11
ATCC 17616	II	N/B	11	5, 11	5, 11
CEP024	III	12	12	2, 12	12
CEP511	III	13	13	N/B	13
C1484	III	13	13	13	13
C4455	III	13	13	13	6, 13
C5424	III	13	13	13	13
CEP103	IV	N/B	N/B	8	8
FC473	IV	N/B	8	8	8
CEP559	IV	N/B	8	8	8
CEP040	V	N/B	N/B	13	13
CEP021	VI	11	11	11	11
L6	VI	11	11	11, 13	11, 13

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 electrophoretotypes (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 H₂O₂ 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 H₂O₂ to the samples, as the catalase activity rapidly produced bubbling, a sign of H₂O₂ degradation. To eliminate

Table 4. SOD activity in *B. cepacia* strains according to growth phase

Strain	Genomovar	Band profile*	SOD activity†	
			Early exponential‡	Late stationary§
CEP509	I	2	43·1	74·5
ATCC 25416	I	2	30·6	45·9
FC124	I	1, 2	52·6	85·5
C5274	II	2	24·2	69·1
C7062	II	2	22·3	168·2
C0514	II	2	35	54·7
CEP484	II	2	22·3	209·5
ATCC17616	II	2, 1	31·9	91
C4455	III	2, 1	35·2	100
CEP024	III	2	17·3	139
CEP511	III	1, 2	39·5	88
C1484	III	2	64	167
C5424	III	2, 1	81	191
FC473	IV	2, 1	41·2	91·3
CEP559	IV	2, 1	27	91·8
CEP103	IV	2, 1	37·7	78·6
CEP40	V	2	51	127
CEP021	VI	2	40·4	67
L6	VI	2	40	147
QC779	<i>E. coli</i>	ND	ND	6

* 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 (OD₆₀₀ 0·5–0·6).

§ Late stationary phase cells (OD₆₀₀ > 2·0).

catalase activity from our samples we took advantage of the information gathered regarding the types of catalase present in each of the isolates. Bifunctional catalase peroxidase enzymes were inactivated by incubation at 65 °C for 1 min while monofunctional enzymes were inactivated by treatment with 3-amino-1,2,4-triazole for 20 min (Marcinkeviciene *et al.*, 1995). Following catalase inactivation, samples were treated with 25 mM H₂O₂ for 20 min prior to loading into the gel, and then stained for SOD activity as described above. Control experiments indicated that neither heat treatment nor the addition of 3-amino-1,2,4-triazole affected the detection of SOD activity by native PAGE (data not shown). Addition of H₂O₂ following heat treatment and/or 3-amino-1,2,4-triazole exposure resulted in the inactivation of all SOD activity in the extracts from all the strains (Fig. 3, lanes 5–8, and data not shown). The inhibition with H₂O₂ was specific for Fe-SOD since the activity of an Mn-SOD from *E. coli* used as a control was not affected by the inhibitor (Fig. 3, lanes 1 and 2) while the activity of an Fe-SOD from *E. coli* was abolished (Fig. 3, lanes 3 and 4). Therefore, we concluded that the SOD form in all the *B. cepacia* strains

examined in this study under our experimental conditions corresponded to an Fe-SOD class.

Bacterial susceptibility to killing by H₂O₂ and superoxide anion

To ascertain whether the differential production of SOD and catalase activities by individual isolates has any biological significance, we evaluated the susceptibility of our isolates to both H₂O₂ and O₂^{·−} exposure. The results were expressed as percentage survival relative to the same bacterial inoculum in the absence of the reactive oxygen species. The level of resistance to killing by H₂O₂ was assessed by exposing bacterial cells to incremental concentrations of H₂O₂ ranging from 2·5 mM to 10 mM, as indicated in Methods. We first conducted pilot experiments with selected strains of each genomovar to determine the survival of cultures grown to exponential and stationary phase following H₂O₂ exposure. In all cases, survival increased dramatically in stationary-phase cultures (data not shown), in agreement with the data showing an increased enzyme activity at this growth stage (Table 2, Fig. 1b). There-

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 H_2O_2 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.

fore, the remaining experiments were conducted using cultures in stationary phase. Strains belonging to genomovar III showed a uniformly high level of resistance to H_2O_2 at all concentrations tested (Fig. 4). Survival of one strain in this group, C5424, was assayed at higher H_2O_2 concentrations, showing that it was capable of surviving in up to 100 mM H_2O_2 (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 H_2O_2 concentrations were those producing a bifunctional catalase/peroxidase. In contrast, only two of the strains producing a bifunctional catalase/peroxidase (FC473 and CEP559) showed poor survival following H_2O_2 exposure. These results suggest that in most cases a bifunctional catalase/peroxidase may be associated with increased resistance to H_2O_2 exposure. The genomovar III strain CEP511 exhibited anomalous behaviour in that its percentage survival was not proportional to the amount of H_2O_2 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 O_2^- exposure was assessed using the xanthine plus xanthine oxidase (X + XO) system and paraquat, which generate extracellular and intracellular O_2^- , respectively. As membrane bilayers are impermeable to O_2^- (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 O_2^- while a cytoplasmic enzyme would provide protection primarily against paraquat-generated O_2^- (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 O_2^- -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 O_2^- 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 O_2^- 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 genomovar 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

Fig. 4. Growth inhibition of *B. cepacia* complex strains exposed to H_2O_2 *in vitro*. Late stationary phase cells were treated with H_2O_2 at concentrations of 2.5 mM, 5 mM and 10 mM as described in Methods. Samples were plated in triplicate for colony counts and percentage survival was calculated relative to colony counts of untreated bacteria. Bars indicate standard deviations. Individual strains are identified only by their numbers as indicated in Table 1. Asterisks indicate the strains producing bifunctional catalase/peroxidases.

Fig. 5. Growth inhibition of *B. cepacia* complex strains exposed to superoxide anion. Late stationary phase cells were exposed to extracellular superoxide using the X+XO superoxide generating system (a) or 10 mM paraquat for generation of intracellular superoxide (b), as described in Methods. Samples were plated in triplicate for colony counts and percentage survival was calculated relative to colony counts of untreated bacteria. Bars indicate standard deviations. Individual strains are identified only by their numbers as indicated in Table 1.

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 *in vitro*, 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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