Characterisation of a Bi-functional Catalase-peroxidase of *Burkholderia cenocepacia*

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Running title: *Burkholderia cenocepacia* catalase-peroxidase

Key words: catalase-peroxidase, *Burkholderia cenocepacia*, cystic fibrosis

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Abstract

Isolates of *Burkholderia cenocepacia* express a putative haem-binding protein (M_r 97kDa) which displays intrinsic peroxidase activity. Its role has been re-evaluated, and we now show that it is a bi-functional catalase-peroxidase, with activity against tetramethylbenzidine (TMB), *o*-dianisidine, pyrogallol, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS). Both peroxidase and catalase activities are optimal at pH 5.5-6.0. The gene encoding this enzyme was cloned and expressed in *E. coli*. We have named it *katG* because of its similarity to other *katG*s including that from *Burkholderia pseudomallei*. It is substantially similar to a previously described catalase-peroxidase of *B. cenocepacia* (*katA*). Mass spectrometric analysis indicated that the initial *katG* translation product may be post-translationally modified in *B. cenocepacia* to give rise to the mature 97kDa catalase-peroxidase.

INTRODUCTION

Burkholderia cenocepacia (genomovar IIIa) isolates are associated with life-threatening lung infections that may progress to septicaemia ("cepacia syndrome") in cystic fibrosis (CF) patients [1]. These bacteria were shown to express a 97kDa putative haem-binding protein [2], but more recent studies [3] showed that this protein does not bind haem dose-dependently, and is peroxidase positive even without prior exposure to exogenous iron(III) protoporphyrin IX. These newer findings lead to the possibility that it is not a true haem-binding protein. In the present study, we re-evaluated the properties of the 97kDa protein and demonstrated it to be a bi-functional catalase-peroxidase. The gene encoding this protein was cloned and expressed in *Esherichia coli*, and a survey of its distribution in bacterial strains of the "*Burkholderia cepacia* complex" was undertaken.

Materials and Methods

Bacterial strains and growth conditions. *B. cenocepacia* clonal isolates strains BC7, C5424, C6433 and J2315 (expressing the 97kDa putative haem-binding protein) were maintained by subculture on horse-blood agar. For peroxidase and catalase studies the cells were sub-cultured three times on Columbia or M9 Minimal Salts Medium agar (Sigma Chemical Company) before growth in bulk on these solid media as lawn growths for 3 days. Cells were harvested into and washed twice in 0.14M NaCl, 0.1M Tris-HCl, pH 7.5 to remove any contaminating growth medium constituents. For molecular genetic studies, *E. coli* strains were grown in Luria-Bertani (LB) broth or agar, supplemented with 100µg ampicillin ml^{-1} where appropriate.

SDS-PAGE and staining for peroxidase and catalase activity. Cell samples of strain J2315 were solubilised in non-reducing sample buffer (37°C for 1h), electrophoresed on 10% acrylamide gels and stained with 3,3',5,5'-tetramethylbenzidine (TMB)/H_2O_2 [2]. Gels were counter-stained with Coomassie Blue [3] to precisely identify the positions of peroxidase bands. Chromogenic peroxidase substrates *o*-dianisidine (3,3'-dimethoxybenzidine), pyrogallol (1,2,3-trihydroxybenzene), 4-chloronaphthol, guaiacol (2-methoxyphenol), and ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (all at 6.3mM) were also tested using the same procedure as for TMB above. Catalase activity was detected in

samples as above using the $K_3Fe(CN)_6/FeCl_3$-H_2O_2 method of Katsuwon and Anderson [4], in which catalase-positive bands appear as clear zones against a dark brown background.

Effect of pH on the peroxidase and catalase activities. Replicate gel tracks of cell samples (strain J2315; 25µg protein) were incubated, after electrophoresis, in 0.14M NaCl buffered at pH 9.0, 8.5, 8.0, or 7.5 with 0.1M Tris-HCl, or at pH 7.0, 6.5, 6.0, or 5.5, buffered with 0.1M sodium acetate/acetic acid, for 1h. The gel strips were developed in $K_3Fe(CN)_6/FeCl_3$-H_2O_2 or TMB-H_2O_2 to reveal catalase and peroxidase bands, respectively.

pH-activity profiles for whole cell catalase and peroxidase. Peroxidase activity was measured by monitoring ΔA_{645nm} after incubation of cell suspensions (J2315; 250µg protein ml^{-1}) in the above buffers at 20°C with 6.3 mM TMB plus 10mM H_2O_2 [5]. Catalase activity was measured at 20°C in the above suspensions by monitoring ΔA_{240nm} [6], using 8mM H_2O_2 as substrate. Catalase and peroxidise activities were expressed as either pmole H_2O_2 degraded or pmole TMB oxidised per minute.

Protein separation and mass spectrometry. Protein bands from Coomassie Blue-stained 1D gels were excised and destained and dehydrated by incubation with 50% acetonitrile/50mM ammonium bicarbonate for 1h at room temperature, followed by vacuum drying in a SpeedVac (Eppendorf). The gel pieces were rehydrated in 50mM ammonium bicarbonate containing 40ng/µL modified trypsin (Promega) and incubated for 16h at 37^{o}C. Peptides were extracted from the gel by incubation with 2 changes of 60% acetonitrile/1% trifluoroacetic acid, and the resulting supernatants were again dried in a SpeedVac. The extracts were desalted using C18 ZipTips according to the manufacturer's instructions (Millipore), and were reconstituted in a final volume of 30µL 5% acetonitrile/0.1% trifluoroacetic acid. Aliquots of 0.5µL sample were spotted onto a MALDI target plate together with an equal volume of 5mg/mL α-cyano-4-hydroxycinnamic acid (LaserBiolabs, France) in 50% acetonitrile/0.1% trifluoroacetic acid. Peptide mass fingerprints were acquired either on a Voyager DE Pro MALDI (Applied Biosystems, California, USA) or a M@LDI (Micromass , Manchester, UK) instrument in positive ion reflector mode. Data were submitted for screening via the Mascot search engine (Matrix Science, London). The mass tolerance was set to 100ppm, and 1 missed cleavage and no

modifications were allowed. For LC-MS/MS analysis, aliquots of 5µL sample were delivered into a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems) by automated in-line liquid chromatography (integrated LCPackings System, 5mm C18 nano-precolumn and 75µm x 15cm C18 PepMap column (Dionex, California, USA)) via a nano-electrospray source head and 10µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 5% acetonitrile/0.05% TFA (v/v) to 48% acetonitrile/0.05% TFA (v/v) in 60mins was applied at a flow rate of 300nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA) (Analyst, Applied Biosystems). Database searching was carried out using Mascot with mass tolerances set to 1.2Da for MS and 0.6Da for MS/MS, and with deamidation as a variable modification.

Cloning of the catalase-peroxidase (*katG*) gene from *B. cenocepacia* strain J2315. Genomic DNA was amplified by PCR using the primers CACCATGTCGAACGAAGGGCAGT and CGATGTACCACCGCTTTT. PCR was performed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles each of 1 min at 94°C, 1 min at 58°C, 3.5 min at 72°C, and a final extension step at 72°C for 5 min. PCR products were electrophoresed on a 1% (w/v) agarose gel and visualised using ethidium bromide to confirm a single product of the correct size before cloning into pET-100/D-TOPO (Invitrogen) to produce pKatG and transformation of One Shot® TOP 10 chemically competent *E. coli* cells (Invitrogen). Successful insertion of the gene in the correct orientation was confirmed by restriction analysis.

Sequencing of *katG*. The *katG* was sequenced from pKatG plasmid DNA using multiple overlapping IRD-700-labelled forward and reverse primers (MWG-Biotech Ltd., UK; details on request) and employing the SequiThermEXCEL™ II DNA Sequencing Kit-LC (Epicentre Technologies) in a LI-COR 4200S auto-sequencer. The sequencing data were viewed using the LI-COR Base ImagIR™ software in conjunction with the Sequencher programme (http://www.genecodes.com).

Expression analysis. BL21 Star™ One Shot *E. coli* cells were transformed with the pKatG plasmid and induction of expression was undertaken by growth at 37°C in the presence of 0.1M isopropyl β-D-thioglucopyranoside (IPTG; Sigma). Pelleted cells were electrophoresed as above and assessed for catalase and peroxidase activity by in-gel staining.

Results

As previously reported [2], a major peroxidase-positive protein of 97kDa was seen for *B. cenocepacia* strain J2315 (Fig. 1, tracks b and h). The 97kDa enzyme also showed peroxidase specificity towards *o*-dianisidine, ABTS and pyrogallol (Fig. 1, tracks c, e, and g), but guaicol (methoxyphenol) and 2-chloronaphthol were not peroxidised (Fig. 1, tracks d and f). Using in-gel $K_3Fe(CN)_6/FeCl_3/H_2O_2$ staining, the 97kDa peroxidase protein was also shown to display catalase activity (Fig 1, track a), a phenomenon also demonstrated by the clonal isolates BC7, C5424 and C6433 (data not shown). The catalase activity of the 97kDa enzyme of strain J2315 was not inhibited by pre-exposure of the cells for 1h to the specific mono-functional catalase inhibitor 3-amino-1, 2, 4-triazole (20mM). Peroxidase activity against substrates TMB, ABTS and *o*-dianisidine was also unaffected by the inhibitors isonicotinic acid and niacinamide (all 20mM) [8] (data not shown). In general, the catalase activity visualised by the in-gel assay was stronger than the peroxidase staining.

Both catalase and peroxidase activities were observed in SDS-PAGE gels over the pH range 5.5 to 8.5, whilst little or no activity was seen at pH 9.0 (Fig. 2). Both enzyme activities were generally low at alkaline pHs, and highest over the acid pH range, and scanning densitometry confirmed maximal catalase and peroxidase activities at pH 6.0 and 5.5, respectively. Peroxidase activity of suspensions of whole cells of strain J2315 against TMB was not detected at neutral or alkaline pHs, but maximal activity was seen at pH 6.0 (Fig. 3a). In contrast, low levels of catalase activity of whole cells were observed at alkaline pH, rising in the acid pH range to a maximum at pH 6.0 (Fig 3b).

Masses of tryptic peptides of the 97kDa catalase-peroxidase were obtained by MALDI-TOF mass spectrometry and matches were obtained to the KatG catalase-peroxidases of *Burkholderia pseudomallei* [9] and *Mycobacterium tuberculosis* [10], and to an archeal catalase-peroxidase of *Haloarcula marismortui* [11]. This genomic data was aligned with the sequence of the J2315 strain (http://www.sanger.ac.uk/Projects/B_cenocepacia/) and used to design primers to a putative open reading frame (ORF) of 2211 bases (768 amino acids) on chromosome 2 which was cloned and re-sequenced (accession number DQ112341). This ORF was subsequently identified in the published *B. cenocepacia* genome (Sanger Institute) and was calculated to have a size of 80.5kDa. A second ORF was also

identified at 3612912-3615098 on chromosome 1, which possessed 73% identity and 81% similarity to the first catalase-peroxidase gene at the amino acid level and may represent another catalase-peroxidase protein.

Paired amino acid alignments (BLASTP) revealed a high degree of homology between the *B. cenocepacia* catalase-peroxidase and the minor catalase-peroxidase described by Lefebre *et al.* [12], those of other selected bacterial species including the cell-surface catalase-peroxidase of *B. pseudomallei* (KatG), and the 77kDa iron (III) protoporphyrin IX monomer binding protein (accession number DQ114424; Smalley *et al.*, 2005) (Table 1). Multiple sequence alignment analysis using CLUSTALW revealed striking similarities between the *B. cenocepacia* catalase-peroxidase and the other selected enzymes in both the C- and N-terminal regions. These included the conserved amino acid triad Arg^{104}-Trp^{107}-His^{108}, and the second haem ligand (His^{270}) of *M. tuberculosis* KatG [13]. Because of the similarities of the catalase-peroxidase to these well-characterised KatG proteins, the gene encoding the *B. cenocepacia* enzyme was named *katG*.

IPTG induction of the *E. coli* BL21Star cells carrying the 80.5kDa catalase-peroxidase gene resulted in a protein product which electrophoresed on 10% gels as a single band with an apparent molecular mass of ~ 80kDa (Fig 4). The recombinant protein stained positively for both peroxidase and catalase showing the gene product to be functionally active (Fig 4). To confirm the identity of the recombinant enzyme expressed in *E. coli* as the product of *katG*, MS/MS analysis was performed after SDS-PAGE and trypsin digestion. On 7% acrylamide gels it was found that the recombinant protein was separated into two bands with calculated molecular masses of 79 and 83kDa, denoted R1 and R2, respectively (Fig 5), both of which were positive for peroxidase (data not shown). The observation of the band R2 is in keeping with the expected size of an initial translation product based on the vector system employed which results in the addition of 36 amino acids to the N-terminus of the expressed protein. We speculate that the lower molecular weight band R1 arises as a result of proteolytic cleavage of the initial translation product. In addition to proteins R1 and R2, a very faint Coomassie Blue stained band of approximately 97kDa (denoted R3) was observed which was not expressed by *E. coli* cells carrying the empty plasmid. This band was peroxidase positive as revealed by TMB staining for a longer time period (data not shown). MS/MS analysis of these three proteins showed them to match *B. cenocepacia* KatG (Table 2). Taken

together these data confirmed that the product of *katG* was the bifunctional catalase-peroxidase. The presence of a higher molecular weight form of the enzyme suggests that the initial translation product may be post-translationally modified to give the mature 97kDa catalase-peroxidase.

Discussion

We have re-evaluated the role of the 97kDa putative haem-binding protein of *B. cenocepacia* [2]. This protein shows peroxidase specificity towards tetramethylbenzidine, *o*-dianisidine, pyrogallol, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), but not against 4-chloronaphthol and 2-methoxyphenol (guaicol). It also has catalase activity, but is not inhibited by the specific mono-functional catalase inhibitor 3-amino-1,2,4,-triazole. This protein does not show dose-dependent binding of iron(III) protoporphyrin IX in either the monomeric or µ-oxo oligomeric form [3], and does not bind to haem-agarose (Smalley et al., unpublished findings). Collectively, these data show this component is not a true haem-binding protein, but a bi-functional catalase-peroxidase, in contrast to the 77- and 149kDa iron(III) protoporphyrin IX-binding, outer-membrane components which do not possess intrinsic catalase activity, and which are only peroxidase-positive after exposure to, and binding of, iron(III) protoporphyrin IX monomers [3].

Multiple amino acid alignment analysis of the translated *B. cenocepacia* catalase-peroxidase gene revealed a strong homology with other bacterial catalase-peroxidases, and supported the above biochemical observations. It possessed the Arg^{88}-Trp^{91}-His^{92} triad which is conserved among all known catalase-peroxidases [14], and displayed the greatest cross-species amino acid homology (70.7% identity and 79% similarity) to that of *B. pseudomallei* KatG, a homo-dimer of subunit size 81.6kDa [15, 16], which plays a role in protecting against hydrogen peroxide [17]. For this reason, the *B. cenocepacia* catalase-peroxidase gene was named *katG*.
A catalase-peroxidase gene *katA* (accession number AF317697) has recently been described in *B. cenocepacia* strain C5424 by Lefebre *et al.* [12], which is similar to the gene identified herein.

MS/MS analysis clearly demonstrated that the recombinant catalase-peroxidase was the product of *katG*. The detection of a higher molecular weight enzyme matching KatG shows that expression of *katG* in *E. coli* is also accompanied

some post-translational processing and suggests that this step may be more efficient in *B. cenocepacia*, giving rise to the mature 97kDa catalase-peroxidase. At present however, the nature of any post-translational modifications is unclear. The electrophoretic mobility of the native 97kDa enzyme from *B. cenocepacia* does not change upon reduction with dithiothreitol, nor does it react with phosphoprotein stains or periodic acid-Schiff reagent (data not shown).

Recent *B. cenocepacia* J2315 sequence database releases indicate that the katG gene (BCAM2107) may actually be extended by 20 amino acids at the N terminus to give a 756 amino acid, 82.6kDa, protein. This, together with other sequence differences that we have noted between our data, the *B. cenocepacia* J2315 database sequence and the *katA* gene of Lefebre *et al.* [12], may indicate that the ORF is not yet correctly identified. Lefebre *et al*. [12] also demonstrated a second catalase-peroxidase gene in *B. cenocepacia* J2315. We confirm the presence of this second gene (BCAL3299) and observe that it is 73% identical and 81% similar to our *B. cenocepacia* katG at the amino acid level.

B. cenocepacia katA mutants are sensitive to H_2O_2 and *katA* also appears to contribute to the normal functioning of the TCA cycle [12], but the extent to which the catalase-peroxidase described herein contributes to growth and survival *in vivo* is not clear. Although the pH of the liquid surface layer of the lung in health is $\approx$ 6.9 [18], endo-bronchial pHs of around 6.5 have been recorded. In addition, respiratory mucins, to which *B. cenocepacia* binds specifically [19], are highly sulphated, especially those produced by CF patients [20, 21, 22], and this also contributes to the acidity of the secretions. The pH is also reduced as a result of defective transmembrane conductance regulator function [23] and the mucopurulent secretions formed in the CF lung during infection also have an acid pH [24, 25]. In view of the above and the acid pH optima of the catalase-peroxidase, it is likely that bacterial cells expressing this enzyme would be advantaged in enduring attack by macrophage-derived H_2O_2 in the slightly acidic conditions prevailing in the lung during chronic infection and inflammation. Although members of the "*B. cepacia* complex" display catalase and peroxidase activities [8], we have generally found that these activities in other species of the complex are very low compared to *B. cenocepacia* strains [26]. Bacterial catalase-peroxidases display wide substrate specificities [14, 27], but it is not known which compounds represent natural peroxidase substrates for the *B. cenocepacia* enzyme or whether it plays any role in

attacking and degrading other host (macro)molecules for defensive or nutritive purposes.

ACKNOWLEDGEMENT

We would like to thank the Cystic Fibrosis Trust (Grant number CF RS 22) and the MRC Proteomics Initiative for financial support.

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Table 1

Amino acid homology analysis of the *B. cenocepacia* catalase-peroxidase KatG (GenBank accession DQ112341) with bacterial and archaeal catalase-peroxidases using paired alignment comparisons performed in BLASTP. Accession numbers are from GenBank (gb) or Swiss-Prot (sp).

Organism	Accession no.	Reference	% Identity	% Similarity
Haloarcula marismortui	gbY16851	Cannac-Caffrey *et al*. [11]	56.8	69.4
Halobacterium salinarum	gbAF069761	Long & Salin [28]	56.4	68.9
Mycobacterium bovis	spP46817	Heym *et al*. [29]	60.0	71.8
Mycobacterium fortuitum	gbY07865	Menendez *et al*. [30]	58.1	65.9
Mycobacterium intracellulare	spQ04657	Morris *et al*. [31]	58.6	69.3
Mycobacterium tuberculosis	spQ08129	Heym *et al*. [32]	59.9	71.6
Escherichia coli	spP13029	Triggs-Raine *et al*. [33]	59.9	71.8
E. coli (0157: H7)	gbX89017	Brunder *et al.* [34]	55.2	66.2
Legionella pneumophila	gbAF078110	Bandyopadhyay & Steinman [35]	57.1	69.3
Salmonella typhimurium	spP17750	Loewen & Stauffer [36]	60.6	71.9
Streptomyces reticuli	gbY14317	Zou *et al.* [37]	64.0	74.0
Yersinia pestis	gbAF135170	Garcia *et al*. [38]	55.6	67.0
Burkholderia pseudomallei	gbAAK72466	Loprasert *et al.* [37]	70.7	79.0
Burkholderia cenocepacia	gbDQ114424	Smalley *et al.* (2005)	71.0	79.0
Burkholderia cenocepacia	gbAF317697	Lefebre *et al.* [12]	94.0	94.0

Table 2

Masses of tryptic peptides derived from the recombinant protein. Analysis was performed with one missed cleavage allowed and deamidation as the only variable modification. A mass tolerance of 1.2Da was allowed for MS and of 0.6Da for MS/MS analysis. R1, R2, and R3 refer to the arrowed bands on Figure 5; m/z, mass/charge; Mr(calc.), calculated relative molecular masses of the sequenced tryptic peptides; (**D**), deamidation.

Protein	**Position**	**m/z**	**Mr(calc.)**	**Sequence**
	29 - 38	564.3347	1126.5982	R.LDLLSQHSSK.T
	39 - 61	1277.6446	2553.2023	K.TDPLDPGFNYAEAFNSLDLDALR.K
	273 - 299	901.4813	2701.3095	K.THGAGPADNVGLEPEAAGLEQQGLGWK.N
R1	333 - 343	700.3453	1398.6819	K.NLFGYEWELTK.S
Overall	383 - 389	449.2351	896.4279	R.FDPVYEK.I
Mascot	520 - 532	681.8758	1361.6575	R.IQGEFNSTQPGGK.K
Score =	534 - 553	955.5686	1909.0883	K.ISLADLIVLAGGAGIEQAAK.R
875	555 - 567	661.3781	1320.6938	R.AGHDVVVPFAPGR.M
	598 - 609	643.9088	1285.7281	K.FAVPAEALLIDK.A
	639 - 655	1005.0381	2007.9690	K.HGVFTDQPETLTVDFFR.N
	690 - 701	645.8812	1288.7139	R.VDLVFGSNAVLR.A
	702 - 715	705.8608	1409.6674	R.ALSEVYASADGEAK.F
	29 - 38	564.3241	1126.5982	R.LDLLSQHSSK.T
	39 - 61	1277.6765	2553.2023	K.TDPLDPGFNYAEAFNSLDLDALR.K
	113 - 127	851.9246	1701.8362	R.FAPLNSWPDNVSLDK.A
R2	273 - 299	901.4947	2702.2935	K.THGAGPADNVGLEPEAAGLEQQGLGWK.N (**D**)
Overall	333 - 343	700.3926	1398.6819	K.NLFGYEWELTK.S
Mascot	383 - 389	449.2540	896.4279	R.FDPVYEK.I
Score =	534 - 553	955.5824	1909.0883	K.ISLADLIVLAGGAGIEQAAK.R
871	555 - 567	661.3666	1320.6938	R.AGHDVVVPFAPGR.M
	598 - 609	643.8862	1285.7281	K.FAVPAEALLIDK.A
	639 - 655	1005.0239	2007.9690	K.HGVFTDQPETLTVDFFR.N
	690 - 701	645.8698	1288.7139	R.VDLVFGSNAVLR.A
	702 - 715	705.8490	1409.6674	R.ALSEVYASADGEAK.F
	29 - 38	564.3127	1126.5982	R.LDLLSQHSSK.T
	39 - 61	852.4210	2553.2023	K.TDPLDPGFNYAEAFNSLDLDALR.K
	253 - 272	1032.0497	2060.9659	R.MAMNDEETVALIAGGHAFGK.T
	273 - 299	901.4964	2701.3095	K.THGAGPADNVGLEPEAAGLEQQGLGWK.N
R3	355 - 368	757.3829	1512.7321	K.NAEPTIPHAHDPSK.K
	370 - 382	737.4313	1472.8272	K.LLPTMLTTDLSLR.F
Overall	393 - 407	876.8862	1751.7725	R.HFMDNPDVFADAFAR.A
Mascot	520 - 532	681.8408	1361.6575	R.IQGEFNSTQPGGK.K
Score =	534 - 553	955.5983	1909.0883	K.ISLADLIVLAGGAGIEQAAK.R
964	555 - 567	661.3550	1320.6938	R.AGHDVVVPFAPGR.M
	598 - 609	643.8859	1285.7281	K.FAVPAEALLIDK.A
	610 - 628	977.5799	1953.1081	K.AQLLTLTAPQMTALVGGLR.V
	639 - 655	1004.9840	2008.9530	K.HGVFTDQPETLTVDFFR.N (**D**)
	690 - 701	645.3592	1288.7139	R.VDLVFGSNAVLR.A
	702 - 715	705.8491	1409.6674	R.ALSEVYASADGEAK.F

Fig 1

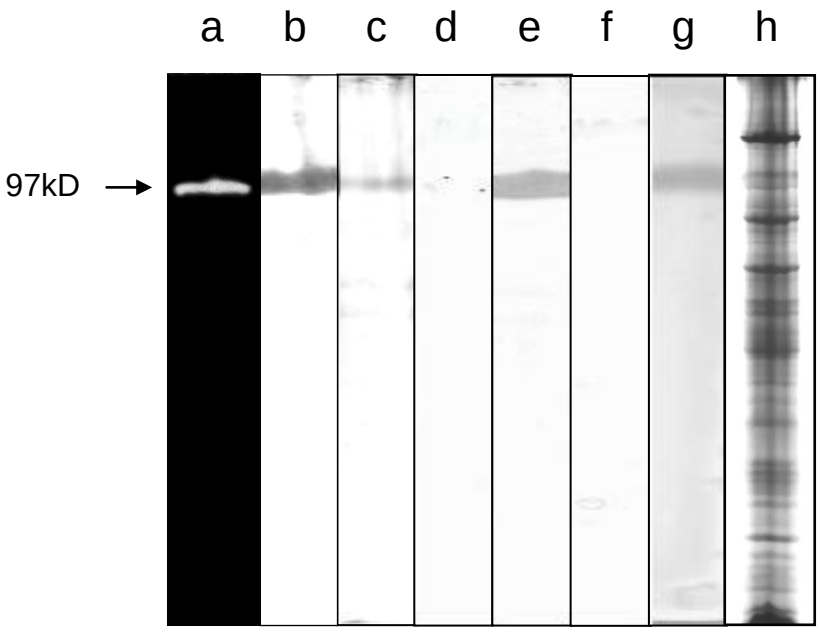

Fig 2

Fig 3

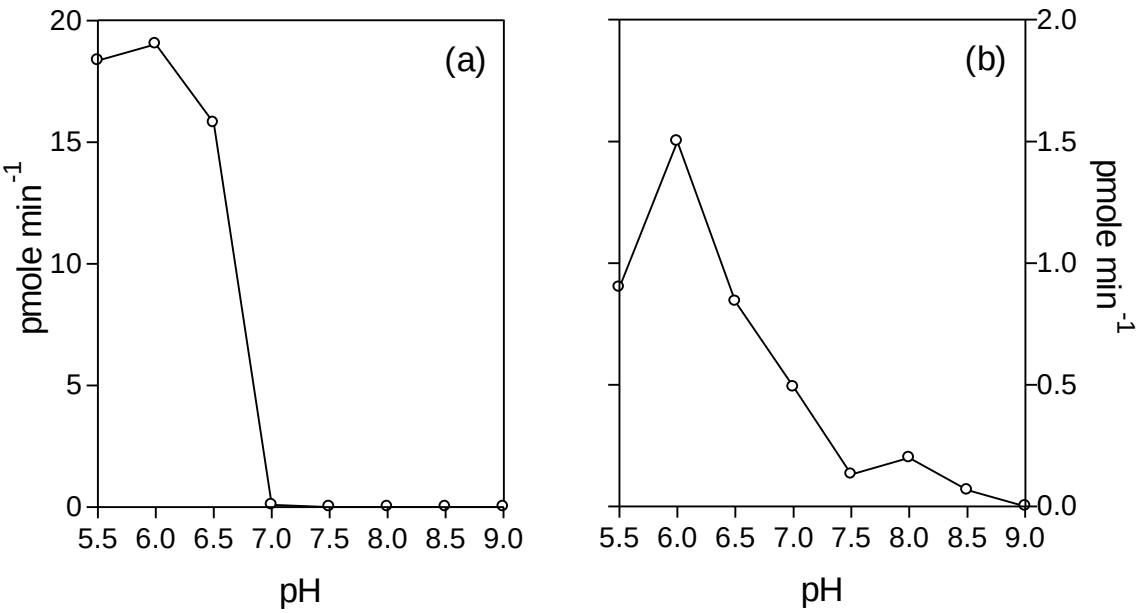
(a)
(b)
pmole min-1
pmole min-1
pH
pH
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0
5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0

Fig 4

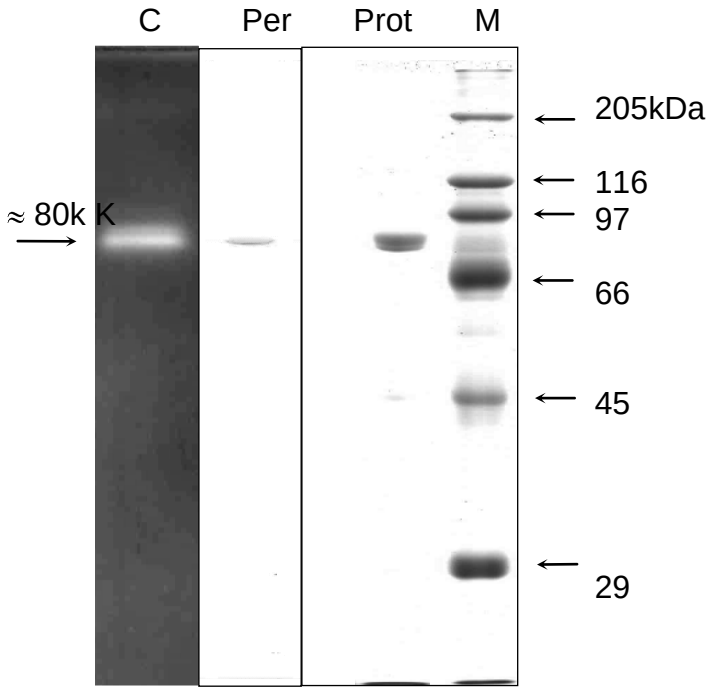

Fig 5

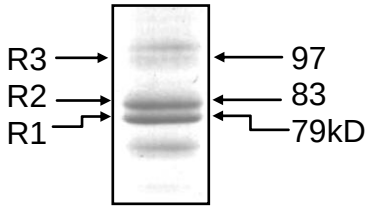

Figure legends

Figure 1: Catalase and peroxidase specificity of the 97kDa protein of *B. cenocepacia* strain J2315 as shown after SDS-PAGE under non-reducing conditions. Catalase activity was demonstrated using the potassium ferricyanide/ ferric chloride-H_2O_2 method (track a), whilst peroxidase activity was assessed against (b) TMB, (c) *o*-dianisidine, (d) 2-methoxyphenol, (e) ABTS, (f) 4-chloronaphthol, and (g) pyrogallol. Track h, Coomassie Blue counterstaining for protein following the peroxidase reaction. See Methods for details.

Figure 2: Effect of pH on the activity of the bi-functional catalase-peroxidase as shown after SDS-PAGE under non-reducing conditions. Peroxidase activity (a) was revealed using tetramethylbenzidine-H_2O_2 whilst catalase was assayed using the $K_3Fe(CN)_6/FeCl_3$-H_2O_2 staining method (b). Gel loadings were 25µg protein per track.

Figure 3: pH activity profile of catalase and peroxidase activities of suspensions of whole cells of *B. cenocepacia* strain J2315 grown on M9 Minimal Salts Medium agar. Peroxidase was measured using tetramethylbenzidine as substrate (shown as ΔA_{645nm}). Catalase activity was assayed by UV absorbance (shown as ΔA_{240nm}). The reactions were carried out at 20°C.

Fig 4: SDS-PAGE on 10% polyacrylamide gels of BL21Star *E. coli* cells expressing the catalase-peroxidase, after growth in the presence of 0.1M IPTG. C, catalase; Per, peroxidase; Prot, protein staining; M, molecular weight markers. The in-gel catalase staining was performed at pH 6.0 in 0.5M sodium acetate.

Fig 5: SDS-PAGE of the recombinant *B. cenocepacia* KatG catalase-peroxidase enzyme on a 7% polyacrylamide gel. Recombinant protein bands R1, R2 and R3 gave mass matches to KatG after trypsinisation and MS/MS analysis (see Table 2). The gel was stained with Coomassie Blue.