Molecular characterization of *katA* from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange

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A gene encoding catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6) from *Campylobacter jejuni* was cloned by functional complementation of a catalase-deficient mutant of *Escherichia coli*. The catalase structural gene, designated *katA*, was assigned by subcloning and its nucleotide sequence determined. The deduced protein product of 508 amino acids, which had a calculated molecular mass of 58346 Da, was found to be structurally and enzymically similar to hydrogen-peroxidases from other bacterial species. The region of DNA containing the structural catalase gene was disrupted by insertion of a tetracycline-resistance marker and the modified sequence then introduced into a strain of *Campylobacter coli* via natural transformation. Genetic and enzymic analyses of a tetracycline-resistant *C. coli* transformant confirmed that catalase-deficient mutants had arisen via interspecific allelic exchange. Compared to the isogenic parental strain the mutant was more sensitive to killing by H_2O_2 .

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, catalase, *katA*, gene replacement

INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are now considered to be the major bacterial cause of human diarrhoeal disease in Britain and probably the developed world (Tauxe, 1992). These Gram-negative, spiral-shaped bacteria are described as being microaerophilic and generally require low levels of O_2 and increased CO_2 concentrations for their growth. The inability of *C. jejuni* and *C. coli* to grow in air is thought to be related to their hypersensitivity to the toxic products of O_2 metabolism (Hoffman *et al.*, 1979a, b). Clearly, the lack of tolerance of these organisms towards O_2 must place serious constraints upon their survival in foods and in environments where they are likely to encounter oxidative stress. However, it is apparent that both *C. jejuni* and *C. coli* are able to survive in food in sufficient numbers to cause infection since this is the main vehicle of their transmission (Griffiths & Park, 1990; Humphrey, 1992). In this context, there are now a number of reports describing aerotolerance in *Campylobacter* spp. (Vercellone *et al.*, 1990; Jones *et al.*, 1993) and

it has even been suggested that *Campylobacter* can adapt to an aerobic metabolism (Jones *et al.*, 1993). It is obvious that prolonged aerobic survival of campylobacters, or indeed any organism, must involve mechanisms through which they are able to eliminate the deleterious effects caused by the generation of toxic O_2 derivatives. In this respect catalase, which catalyses the dismutation of H_2O_2 to H_2O and O_2 , is considered to be part of a defence system against oxidative stress and, accordingly, is present in most aerobic organisms. In addition, because macrophage killing is associated with a respiratory burst that involves the generation of H_2O_2 and superoxide, the antioxidative effects of catalase are thought to make an important contribution to the survival of several bacterial pathogens during infection (Mandell, 1975; Haas & Goebel, 1992).

As a first step to defining the role of catalase in the survival of *C. jejuni* and *C. coli* under conditions of oxidative stress, we describe the cloning and phenotypic expression of the gene (*katA*) encoding catalase, from *C. jejuni*, in *Escherichia coli*. In addition, a copy of the *C. jejuni* structural catalase gene, disrupted by the insertion of an antibiotic marker, was used to transform a strain of *C. coli* to a catalase-negative phenotype demonstrating the oc-

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currence of allelic exchange between DNA originating from two separate species of *Campylobacter*. In order to assess the role of catalase in the oxidative stress resistance of *Campylobacter* spp., the *C. coli* catalase-deficient mutant was compared with the isogenic parental strain with respect to their sensitivity to H_2O_2 .

METHODS

Bacterial strains and media. *Campylobacter jejuni* NCTC 11351 and other strains of *C. jejuni* were obtained from the National Collection of Type Cultures (Colindale) and grown microaerophilically as described previously (Purdy & Park, 1994). The strain *Campylobacter coli* UA585 was a generous gift from D. E. Taylor (University of Alberta, Edmonton, Canada). *E. coli* UM255, a catalase double mutant, was kindly provided by P. C. Loewen (University of Manitoba, Canada). *E. coli* strains were grown at 37 °C on LB agar supplemented with 100 µg ampicillin ml^{-1} when necessary.

Construction and screening of a *C. jejuni* genomic library. Chromosomal DNA from *C. jejuni* was extracted according to the method of Pitcher *et al.* (1989) and partially digested with *Hind*III. Fragments of 3–7 kb in size were purified from an agarose gel and ligated into the cloning vector pTZ19R (Pharmacia) using standard procedures (Maniatis *et al.*, 1982). The library was introduced into *E. coli* UM255 by electroporation (Dower *et al.*, 1988) and cells plated on to LB agar supplemented with ampicillin. Transformants were screened for catalase activity by flooding the plates with 3% H_2O_2 and looking for colonies producing bubbles of O_2 . Colonies exhibiting catalase activity were then rapidly transferred to fresh agar plates.

DNA analysis. Plasmid DNA from colonies containing the recombinant catalase gene was isolated by miniprep and the gene assigned by subcloning (Maniatis *et al.*, 1982) to a recombinant plasmid termed pCKG2, which contained a 1.8 kb *Hind*III DNA fragment derived from *C. jejuni*. To facilitate nucleotide sequencing the insert in pCKG2 was cut into three smaller fragments with *Eco*RI and ligated into pTZ19R. DNA sequencing was performed on an Applied Biosystems model 373A DNA sequencing system. A Taq Dye Deoxy Terminator Cycle Sequencing kit (ABS) was used to generate fluorescence-based dideoxysequence reactions containing pCKG1 and its derivatives as template. Primers were designed to bind to opposing strands at approximately 200 bp intervals to allow sequence data to be obtained from both strands separately. Nucleic acid and protein and sequence data were analysed using the Wisconsin molecular biology software package (Devereux *et al.*, 1984).

Protein extraction and enzymic analysis. In order to characterize the protein product of the cloned *C. jejuni* catalase gene and the enzymic activity of a *C. coli* catalase-deficient mutant, total cell proteins, prepared as described previously (Purdy & Park, 1994), were analysed by SDS-PAGE (Laemmli, 1970). To demonstrate the expression of catalase activity protein samples were resolved on 7.5% (w/v) polyacrylamide gels, under non-denaturing conditions, and then assayed for catalase activity *in situ* using the ferricyanide-negative stain described by Woodbury *et al.* (1971). Staining for 3,3'-diaminobenzidine-peroxidase activity was as described by Wayne & Diaz (1986).

Allelic exchange and natural transformation. The *katA* sequence present in pCKG2 was disrupted by the introduction of a 2.3 kb *Bgl*II tetracycline-resistance cassette (Dickinson *et al.*,

1995) into the unique *Bgl*II site at position 1145 (Fig. 1). The resulting plasmid, pCKG3, was introduced into *C. coli* UA585 by natural transformation (Wang & Taylor, 1990). Transformants were recovered after 48 h following microaerophilic incubation at 37 °C on Mueller–Hinton agar (MHA) containing 10 µg tetracycline ml^{-1} .

DNA/DNA hybridizations. Genomic DNA, isolated from both wild-type and a catalase-deficient mutant of *C. coli*, was digested with various enzymes and electrophoresed on a 0.7% agarose gel. Following depurination with 250 mM HCl, the DNA was transferred from the gel to a Hybond N+ nylon membrane (Amersham) using 0.4 M NaOH. Hybridizations were performed using a non-radioactive ECL gene detection kit (Amersham) according to the manufacturer's instructions using the purified insert from pCKG2 as a gene probe.

H_2O_2 sensitivity assay. Cell suspensions, prepared from overnight cultures of *C. coli* UA585 or CK100 on agar plates, were inoculated into pre-warmed Mueller–Hinton broth to an $OD_{600} = 0.3$. H_2O_2 was added to a final concentration of 1 mM and cells incubated microaerophilically at 37 °C. During the course of the experiment the ability of cells to replicate was assessed by plating diluted aliquots onto MHA plates containing bovine catalase (100 U ml^{-1}). At the same time the concentration of H_2O_2 remaining in the media was determined using the peroxidase/phenol red assay (Pick & Keisari, 1980).

RESULTS

Cloning of *katA* from *C. jejuni* by complementation of *E. coli* UM255

Phenotypic complementation of the *E. coli* double catalase mutant UM255 (Mulvey *et al.*, 1988) was used as a screen to identify derivatives of a *C. jejuni* genomic library that contained potential catalase genes. Screening of approximately 5000 transformants yielded eight clones which produced bubbles of O_2 when exposed to 3% H_2O_2 . All eight of these derivatives were later found to contain an identical 4 kb insert of *C. jejuni* DNA (data not shown). To facilitate further characterization of the putative catalase gene, one derivative, designated pCKG1, was digested with *Hind*III, and the resulting fragments ligated into pTZ19R. The recombinant plasmids were transferred into *E. coli* UM255 and the transformants screened for catalase activity. In this manner, the gene encoding the putative catalase was assigned to a 1.8 kb *Hind*III fragment present in a plasmid designated pCKG2.

Nucleotide sequence analysis of the *katA* gene

The nucleotide sequence of the 1.8 kb fragment of *C. jejuni* DNA in pCKG2 was determined. This sequence was found to contain an untruncated ORF. It was assumed, therefore, that the *Hind*III site used to generate this fragment was internal to the 3' end of the catalase gene. Consequently, the sequence of the 3' terminus of the gene was determined using pCKG1 as a template as this plasmid was predicted to contain an intact ORF. Accordingly, the ORF in pCKG2 was found to extend beyond a *Hind*III site, present at position 1838, and internal to the termination codon for the intact gene (Fig. 1). The region of DNA encoding the catalase activity was

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atacagcaacttacactctttttgcacaaactaatggaattttacgctttttagcttcatttttgattttttcatagattattgatttaaaagcttcttaacattactatcacatc 120
tgatcacaggaatcgggttgcggttaactctattttcattacgcgcacccagtggtctctatttttttagcacctaaatcatgtaaaacagctctaataggagtgatttcatctgcacc 240
-----g--a--c--t--c
aataacaaaactgcataaattctcctttgtgatttttaaatatattttgatattattatataataaaaaaactaatatttttgaattttatcatcaataaaatgcaattat 360
-10 SD
tcagtttaatttttaattttatagatataatttactagtttaataaaattttatttttagcagaaaacaatgaaaaaataactaacgatttttgaaacattatagcogataatcaaaatt 480
M K K L T N D F G N I I A D N Q N S
cattaagtgcagggcacaagaagaccttttacttatgcaagattatctttttgctgaaaaaactgtcatcaaatagagaagaattccagaagaacccgttcattgctaaagggaagtggag 600
A H Q N R E R I P E R T V H A K G S G A L S A G T K G P L L M Q D Y L L L E K L
cttatggcgaataaaaatcactgctgatttatctgcttatactaggcaaaatatttcaaaaaggagaatcaactcctctttttctacgcttttcaacagtgcagctgaagcaggt 720
Y G E I K I T A D L S A Y T K A K I F O K G E I T P L F L R F S T V A G G E A G A
cagcagatgctgaacgcgagctgagaggttttctgatttaattttacacaaaagaaggaaactgggacttggtaggaaataaactccgacattctctcogtgatgcttataaatttc 840
A D A E R D V R G F A I K F Y T K E G N W D L V G N N T P T F F I R D A Y K F P
ctgatttccactactcaaaaagagatccaagaactcatcaagaagtaataatgctgctgggatttttggagtttatgtcctgaaagtttcatcaagtaaccattcttatgagag 960
D F I H T Q K R D P R T H L R S N N A A W D F W S L C P E S L H Q V T I L M R D
atagaggaattcctgcaagttatcgtcatatgcatggatttgaagccatacttattagttttattaatgataaaaaatgaaagattttgggtgaaattccatttttaaaaccaacaaggga 1080
R G I P A S Y R H M H G F G S H T Y S F I N D K N E R F W V K F H F K T Q Q G I
ttaaaaatcttaccacaagaagctgcccagcttatagcaaaagatagagaagctcatcaaaagagatctctataatgctatagaanaatgaagattttccaaatggaagttcaagttc 1200
K N L T N O E A A E L I A K D R E S H Q R D L Y N A I E N K D F P K W K V Q V Q
aaattctgtgaaaaagatataaaaaaacttggatttattccttttgatttaacaaaaatttggcctcatagctctgtgctttgatggatataaggcgaatgattctaacaaaaatc 1320
I L A E K D I E K L G F N P F D L T K I W P H S L V P L M D I G E M I L N K N P
ctcaaaattatttttaagagttgaacaagctgcttttagtccaagcaatctgttccctggaattggcttttagcctgataaaaattgttgaagctagaattttttcatatcctgatgcac 1440
Q N Y F N E V E Q A A F S P S N I V P G I G F S P D K M L Q A R I F S Y P D A Q
aaagatatagaataggaactaattatcatcttttgcctgaaatcgtgcaaaaagogaagtgaaactactcaatgtcgtgctgctatgaatttttgatagttataaaaaatgatgcagctt 1560
R Y R I G T N Y H L L P V N R A K S E V N T Y N V A G A M N P D S Y K N D A A Y
attatgaacaaacagctatgatataatagcccaaaaagaagcaaaagctatcttggaactgatttagtcttagaaggcgtagccacaagatagctccactagataatgacttttatactc 1680
Y E P N S Y D N S P K E D K S Y L E P D L V L E G V A Q R Y A P L D N D F Y T Q
aaaccaagagctttatttattatgaatgatgatcaaaaactcaactttttcataatagccactctctatggagggaattgagtaaaaaattatcactagagctttagaacattttg 1800
P R A L F N L M N D Q K T Q L F H N I A T S M E G V D E K I I T R A L E H F E
aaaaaattccactgatatacaaaaaggaattaaaaaagcttccctatagtgagctgtattagagcttggcgtaatcatggtcatagctgttctctgtgaaatttgttatccgtcac 1920
K I S P D Y A K G I K K A F P I V S R I R A W R N H G H S C F L C E I V I R S Q
aatccacacacatcagagcgggaagcataaagtgtaaagcctggggtgctaatga
F H T T Y E P E A *

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Fig. 1. Nucleotide sequence of the *C. jejuni* NCTC 11351 *katA* gene. The nucleotide sequence of a 1978 bp region of pCKG1 is shown. The predicted amino acid sequence is shown below the nucleotide sequence and the termination codon is represented by an asterisk. A potential ribosome-binding site (SD) and a putative -10 promoter region are underlined. The region which shows high homology to the Fur protein binding sequence is also shown above the nucleotide sequence. Conserved nucleotides are represented by dashes. Recognition sites for the restriction enzyme *HindIII* are underlined.

found to contain an ORF of 1521 bp (designated *katA*) with the potential to encode a 508 amino acid protein with a predicted molecular mass of 58346 Da (Fig. 1). A putative ribosome-binding site (AGGAG; Gold, 1988) exists 6 bp upstream of the ATG start codon. A potential -10 region (conserved in all six residues; Hawley & McClure, 1983) of an *E. coli* consensus promoter sequence was present 37 bp upstream of this point although no sequence with homology to the corresponding -35 region was apparent. However, the sequence GATA-ATtATtATtATaATa, which starts at nucleotide position 292, matches 14 of 19 nucleotides found in the consensus sequence for Fur-binding sites involved in the transcriptional regulation of iron-repressible genes (de Lorenzo *et al.*, 1987; conserved residues shown in uppercase). The G + C content of the coding region is 33%, which is consistent with the average G + C content for DNA from *Campylobacter* species (Belland & Trust, 1982).

A search of the GenBank database revealed that the predicted protein product of the *katA* gene had an

extensive degree of homology to catalases from various sources (Fig. 2). The highest degree of homology was seen with bacterial hydroperoxidases, there being 65% identity with *Brucella abortus* catalase (Sha *et al.*, 1994), 60% identity with *Bordetella pertussis* catalase (DeShazer *et al.*, 1994), 58% identity with *Haemophilus influenzae* HktE catalase (Bishai *et al.*, 1994) and 54% identity with *Bacillus subtilis* Kat19 catalase (Bol & Yasbin, 1991). In addition, the predicted *C. jejuni* catalase protein was found to be 42% identical to the *E. coli* HP II catalase (von Ossowski *et al.*, 1991).

Expression and enzymic activity of the cloned *C. jejuni katA* gene product in *E. coli*

To determine the size of the proteins specified by the derivative plasmids containing the cloned *C. jejuni katA* gene, total cell proteins from the various plasmid-containing strains were analysed by SDS-PAGE. Cells containing the plasmid pCKG1 produced a 57.5 kDa

1	50	301	350
Cj.MKKLTNDPNTIADNQNLSAQTGKPLLMQDYLLLEKLA		Cj. PLMDIGEMILNKNPNQNYFNEVEQAAPSPSNIVPGIGFSDKMLQARIFY	
Bp.MNAMTNKTLTAAAGAPVADNNNTMTAGFGFALLQDVWFLEKLA		Bp. PLIEVGVLNKNPNENYFAEVEQAAPTPANVVPVIGFSDKMLQGRIFY	
Hi. MSSQCPFSHLAATNLTMGNGAPVADNQNLSLAGRGFLLAQDLWLNEKLA		Hi. PLIEVGFELENPNENFFADVEQSAFAPENLVPGIGFSDKMLQARIFY	
Ba. MKLQEDYSMTDRPIMTTSAGAPIFDNQNLSLAGRGFLMQDYQLIEKLS		Ba. PPDIGVMELNKNPNENYFTEVENAAPSNIIVPGIGFSDKMLQARIFY	
Bs.MSSNKLTTSWGAFAVDNQNLSMTAGSRGPTLIQDVHLEKLA		Bs. PLIEVGRMVLDPNPNENYFAEVEQAATSPGTLVPGIDVSDKMLQGRIFY	
Ec.LTNQGVRIADDONSLRAGSGFTLLEDFILREKIT		Ec. PVQRVGKMLNKNPNENFFAENQAAPFPGHIVPGLDFTNDPLLQGRIFY	
51	100	351	400
Cj. HQNEERIPERTVHAAGSGAYGEIKITADLSAYTHAKIF.QKGEITPLFLR		Cj. FDAQRYRIQT.NYHLLPVNRKSEVNTYNVAGAMNFDYSK...NDAAYYE	
Bp. HFDRERIPERVVHAAGSGAYGFTFTVTHDISRYTHARIFAEVGKQTPFLR		Bp. GDTHRYRLGI.NHHQIPVNAAPCPFHSHRDGMGRVDG...NGGATLNYE	
Hi. DFVEEVIPEERMHAAGSGAGFTFTVTHDITKNTAKIFSEVGKNTFMFAR		Hi. ADAQRYRLGV.CYRQIPVNRPCPVHSHNRDQGRVDG...TYGSLPHYE	
Ba. HQNEERIPERAVHAAGSGAYGTLTITGDISRYTHAKVL.QPGAQTPMLAR		Ba. ADAHRRLGT.HYESIPVNPQKCPVHHYHRDGMNVYGGINTGNPDAYYE	
Bs. HFNEERVPERVVHAAGGAGAGYFPEVINDVTKYTHAFLSEVGKRTPLFIR		Bs. HDAHRYRGA.NHQALPINRARNKNVNYQRDGMRFDD...NGGGVYYE	
Ec. HFDERIPERIVHAAGSAAGGYQFYKELSDITADFLSDPNKITFVFVR		Ec. TDTQISRLGGPNFHEIPINRPTCFYHNFQDGMHRMG...IDTNPANYE	
101	150	401	450
Cj. FSTVAGEAGAADAERDVRCFALKFYTEGNNDLVGNNTPTFFIEDAYKFP		Cj. PMSYDNSPKEDKSYLEPDLVLEG.VAQRYA.PLDN....DFYTOPRAL	
Bp. FSTVAGEAGAADAERDVRCFALKFYTEGNNDLVGNNTPTFFIEDPLKFP		Bp. PMSFGWE.REAKHAAEPPLALDQGAADRWNHRVDE....DYSQPGAL	
Hi. FSTVAGEAGAADAERDVRCFALKFYTEGNNDLVGNNTPTFFIEDPEKFP		Hi. PMSFSQW.QQPDFAEPPLRISGDAA.HWDYRTDDN....DYSQPRAL	
Ba. FSTVAGEAGAADAERDVRCFALKFYTEGNNDLVGNNTPTFFIEDPLKFP		Ba. PMSFNGP.VEQPSAKEPPLCISGN.ADRYNHRIGN....DYSQPRAL	
Bs. FSTVAGEAGSADTTRDFRGFAKPYTEGNYDIVGNNTPTFFIEDAIKFP		Bs. PMSFGG.PKESPEDKQAAYPVQGIADSVSYDHYDH....YTOAGDL	
Ec. FSTVQGGAGSADTVRDTRGFATKPYTEGIFDLVGNNTPTFFIQDAHKFP		Ec. PMSINDNWPRETTPPGPKRGGFESYQERVEGNKVRERSPSFGYYSHRPLF	
151	200	451	500
Cj. DFIHTQKRDPRTHLRSNNA...WDFWSLCPESLHQVTILMPDRGIPAS		Cj. FNLMNDQKQTQLFHNIATSMEGVD.EKIIITRALEHFENISPDYAKGIKKA	
Bp. DFIHTQKRDPKTNLRNATA...WDFWSLNPESLHQVTILMSDRGLPQN		Bp. FRLMNDQKQQLFGNIGRHMAGVP.EEIQRRLQEHFRADPAYAAGVAKA	
Hi. DLNKAVKRDPRTNMRSATNN...WDFWTLLEALHQVTIVMSDRGIPAS		Hi. FNLMNAEQKQSLFNNTAAAMGDAP.DFIKYRHIRNCHWCDAAYGEGVAKA	
Ba. DFIHTQKRDPRTHLRSATAM...WDFWSLCPESLHQVTILMSDRGLPTD		Ba. FNLFDAQAQKRLFSNIAAAMKGVF.GFIVERQLGHFKLIHPEYEAGVRKA	
Bs. DFIHTQKRDPKTHLKNPTAV...WDFWSLCPESLHQVTILMSDRGIPAT		Bs. YRLMSDERTRLVENIVNAMKPVKEEIKLQIEHFKADPEYGRVRAEG	
Ec. DVFHAVKPEPHWAIQQGSAHDTFDWYVSLQFETLHNVMWAMESDRGIPRS		Ec. WLSQTPFEQRHIVDGFSELSKVVRFYIRERVDQLAHIDLTLAQAVAKN	
201	250	501	550
Cj. YRHHMGFGSEHTYSFINDKNERFWVVFHFKTQQGIKNLTNQEAELIAKDR		Cj. FPIVSRIRAWRNHGHSCFLCEIVIRSQFHTTYEPA.....	
Bp. YRQQHGFSGHTYSFVNDAGERFVYVVFHFKSQQGIACYTDGEAAELVGRDR		Bp. LGL.....	
Hi. YRHHMGFGSEHTYSFVNEAGERFWVVFHFKTQQGIKNLTDAEAAEIIANDR		Hi. LGLTVEDALKARDTDFALGQCGLL.....	
Ba. VRHINGFGSEHTYSFVNDAGERFVYVVFHFKTQQGKHWTNAEAEQVIGRTR		Ba. LKDAHGYDANTIALNEKITAAE.....	
Bs. LRHHMGFGSEHTFKWTNAEPEGVWIKYHFKTEQGVKNLDVNTAAKIAGENP		Bs. LGLPIKKDS.....	
Ec. YRTMEGFGIHTFRLINAEAGKATFVRFHWKPLAGKASLVWDEAQKLTGRDP		Ec. LGIELTDDQLNITPPPDVNLGKKDPSLSLYAIPDGDVGRVVAILLNDEV	
251	300		
Cj. ESHQRLDYNAIENKDFPKWKVQVQILAEDKIEKLGFPDFTKIWPVHSLV			
Bp. ESAQRLDFQNIIEGQFFPRWTLKQVQVMPAEAAATYHINPFDLTKVWPHADY			
Hi. ESHQRLDYEAIEERGDFPKWTLFVQIMPEADAERKVPYHFDLTKVWSKKDY			
Ba. ESTQEDLPSAIEENGDFPKWKVQVQIMPELDADKTFYNPFDLTKVWPHADY			
Bs. DYHTEDLFNAIENGDFPAWKLVVQIMPLEDANTYRFPDFDVTKVWSQKDY			
Ec. DFHRAELWEAIEAGDFPEYELGQLIPEDEDFKDFDLDLPTKLIPEELV			

Fig. 2. Comparison of the catalase from *C. jejuni* with those from other bacterial species. The derived amino acid sequence from the *C. jejuni* *kata* gene (Cj) is aligned with that from *B. pertussis* (Bp), *H. influenzae* (Hi), *B. abortus* (Ba), *B. subtilis* (Bs) and *E. coli* (Ec). Residues conserved between all six enzymes are shown in bold.

protein that was not seen in cells containing the parental vector pTZ19R (Fig. 3). The size of this protein is very similar to that predicted for the product of the *kata* gene and it is most likely that this band represents KatA. In contrast, the plasmid pCKG2 uniquely specified a 60 kDa protein (Fig. 3). This is consistent with the nucleotide sequence data, which shows that the ORF is incomplete and reads into the plasmid-derived *lacZ* gene. It is likely, therefore, that this band represents a protein in which a truncated KatA is fused, via its C-terminus, to β -galactosidase. The calculated size of the fusion protein, which is 62 kDa, is in good agreement with this.

To confirm that KatA did indeed possess catalase activity, lysates from *E. coli* recombinants were analysed by non-denaturing PAGE and stained for catalase activity. As

expected no bands of catalase activity were seen in lysates from strain UM255 harbouring the parental vector pTZ19R (Fig. 4). In contrast, when pCKG1 was present in UM255 a single band of catalase activity was apparent. The achromatic band of catalase activity specified by pCKG2 appeared to be the result of protein that was larger than that produced by pCKG1 (Fig. 4). Again, this result is consistent with this protein being a KatA- β -galactosidase fusion. It is interesting to note that, although this protein lacks 38 amino acids from the C-terminus of the catalase, it is still enzymically active. When protein extracts from *C. jejuni* were analysed for catalase activity, in a similar fashion, a single band of catalase was evident. The R_F value for this band of activity was equivalent to that specified by pCKG1 in *E. coli*, suggesting that the same catalase enzyme is responsible for the production of

Fig. 3. Expression of the catalase from *C. jejuni* in *E. coli*. Total soluble proteins from recombinant strains of *E. coli* UM255 were analysed by electrophoresis on 10% SDS-PAGE gels and visualized by Coomassie blue staining. Lanes: A, lysate from cells containing pTZ19R; B, lysate from cells containing pCKG1; C, lysate from cells containing pCKG2. Molecular mass standards are given in kDa and proteins produced uniquely from specific plasmids are indicated by arrows.

Fig. 4. Non-denaturing polyacrylamide gels stained for catalase activity. Total soluble proteins were analysed by electrophoresis on 7.5% polyacrylamide gels run under non-denaturing conditions and visualized following specific staining for catalase activity. Catalase activity appears as an achromatic band against a dark background. Lanes: A, lysate from *E. coli* cells containing pTZ19R; B, lysate from *E. coli* cells containing pCKG2; C, lysate from *E. coli* cells containing pCKG1; D, lysate from *C. jejuni* NCTC 11351; E, lysate from *C. coli* UA585; F, lysate from *C. coli* CK100. (Note that lanes E and F represent a separate gel so the mobility of the achromatic band should not be compared with those in other lanes.)

both bands. To determine whether or not KatA also possessed peroxidase activity, equivalent non-denaturing PAGE gels were assessed for 3,3'-diaminobenzidine-peroxidase activity as described by Wayne & Diaz (1986). Proteins with peroxidase activity generally stain black against a light background. No bands of staining were detected for any protein sample, including one prepared from *C. jejuni* (data not shown).

Generation of a catalase-deficient mutant of *C. coli* by interspecific allelic exchange

As a first step towards constructing isogenic catalase-deficient mutants the structural *C. jejuni* catalase gene present in pCKG2 was disrupted by the insertion of a *Bgl*/II tetracycline-resistance cassette into the unique *Bgl*/II site at position 1145 (Fig. 1) to generate pCKG3. The strain *C. jejuni* NCTC 11351 from which *katA* was originally cloned has proven refractory to the introduction of recombinant DNA either by electroporation or natural transformation (unpublished data) and is, therefore, an unsuitable host for the construction of mutants via gene replacement. The opportunity arose, therefore, to determine whether pCKG3, containing DNA derived from *C. jejuni*, could be used to generate a catalase-

deficient mutant of *C. coli* via interspecific allelic exchange. Accordingly, pCKG3 was introduced into *C. coli* UA585 by natural transformation. Tetracycline-resistant transformants were obtained with a low frequency (up to 20 transformants per μ g DNA).

Genetic and phenotypic characterization of *C. coli* *katA* isogenic mutants

To examine the phenotype of the tetracycline-resistant colonies, catalase activity was assessed using the qualitative plate assay. Approximately 50% of the transformants exhibited a catalase-negative phenotype consistent with allelic exchange resulting from a double crossover recombinational event. In the catalase-positive transformants it is likely that integration of pCKG3 had occurred via a single crossover which would regenerate an intact copy of the catalase structural gene (Dickinson *et al.*, 1995). To confirm the nature of the genetic event which led to the catalase-deficient phenotype, a DNA fragment containing the *katA* gene (the 1.8 kb insert in pCKG2) was labelled and hybridized to digested total DNA extracted from one *Kat*⁻ *C. coli* transformant designated CK100. For the DNA from the parental strain of *C. coli* the probe hybridized to single *Eco*RV and *Cla*I

Fig. 5. Southern analysis of CK100, a tetracycline-resistant *Kat*⁻ transformant. A *Hind*III fragment from pCKG2 was labelled and used to probe genomic DNA isolated from: *C. coli* UA585 digested with *Eco*RV (lane A), *Cla*I (lane C) and *Bgl*II (lane E), and *C. coli* CK100 digested with *Eco*RV (lane B), *Cla*I (lane D) and *Bgl*II (lane F). Molecular size standards are shown in kb.

fragments (Fig. 5). This is expected since there are no sites for these enzymes internal to *katA*. For CK100, however, although the probe hybridized to single bands in the *Eco*RV and *Cla*I digests, the size of these fragments was 2.3 kb larger when compared to the wild-type. This is consistent with the exchange of the wild-type *katA* DNA with the disrupted sequence present in pCKG3, containing the 2.3 kb tetracycline-resistance marker. Digestion of the DNA with *Bgl*II produced the same pattern of hybridization in the DNA from both mutant and wild-type cells. Again this is expected if allelic exchange has occurred, since the *Bgl*II tetracycline-resistance marker cassette, internal to the disrupted *katA* gene in the mutant strain, will be released and the regions of DNA which flank this site will be unchanged relative to the wild-type DNA. No hybridization occurred between the labelled plasmid backbone (pTZ19R) and DNA from CK100 (data not shown) confirming the loss of the non-homologous plasmid-derived sequence during allelic exchange. To confirm the *Kat*⁻ phenotype of CK100, lysates from the strain were analysed by non-denaturing PAGE and stained for catalase activity. Although a single band of catalase activity was apparent in lysates from the isogenic parental strain of *C. coli* no achromatic bands were seen in lysates prepared from the mutant (Fig. 4).

H₂O₂ sensitivity of *Kat*⁺ and *Kat*⁻ *C. coli*

To assess the contribution of the catalase, encoded by *katA*, in the oxidative stress resistance of *C. coli* the sensitivity of the wild-type strain and CK100 (*Kat*⁻) to H₂O₂ was assessed. In 1 mM H₂O₂ the survival of the parental *Kat*⁺ strain decreased by 50% over the first

Fig. 6. H₂O₂ sensitivity of *C. coli* UA585 (*Kat*⁺) and CK100 (*Kat*⁻). (a) Cells of *C. coli* UA585 (●) and CK100 (○) were exposed to 1 mM H₂O₂ and the percentage survival determined from viable counts. (b) The persistence of H₂O₂ in the growth media during the survival of *C. coli* UA585 (●) and CK100 (○) was also determined. Similar results were reproducibly obtained in several experiments.

30 min, after which point cell growth appeared to resume (Fig. 6). In contrast, the survival of the *Kat*⁻ mutant dropped to just 0.8% after the first 15 min and thereafter was undetectable (less than 10 c.f.u. ml⁻¹). To determine whether the destruction of H₂O₂ by catalase or the persistence of this microbicidal agent could account for the difference in resistance of the wild-type cells compared to *Kat*⁻ cells, H₂O₂ concentration was assessed during the survival studies. In the presence of *Kat*⁺ cells the concentration of H₂O₂ fell to undetectable levels (less than 1 μM) after just 5 min. Conversely, when H₂O₂ was exposed to *Kat*⁻ cells its concentration fell gradually to 68% of the original level over the first 60 min and then did not decrease further over the next 60 min.

DISCUSSION

Catalases, which degrade H₂O₂ to H₂O and O₂, are present in most aerobic organisms and are considered to be part of a defence system against oxidative stress (Haas & Goebel, 1992). In this study we describe the cloning of

a gene (*katA*) encoding catalase from the microaerophilic pathogen *C. jejuni*.

The product of the *C. jejuni katA* gene appeared as a 57.5 kDa protein in recombinant strains of *E. coli*, which was in close agreement to the predicted *katA* gene product of 58.3 kDa. The amino acid sequences of a number of bacterial catalases have now been deduced and most have been found to share homology with eukaryotic haem-catalases (von Ossowski *et al.*, 1993). The notable exception is the *E. coli* HPI catalase. This is not surprising, however, since it possesses both a catalase activity and broad-range peroxidase activity (Claiborne & Fridovich, 1979). The amino acid sequence of KatA from *C. jejuni* showed no homology to HPI but considerable homology to the group of bacterial hydroperoxidases. Moreover, although hydroperoxidase activity was evident in samples containing KatA, we were unable to detect any peroxidase activity. The catalase from *C. jejuni* appears, therefore, to be both structurally and enzymically similar to bacterial hydroperoxidases (von Ossowski *et al.*, 1993). Furthermore, since only one protein band corresponding to catalase activity, and no bands of peroxidase activity, was detected in cell lysates from *C. jejuni*, it is possible that the hydroperoxidase activity encoded by *katA* is the only catalase possessed by this strain. We cannot, however, exclude the possibility that this strain contains additional inducible catalases that are not expressed under the microaerophilic conditions used to culture the cells. If this was the case though we would have expected to identify these catalases in our complementation studies.

The catalase gene was expressed at high levels in *E. coli* in the absence of the *lac* promoter inducer IPTG and independently of the orientation of the inserted DNA (data not shown), suggesting that expression in *E. coli* is directed by sequences present on the cloned *C. jejuni* DNA. In this respect, we have identified a region upstream of the catalase structural gene with homology to the *E. coli* consensus Fur-binding site. Although further experimentation is needed to determine whether iron-dependent regulation of the *C. jejuni katA* gene occurs, it is notable that Fur-binding sites have been identified upstream of another gene whose product is involved in oxidative stress resistance, namely superoxide dismutase (Purdy & Park, 1994; Pesci *et al.*, 1994). In addition, it is likely that *C. jejuni* has the appropriate mechanisms to facilitate iron-dependent Fur-regulation since a gene encoding a potential iron-responsive Fur-binding regulatory protein has recently been identified (Wooldridge *et al.*, 1994).

The strain *C. jejuni* NCTC 11351, from which *katA* was originally cloned, has proven refractory to the introduction of recombinant DNA either by electroporation or natural transformation and was, therefore, an unsuitable host for the construction of catalase mutants via gene replacement. We chose, therefore, to assess whether it was possible to use the *C. jejuni* derived *katA* sequence to generate isogenic mutants by allelic exchange in a closely related species, namely *C. coli*. Following the introduction of the disrupted *C. jejuni katA* sequence into *C. coli* via

natural transformation both Kat⁻ and Kat⁺ clones were isolated. Genetic and enzymic analysis of a Kat⁻ tetracycline-resistant *C. coli* clone confirmed that allelic exchange had occurred between the DNA sequences of different origin. It is interesting to note that the process of homologous recombination, which apparently mediated the allelic exchange, occurred even in the absence of complete homology. Partial sequence analysis (data not shown) has shown the *C. coli katA* gene to be 92% identical to that from *C. jejuni*.

Catalase is thought to provide protection against oxidative stress by converting H₂O₂ to H₂O and O₂. To establish a role for catalase in the oxidative stress resistance of *Campylobacter* the resistance of the catalase-deficient isogenic mutant of *C. coli* to H₂O₂ was assessed in comparison to the wild-type. The Kat⁻ mutant appeared to be much more sensitive to H₂O₂ than the parental strain. Furthermore, the sensitivity of CK100 to H₂O₂ appeared to be a consequence of its failure to detoxify this molecule, since more than 60% of the H₂O₂ persisted throughout the duration of the experiment. In contrast, wild-type cells were able to reduce the concentration of H₂O₂ to undetectable levels within just 5 min of exposure. In view of the persistence of H₂O₂ in the presence of the Kat⁻ mutants, the death of cells is perhaps not surprising since H₂O₂ diffuses across membranes and damages a variety of cellular targets (Farr & Kogoma, 1991). Although it is not possible to rule out the possibility that the disrupted *katA* sequence has a polar effect on the expression of downstream genes, it seems very likely that the differential in the H₂O₂ sensitivity of the mutant compared to the wild-type strain is attributable to the absence of catalase.

As part of the defence mechanism which protects cells against oxidative damage, catalase is likely to play a crucial role in the survival of campylobacters in aerobic environments in which H₂O₂ may arise in the course of aerobic metabolism. In addition, it has been postulated that catalases might be an important means for bacterial pathogens to survive the oxidative bursts generated by eukaryotic inflammatory cells (Mandell, 1975; Haas & Goebel, 1992). The genetic analysis of the gene encoding the catalase from *C. jejuni* and the characterization of an isogenic catalase-deficient mutant of *C. coli* may represent an important first step in establishing a role for this enzyme in the physiology and virulence of these clinically important pathogens.

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