KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7

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A gene coding for a catalase-peroxidase activity was identified on a 9·7 kb *Sma*I DNA fragment derived from the large plasmid pO157 of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 strain EDL 933. Nucleotide sequencing revealed an ORF of 2208 bp and predicted a 736 amino acid polypeptide with a molecular mass of 81·8 kDa. This putative protein was found to be highly homologous to members of the bacterial bifunctional catalase-peroxidase family. Analysis of its amino acid sequence revealed the presence of characteristic peroxidase 1 and 2 motifs. In addition, an N-terminal signal sequence was found, suggesting that the catalase-peroxidase is transported through the cytoplasmic membrane. EHEC catalase-peroxidase activities were investigated in cytoplasmic and periplasmic crude extracts as well as in culture supernatants from wild-type and recombinant *E. coli* strains. EHEC-specific catalase-peroxidase activity was detected primarily in the periplasm in strain EDL 933. The newly discovered enzyme was designated KatP, to indicate its plasmid origin. PCR analysis of representative strains of all enteric *E. coli* pathogroups (i.e. enterohaemorrhagic, enterotoxigenic, enteropathogenic, enteroaggregative and enteroinvasive *E. coli*) revealed a close association between the occurrence of EHEC-haemolysin and the *katP* gene in Shiga-like-toxin-producing *E. coli* O157 strains.

Keywords: enterohaemorrhagic *Escherichia coli* O157, plasmid pO157, plasmid-encoded catalase-peroxidase, *katP*

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157 is the predominant causative agent of haemorrhagic colitis (HC) and the haemolytic uraemic syndrome (HUS). Foodborne outbreaks and sporadic cases of HC and HUS have been increasing worldwide (Griffin & Tauxe, 1991; Tarr, 1995; Griffin, 1995). Besides diagnostic and epidemiological approaches, most molecular studies on this pathogen have focused on the function of Shiga-like toxins (O'Brien & Holmes, 1987; O'Brien *et al.*, 1992; Tesh & O'Brien, 1991), intimin_{O157} (Louie *et al.*, 1993; Yu &

Kaper, 1992) and the large plasmid pO157 (Fratamico *et al.*, 1993; Hales *et al.*, 1992; Karch *et al.*, 1987; Toth *et al.*, 1990; Tzipori *et al.*, 1987). While the outer-membrane protein Intimin_{O157} is involved in the intimate bacterial attachment-effacement of enterocytes in the large intestine (Louie *et al.*, 1993; Griffin, 1995), Shiga-like toxins (SLT) may primarily contribute to the extraintestinal symptoms by receptor-mediated cytotoxic activity to target cells (Tesh & O'Brien, 1991; Griffin, 1995). Plasmid pO157 is present in almost all clinical EHEC O157 isolates and was first detected in strains causing an outbreak of HC (Wells *et al.*, 1983). Plasmid pO157 has been the subject of several investigations regarding adherence to cultured cells (Dytoc *et al.*, 1993; Karch *et al.*, 1987; Toth *et al.*, 1990), pathogenicity in animal models (Tzipori *et al.*, 1987, 1988; Wadolkowski *et al.*, 1990), and plasmid-encoded functions (Fratamico *et al.*, 1993; Dytoc *et al.*, 1993; Hales *et al.*, 1992). However, the occurrence of this plasmid in clinical isolates could not be linked conclusively to the pathogenic properties of EHEC O157 strains. Recently, we have

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; SLTEC, Shiga-like-toxin-producing *E. coli*; EPEC, enteropathogenic *E. coli*; EAaggEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli*; HC, haemorrhagic colitis; HUS, haemolytic uraemic syndrome; HP, hydroperoxidase; SLT, Shiga-like toxin.

The EMBL accession number for the sequence reported in this paper is X89017.

characterized plasmid pO157 of the EHEC O157:H7 strain EDL 933 by restriction mapping and described a pO157-encoded determinant, designated EHEC-haemolysin (Schmidt *et al.*, 1995, 1996). EHEC-haemolysin is a member of the RTX (Repeats in ToXin) family of pore-forming cytotoxins and was recently shown to be cytotoxic for human and bovine cell lines (Bauer & Welch, 1996).

In the present study, we describe a second pO157-encoded determinant, the EHEC KatP catalase-peroxidase, which is produced in addition to the two chromosomally encoded catalases or hydroperoxidases (HP) of *E. coli*, HPI and HPII. HPI, which is encoded by the *katG* gene, shows bifunctional catalase and peroxidase activity and acts as homotetramer, each subunit of which has a molecular mass of approximately 80 kDa (Triggs-Raine *et al.*, 1988; Triggs-Raine & Loewen, 1987; Loewen *et al.*, 1985a). *E. coli* HPI, like other bacterial bifunctional catalase-peroxidases, is a haem-binding enzyme that carries out a variety of functions using hydrogen peroxide as the electron acceptor. In peroxidases, the haem prosthetic group is protoporphyrin IX and the fifth ligand of the haem iron is a histidine. Another histidine residue serves as an acid-base catalyst in the reaction between peroxide and the enzyme. The regions around these two active sites are conserved in a majority of peroxidases (Welinder, 1991).

HPII is encoded by the *katE* gene and functions as a catalase only (von Ossowski *et al.*, 1991). Catalases are part of bacterial defence mechanisms against oxidative stress, which involve at least 30 proteins, depending on the stimulus applied (Farr & Kogoma, 1991). Reactive oxygen molecules, produced as by-products of oxygen metabolism, may damage bacterial cells in many ways (Farr & Kogoma, 1991). In recent years, superoxide dismutases and catalases expressed by bacterial pathogens have been increasingly thought to protect these pathogens from oxidative damage caused by reactive oxygen molecules produced by phagocytes or other host cells during the infectious process (Welch, 1987; Bortolussi *et al.*, 1987; Beaman & Beaman, 1984).

METHODS

Bacterial strains, plasmids and culture conditions. Enterohaemorrhagic *E. coli* O157:H7 strain EDL 933 was isolated from a patient with HC and harbours the 93.6 kb plasmid pO157 as well as one smaller plasmid of 3.3 kb (O'Brien *et al.*, 1983; Schmidt *et al.*, 1996). Strain EDL 933-cu was cured of pO157, but not of the smaller plasmid (Tzipori *et al.*, 1987). HPI-negative *E. coli* mutant UM202 (*katG17::Tn10*) was kindly provided by P. C. Loewen, University of Manitoba, Winnipeg, Manitoba, Canada (Loewen *et al.*, 1985b). *E. coli* HB101 and *E. coli* DH5 α (Gibco BRL) were used as host strains for recombinant plasmids. Diarrhoeagenic *E. coli* isolates used for PCR analysis were from our strain collection. The seven EHEC O157:H7 strains all produced SLT-I, SLT-II, SLT-IIc or combinations of these toxins. The twelve non-O157 Shiga-like-toxin-producing *E. coli* (SLTEC) belonged to serogroups O26 (three strains), O111 (three strains), O128 (two strains), O2, O22, O52 and O55 (one strain each) and also expressed different SLT genotypes. The enteropathogenic *E. coli* (EPEC) strains

were of classical EPEC serogroups O55, O111, O114, O119 and O128, and were all non-toxicogenic. The enteroaggregative *E. coli* (EAggEC) strains all fulfilled the criterion of 'stacked brick'-like adherence to HEp-2 cells. The five enterotoxigenic *E. coli* (ETEC) strains expressed heat-stable toxin (ST), heat-labile toxin (LT) or a combination of both toxins. The four enteroinvasive *E. coli* (EIEC) strains were previously identified in our laboratory by the presence of invasion genes located on the large invasion plasmid (data not shown). Plasmids pB26 and pB9 contain 25.9 kb and 8.6 kb *Bam*HI fragments, respectively, which were derived from pO157 (this study) and cloned in vector pK18 (Pridmore, 1987). Plasmid pUC4K (Pharmacia LKB) was used as donor of the kanamycin-resistance cassette. Cloning of a 9.7 kb *Sma*I fragment from pO157, which carries the entire EHEC catalase-peroxidase gene in phagemid vector pBluescript IKS (Stratagene) generated recombinant plasmid pSm10 (this study).

For routine purposes, *E. coli* strains were grown in L-broth (1%, w/v, tryptone, 0.5%, w/v, yeast extract, 1%, w/v, sodium chloride, pH 7.5). For preparation of cell extracts, cells were grown in N-broth (nutrient broth), pH 6.7 (Difco), supplemented with 0.5% (w/v) sodium chloride. If necessary, 20 μ g tetracycline ml⁻¹, 100 μ g ampicillin ml⁻¹ or 50 μ g kanamycin ml⁻¹ was added to the culture media.

General recombinant DNA methods. Restriction enzymes and T4 DNA ligase were purchased from Gibco BRL, New England Biolabs and Boehringer Mannheim. Plasmid DNA was purified with a Qiagen plasmid Midi kit. For cloning and restriction enzyme analysis, standard procedures were used (Sambrook *et al.*, 1989). Transformation of plasmid DNA was performed according to the method of Hanahan (1983). Purification of DNA fragments from agarose gels was carried out with a Prep-a-Gene kit (Bio-Rad). Hybridization experiments were conducted with a digoxigenin labelling and detection kit (Boehringer Mannheim) according to the manufacturers' instructions.

Nucleotide sequencing and sequence analysis. The nucleotide sequence was determined with an automatic sequencer (model 373A; Perkin-Elmer-Applied Biosystems) by *Taq* cycle sequencing using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer-Applied Biosystems) as described previously (Schmidt *et al.*, 1995).

Nucleotide sequence data were obtained by stepwise sequencing. Initially, universal and reverse primers for M13/pUC18 (Boehringer) were used for sequencing recombinant plasmids pB26 and pB9. As new sequence data became available, customized synthetic primers (18- to 22-mers) were utilized (Roth, Karlsruhe, Germany). Both strands of DNA were sequenced completely and each base position was determined on average four times.

Nucleotide sequence analyses and the searches for homologous DNA sequences in the EMBL and GenBank database libraries were performed with the program package HUSAR (Heidelberg Unix Sequence Analysis Resources; German Cancer Research Centre, Heidelberg, Germany), as well as with the DNAsis program (Hitachi Software).

Bacterial cell fractionation. Periplasmic proteins were isolated by the method of Jacobs *et al.* (1992) with minor modifications. Briefly, bacterial cells were cultured in N-broth at 37 °C without shaking to an OD₆₀₀ of 0.15–0.3 (approximately 10⁸ cells ml⁻¹). Cells were harvested by centrifugation (3800 g, 15 min, 4 °C) and resuspended in 30 mM Tris/HCl, pH 8.0, containing 27% (w/v) sucrose, 0.2 mM EDTA and 1% (v/v) 2-phenylethanol (Merck). The suspension was submitted to three cycles of freezing (liquid nitrogen) and thawing (37 °C, water bath) to

permeabilize the outer membrane of the bacteria. After centrifugation (20000 *g*, 30 min, 4 °C), the supernatant was used as periplasmic fraction.

The pellet was resuspended in 20 mM sodium/potassium phosphate buffer, pH 7.0, containing 0.5 mg lysozyme ml⁻¹. After incubation for 10 min at 30 °C, the cells were lysed by three cycles of freezing (liquid nitrogen) and thawing (37 °C, water bath). DNA was broken down by 50 µg DNaseI ml⁻¹ for 5 min at 30 °C. Cell debris was removed by centrifugation (20000 *g*, 30 min, 4 °C) and the supernatant was used as cytoplasmic fraction. Protein concentrations were estimated by the method of Markwell (1978) with BSA as the standard.

Precipitation of culture supernatants. For precipitation of proteins from culture supernatants, with either ammonium sulfate or trichloroacetic acid (TCA), cells were grown to an OD₆₀₀ of 0.15–0.3 and centrifuged as described above. For ammonium sulfate precipitation, culture supernatants were filtered through a 0.22 µm filter, mixed with ammonium sulfate by slow addition of the same volume of a saturated ammonium sulfate solution and stirred for 1 h at 4 °C. The precipitate was separated by centrifugation (20000 *g*, 30 min, 4 °C), dissolved in 20 mM sodium/potassium phosphate buffer and then used for enzyme assays. For TCA precipitation, TCA was added to a final concentration of 10% (w/v) to the filtered culture supernatants. After incubation on ice for 1 h and centrifugation as described above, the TCA precipitate was dissolved in Laemmli sample buffer (Laemmli, 1970) and analysed by SDS-PAGE.

Enzyme assay. Peroxidase activity was determined by following the rate of oxidation of the substrate *o*-dianisidine dihydrochloride (Sigma). Measurements were performed in a Hitachi U-2000 spectrophotometer at a wavelength of 460 nm at room temperature. The reaction mixture contained 10 mM sodium/potassium phosphate buffer, pH 7.0, 0.3 mg *o*-dianisidine dihydrochloride ml⁻¹, 18 mM H₂O₂ and an appropriate amount of protein extract. One enzyme unit is equivalent to an increase in *A*₄₆₀ of 11.3 units min⁻¹, assuming an absorption coefficient of 11.3 mM⁻¹cm⁻¹ for *o*-dianisidine (de Mendez *et al.*, 1991). Specific activity was expressed as units per mg total protein in the assay. Values of enzymic activity were means of triplicate measurements.

Visualization of catalase and peroxidase activities on polyacrylamide gels. Electrophoresis was performed on non-denaturing 8.5% (w/v) polyacrylamide gels in a Mini-PROTEAN II Dual Slab Cell (Bio-Rad) at pH 8.8, according to the method of Laemmli (1970). Twenty microgram samples of the protein extracts were adjusted with glycerol and bromophenol blue to final concentrations of 10% (v/v) and 0.02% (w/v), respectively, and applied to the gel. Electrophoresis was carried out for 5 h at a constant current of 10 mA. Staining for peroxidase activity was performed as described by Wayne & Diaz (1986) but instead of diaminobenzidine, *o*-dianisidine (0.3 mg ml⁻¹) was used as electron donor. The activity staining for catalase was carried out as described by Woodbury *et al.* (1971) with minor modifications: after three washing steps for 15 min in H₂O, the gels were soaked in 0.03% H₂O₂ for 10 min and rinsed with H₂O for 2 min; after this, the gels were incubated in a moist chamber for 15 min at room temperature to allow consumption of H₂O₂ by the catalase bands. The gels were then stained with a freshly prepared solution of 1% (w/v) ferric chloride and potassium ferricyanide.

PCR. PCR for detection of EHEC *katP* sequences was performed with the GeneAmp PCR System 9600 (Perkin-Elmer-Applied Biosystems). Primers wkcat-B (5' CTTCCTGTTCTGATTCTTCTGG 3') and wkcat-F (5' AACTTATTTCTCGCATCATCC 3') were designed to amplify a 2125 bp fragment of the

EHEC *katP* gene. Briefly, 10⁴ bacterial cells derived from a single colony were suspended in 40 µl saline (0.85% sodium chloride). PCR was performed in a total volume of 50 µl containing 5 µl of the bacterial cell suspension, 30 pmol of each primer, 200 µM of each deoxyribonucleoside triphosphate, 5 µl GeneAmp 10-fold concentrated PCR buffer II, 3 µl 25 mM MgCl₂ and 2 units of AmpliTaq DNA polymerase (Perkin-Elmer-Applied Biosystems). After an initial denaturation of 5 min at 94 °C, the samples were subjected to 30 cycles of amplification, each of which consisted of 30 s at 94 °C, 60 s at 56 °C and 150 s at 72 °C. The reaction was completed with a final extension step of 5 min at 72 °C. Ten microlitres of the PCR products was mixed with gel-loading buffer and electrophoresed on 0.6% (w/v) agarose gels.

RESULTS

Cloning and sequence analyses of the EHEC *katP* gene

In order to characterize the large plasmid of *E. coli* O157:H7 strain EDL 933 at a molecular level, we constructed several subclones in vector pK18 and performed partial sequence analysis of these clones with universal and reverse sequencing primers. By using subclones pB26 and pB9, which harbour pO157-derived 25.9 kb and 8.6 kb *Bam*HI restriction fragments, we found nucleotide sequence similarities (> 50%) to the catalase-peroxidase genes of *E. coli*, *Salmonella typhimurium*, *Mycobacterium* spp., *Bacillus stearothermophilus* and *Rhodobacter capsulatus*. This prompted us to investigate these putative catalase sequences more systematically, since no plasmid-borne catalase of an obligate bacterial pathogen has been described to date.

Fig. 1. Maps of recombinant plasmids and strategy of disruption of the *katP* gene. The restriction sites for *Bam*HI (B), *Hind*III (H), *Kpn*I (K), *Rsr*II (R), *Sal*I (S) and *Sma*I (Sm) are indicated. The position and direction of transcription of the EHEC *katP* gene is depicted by the hatched arrow. In plasmid pSmK9, the 1.1 kb *Kpn*I fragment which is present in pSm10 is deleted. The kanamycin-resistance cassette (*kana*, dark box) is inserted in the single *Sal*I site of pSmK9 to obtain pSmK9::k_{ana}+. The position of the 9.7 kb *Sma*I fragment (in kb, clockwise from the starting point of the map) on wild-type plasmid pO157 is indicated above the pSm10 map.

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1  GTTGGCGCTTGGAAACCCTTAGTATTTTGTCTGTAGTATCTATCCAGCAATAGGTATATCCTGTTGCATCAATAAAGTTGACTTTTGTATACAACAT
101  GCGAATTTCCCTTAATCCGAGCTATTCTCGTATGATAAAAAAACTCTTCCTGTTCTGATTCTTCTGCGCTATCGGGAGCTTTTCTACCGCTGTAGCCG
1  SD M I K K T L P V L I L L A L S G S F S T A V A A
201  CTGATAAAAAAGAGACTCAAAATTTCTACTATCCAGAAACACTGGATTAACTCCTCTGAGATTACACAGCCCTGAATCAAAATCCCTGGGGGGCTGATT
25  D K K E T Q N F Y Y P E T L D L T P L R L H S P E S N P W G A D F
301  TGATTATGCCACCAGATTTCACAGCTGGATATGGAGGCTCTGAAAAAGATATCAAGATTGCTGACAACCTCCAGGATTGGTGGCTGCGGATTAT
58  D Y A T R F Q Q L D M E A L K K D I K D L L T T S Q D W W P A D Y
401  GGTCAATTATGGTCTTTCTTTATTCTGATGGCTGGCAGGCTGCCGGAACATACAGGACATATGATGGCCGGGAGGCCAGTGGTGGTCAGCAACGTT
91  G H Y G P F F I R M A W H G A G T Y R T Y D G R G G A S G G Q Q R F
501  TTGAACCGCTGAACAGCTGGCCGGGATAACGTTAATCTGGATAAAGCCGCTGATGCTGTGGCCAGTCAAGAAAAATACGGCTCCAGTATTTCTGGGG
125  E P L N S W P D N V N L D K A R R L L W P V K K K Y G S S I S W G
601  AGACCTGATGGTCTGACTGGTAATGTTGCCCTGAATCCATGGGATTAAAAAGCTGGGATTGCTGGCGGAAGAGAAGATGACTGGGAGTGGGACCTG
158  D L M V L T G N V A L E S M G F K T L G F A G G R E D D W E S D L
701  GTATACTGGGGGCTGACAACAGCCTCTTGAGATAACCGGATAAAAAAGGAACTTCAGAAACCTCTTGCCGCCAGCAGATGGGACTTATTATG
191  V Y W G P D N K P L A D N R D K N G K L Q K P L A A T Q M G L I Y V
801  TCAATCTGAAGGCCCGGTGGAAACAGATCCTCTGGCTTCCGGAAGATATCAGGGAAGCTTTTTCAGTATGGCCATGGATGATGAGGAGACTGT
225  N P E G P G G K P D P L A S A K D I R E A F S R M A M D D E E T V
901  GGCCCTGATCGCGGAGGGCATACTTTGGTAAAGCACATGGTCAGCGTCTCCTGAAAAATGATTGGCGCAGGGCTGATGGTGCACCTGTGGAGGAG
258  A L I A G G H T F G K A H G A A S P E K C I G A G P D G A P V E E
1001  CAGGGACTGGGATGGAAAAATAATGTGTACAGGAAACGGCAATATACCATCACCAGTGGCTGGAAGGAGCCTGGTGCATCGCCAAACCCAGTTCA
291  Q G L G W K N K C G T G N G K Y T I T S G L E G A W S T S P T Q F T
1101  CAATCGAGTATCTGAAGAATTATATAAATATGAATGGGAGCTGCACAAGAGTCTGCGCGTCTTATCAGTGAAGCCTAAAAAGCGCAAAATATAGT
325  M Q Y L K N L Y K Y E W E L H K S P A G A Y Q W K P K K A A N I V
1201  TCAGGACGCCATGATCCGCTCTGCTGATCCGTTGATGATGTTTACGACGGATATGCTCTTAAAGTTGATCCTGAATATAAGAAAAATACCCCGT
358  Q D A H D P S V L H P L M M F T T D I A L K V D P E Y K K I T T R
1301  TTCCTGAATGATCAAAAGCTTTTGAGCAGGCATTGCAAGAGCATGGTTAAACTGACCCACCGGATATGGGACCGGCAGCCGATATCTGGTAATG
391  F L N D P K A F E Q A F A R A W F K L T H R D M G P A A R Y L G N E
1401  AAGTTCCTGCAAGATCATTTATCTGGCAGGATCCTCTTCTGCGCGGATATACAATGATTGATGGTAAAGACATTAAGTCGTAAGAGAGAGGTTAT
425  V P A E S F I W Q D P L P A A D Y T M I D G K D I K S L K E Q V M
1501  GGATTGGGTATCCCTGCATCTGAGCTGATAAAGACAGCCTGGGCTTCAGCTTCCACATTTCTGTGACTGATTATCGTGGGGGAAATATGGTGGCCGC
458  D L G I P A S E L I K T A W A S A S T F R V T D Y R G G N N G A R
1601  ATCAGGTTACAGCCCGAAATTAAGTGGGAAGTTAATGAGCCTGAAAAAGTGAAGAAAGTACTGGCATCCCTGACCTCATTACAGCGTGAATTTAACAATA
491  I R L Q P E I N W E V N E P E K L K K V L A S L T S L Q R E F N K K
1701  AACAGTCTGACGGAAGAAAGTGTGCTGGCTGATTTAATGTTCTTTCGGGTAATGCTGCAATCGAAGATGCGGCCAGAAAGCCGGGTGGAAGTTGA
525  Q S D G K K V S L A D L I V L S G N A A I E D A A R K A G V E L E
1801  GATTCCTTTTACTCCGGGAAGAACTGACGCCTCTCAGGAGCAGACGGATGTTGCCCTCATTGAGTACTGGAGCCGACAGCAGATGGATTGAGAAATTA
558  I P F T P G R T D A S Q E Q T D V A S F S V L E P T A D G F R N Y
1901  TACTCAAAAGCAGAAGTCATATATCGCCGTTGAAAGCCTCATTGATAAAGCCAGTCAGCTGATCTCACCCTTCTGAAATGACGGCATTACTGGTG
591  Y S K S R S H I S P V E S L I D K A S Q L D L T V P E M T A L L G G
2001  GTCTCGGGTAATGGATATTAATACAAATAATTCTCGTTGGGAGTGTTTACCGATACCCCTGGTGTCTGGATAACAAGTTTTTGTGTAATCTGCTGGA
625  L R V M D I N T N N S S L G V F T D T P G V L D N K F F V N L L D
2101  TATGTCAACACGATGGAGTAAAGCAGATAAAGAAGATACATAAATGGATTTCGATCGTAAACCGGAGCATTAAATGGAAAGCATTCTCTGTTGATTTA
658  M S T R W S K A D K E D T Y N G F D R K T G A L K W K A S S V D L
2201  ATCTTCAGTTCAAAATCCTGAATTACGTGCGGTGGCAGAAGTATATGCTCGGATGATGCGAGAAATAAGTTTATTCATGATTTGTTAAATCGTGAATA
691  I F S S N P E L R A V A E V Y A S D D A R N K F I H D F V K S W N K
2301  AAGTTATGAATAGCGATCGGTTTGATTAAACAATAAATGATAAACTGATGAGGGAGGGCACCTCCCTCATGAAGGAGAAATATGCAGAAAAAGCAATA
725  V M N S D R F D L N N K
terminator
2401  AAGTTAT

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Fig. 2. Nucleotide sequence of the EHEC *katP* gene. The DNA translation is shown in the one-letter code below the nucleotide sequence, at the first nucleotide of every codon. The putative Shine-Dalgarno (SD) and terminator sequences are indicated. The locations of the PCR-primer-binding sites are depicted by arrows above the nucleotide sequence.

Nucleotide sequencing of the two aforementioned DNA fragments with customized primers revealed further catalase-peroxidase-specific nucleotide sequences. Therefore, it was concluded that the putative catalase gene overlapped both the 25.9 kb and 8.6 kb *Bam*HI restriction fragments which are located consecutively on pO157

(Schmidt *et al.*, 1996). To clone the entire gene on a single fragment, we ligated a 9.7 kb *Sma*I fragment, which included the respective *Bam*HI site, into the vector pBluescript IKS. The resulting plasmid was designated pSm10 (Fig. 1). The *Sma*I fragment covered the region from 38.5 to 48.2 kb in the pO157 map (Schmidt *et al.*,

Table 1. Amino acid sequence identities and weighted similarities of the EHEC O157:H7 plasmid-encoded catalase-peroxidase KatP to other bacterial bifunctional catalase-peroxidases

Values of percentage identity, percentage similarity and residues aligned were calculated with the program GAP from the HUSAR software package.

Organism	Enzyme	Identity (%)	Similarity (%)	Residues aligned	Reference
<i>Bacillus stearothermophilus</i>	PerA	59	69	708	Loprasert <i>et al.</i> (1989)
<i>Mycobacterium intracellulare</i>	MI85	56	67	713	Morris <i>et al.</i> (1992)
<i>Escherichia coli</i>	KatG (HPI)	55	67	689	Triggs-Raine <i>et al.</i> (1988)
<i>Mycobacterium tuberculosis</i>	KatG	55	66	715	Heym <i>et al.</i> (1993)
<i>Salmonella typhimurium</i>	KatG (HPI)	54	66	689	Loewen & Stauffer (1990)
<i>Rhodobacter capsulatus</i>	CpeA	43	47	562	Forkl <i>et al.</i> (1993)

1996). By further use of customized oligonucleotides, we sequenced 2407 bp from both strands of this fragment (Fig. 2). This sequence was analysed for ORFs with the DNAsis software package. A single ORF of 2208 bp was found beginning with ATG at position 131 and ending with TGA; the last nucleotide (A) is found at position 2341. This ORF is preceded by a potential ribosome-binding site with the sequence GGAG spanning position 119–122, 8 bp upstream from the ATG start codon. A potential rho-independent terminator sequence (Rosenberg & Court, 1979) containing a 10 bp hyphenated dyad with a calculated ΔG^0 of $-76.6 \text{ kJ mol}^{-1}$ was found 8 bp downstream from the translational stop codon (Fig. 2).

Restriction analysis of the 9.7 kb *Sma*I fragment with the enzymes *Bam*HI, *Hind*III, *Kpn*I, *Rsr*II and *Sa*I, and comparison of the fragment patterns obtained with the restriction sites deduced from the nucleotide sequence, as well as hybridization with specific probes, allowed us to determine the position of the ORF within the fragment. All of the restriction enzyme sites expected from the nucleotide sequence were found (Fig. 1).

The nucleotide sequence of the ORF predicted a putative protein of 736 amino acids with a molecular mass of 81.8 kDa and a calculated isoelectric point of 5.97. By comparing this amino acid sequence with sequences of the SwissProt database library, significant identities and similarities were found with several bacterial bifunctional catalase-peroxidases (Table 1). The newly discovered EHEC catalase was termed KatP because of its genetic determination on plasmid pO157 (*katP*) as well as to distinguish it from the *E. coli* chromosomal HPI (KatG) and HPII (KatE) catalases.

We investigated the KatP amino acid sequence for the presence of catalase-peroxidase-specific signatures and found that the peroxidase 2 motif covered positions 94–105, and the peroxidase 1 motif positions 256–266 (Welinder, 1991).

By comparing the amino acid sequences of different bacterial catalase-peroxidases with that of the putative

EHEC KatP protein, we found the N termini of all investigated proteins to be highly variable. Analysis of the first 20–30 amino acids with the method developed by von Heijne (1986) revealed a typical signal peptide for EHEC KatP with the sequence MIKKTLPLVILLALSGSFST-AVA. The signal sequence includes two positively charged lysine residues close to the amino terminus (residues 3 and 4) and a non-polar core region spanning residues 6–14. The putative cleavage site is located between position 23 and 24 of the amino acid sequence.

Quantitative determination of the peroxidase activity of wild-type and cloned KatP

Since KatP possesses an amino-terminal signal peptide, it was likely to be transported through the cytoplasmic membrane. Therefore, periplasmic and cytoplasmic fractions, as well as culture supernatants, were investigated separately. First, the expression of EHEC *katP* was investigated by determining the specific periplasmic and cytoplasmic peroxidase activity of *E. coli* DH5 α /pSm10 (KatP⁺) using *o*-dianisidine as the substrate. The cytoplasmic peroxidase activity of this strain was twofold higher, whereas its periplasmic peroxidase activity was 20–30-fold higher when compared with vector control *E. coli* DH5 α /pBluescript IKS. This increased peroxidase activity could be caused by (i) the enzymic activity of KatP, or (ii) a regulatory effect of the pSm10 DNA on the expression of the chromosomal *katG* gene.

In order to prove that the increased peroxidase activity of DH5 α /pSm10 is determined by the *katP* gene and not by other putative genes that may be located on the 9.7 kb *Sma*I fragment of pSm10, we inactivated *katP* by insertion of a kanamycin-resistance cassette derived from pUC4K in the single *Sa*I site of the gene. Moreover, to exclude possible polar effects of the kanamycin-resistance cassette on DNA located downstream of *katP*, it was inserted in both orientations. Since plasmid pSm10 contains two further *Sa*I sites (Fig. 1), these had to be removed prior to insertion of the kanamycin-resistance cassette in the *katP* gene. To do this, we restricted pSm10 with *Kpn*I, removed the 1.1 kb *Kpn*I fragment by agarose gel electrophoresis,

Fig. 3. Specific peroxidase activities of test strains. Values of specific peroxidase activity [mU (mg total protein)⁻¹] are depicted above the respective columns together with standard deviations. Values are means of triplicate measurements.

and religated the remaining plasmid to obtain pSmK9 (Fig. 1). After excision of the kanamycin-resistance cassette from pUC4K with *Sa*I, it was inserted in the single *Sa*I site of pSmK9. The resulting clone was designated pSmK9::kana⁺ (Fig. 1). The correct insertion of the kanamycin-resistance cassette was verified by *katP*-specific PCR, restriction endonuclease digestion and DNA sequencing.

To overcome the effects of chromosomal peroxidase HPI of host strain DH5α and to exclude regulatory effects of pSmK9 on the chromosomal *katG* gene, HPI-negative *E. coli* mutant UM202 was used for additional experiments. Fig. 3 shows the results obtained when specific peroxidase activity was determined in periplasmic and cytoplasmic crude extracts of cloned *katP* in several *E. coli* hosts and controls. In the control strain DH5α, peroxidase activity was primarily found in the cytoplasmic fraction (Fig. 3). Following transformation with pSmK9 (*katP*⁺), cytoplasmic activity increased twofold. In contrast to DH5α, high periplasmic peroxidase activity could be observed (Fig. 3), which is in the same range as for pSm10. The specific peroxidase activity of vector control *E. coli* UM202/pBluescript IKS was very weak, as expected from the mutation in the *katG* gene (Fig. 3). In UM202/pSmK9 (*katP*⁺), cytoplasmic peroxidase activity increased 60-fold, whereas the periplasmic activity increased 40-fold (Fig. 3). Disruption of the *katP* gene in UM202/pSmK9::kana⁺ resulted in low cytoplasmic and periplasmic peroxidase levels, which were similar to those of the vector control strain UM202/pBluescript IKS. Furthermore, measurements of peroxidase activities of strain UM202/pSmK9::kana⁻, carrying the kanamycin-

resistance cassette inserted in the opposite orientation, were in the same range as in UM202/pSmK9::kana⁺ (Fig. 3). When the peroxidase activity of wild-type strain EDL 933 and EDL 933-cu was determined, the same high levels of cytoplasmic activity were found in both strains. However, there were differences in the periplasmic fraction. Periplasmic peroxidase activity in EDL 933 was clearly higher than in strain EDL 933-cu. These experiments indicated that the enhanced peroxidase levels observed in strains DH5α/pSmK9, UM202/pSmK9 and wild-type EDL 933 are the result of the enzymic activity of KatP.

Peroxidase activity was also determined in culture supernatants of the test strains. The tests were performed with native culture supernatants and with concentrated culture supernatants prepared by ammonium sulfate precipitation. Furthermore, these fractions (and in addition TCA-precipitated supernatants) were investigated for the KatP protein by SDS-PAGE. None of the supernatants and precipitates showed either peroxidase activity or the 80 kDa band (as expected in the presence of the KatP protein). No activity was detected by peroxidase and catalase stainings of culture supernatants and precipitates in non-denaturing gels (data not shown).

Visualization of catalase and peroxidase activities of recombinant strains on non-denaturing gels

Cytoplasmic and periplasmic crude extracts from the strains described above were separated on polyacrylamide gels under non-denaturing conditions. The gels were stained with either *o*-dianisidine (for peroxidase activity)

Fig. 4. (a) Peroxidase activity stain after non-denaturing gel electrophoresis of periplasmic (pp) and cytoplasmic (cp) fractions from test strains. Lane 1, *E. coli* DH5 α (cp), lane 2 (pp); lane 3, DH5 α /pSmK9 (cp), lane 4 (pp); lane 5, UM202/pBluescript IKS, (pp); lane 6, UM202/pSmK9 (pp); lane 7, UM202/pSmK9::kana⁺ (pp). (b) Catalase activity stain after non-denaturing electrophoresis of periplasmic fractions from strains UM202/pBluescript IKS (lane 1) and UM202/pSmK9 (lane 2). The gels shown in (a) and (b) were run together and stained separately for enzymic activity. (c) Peroxidase activity stain after non-denaturing gel electrophoresis of periplasmic (pp) and cytoplasmic (cp) fractions from *E. coli* O157:H7 strain EDL 933, lane 1 (pp), lane 2 (cp) and EDL 933-cu, lane 3 (pp), lane 4 (cp). HPI, HPII, KatP and the additional peroxidase band are indicated by arrows.

or ferricyanide (for catalase activity). Fig. 4(a) shows peroxidase-activity-stained gels with cytoplasmic and periplasmic crude extracts from our test strains.

Crude extracts from strains harbouring pSmK9 demonstrated an additional slow-migrating band (Fig. 4a, lanes 3, 4 and 6), which was not present in control strains without pSmK9 (Fig. 4a, lanes 1, 2 and 5). This band was detectable in both cytoplasmic and periplasmic fractions. The occurrence of this band seemed not to be influenced by simultaneously expressed *katG* (HPI).

Disruption of the *katP* gene in strain UM202/pSmK9::kana⁺ resulted in a lack of the additional band which was observed in KatP⁺ strains (Fig. 4a, lane 7). Therefore, this additional band with peroxidase activity was concluded to be the KatP protein. Interestingly, chromosomal HPI peroxidase activity of *E. coli* DH5 α was detected only in the cytoplasmic fraction (Fig. 4a, lanes 1 and 3).

Fig. 4(b) shows catalase activity staining of periplasmic crude extracts of UM202/pBluescript IKS (lane 1) and UM202/pSmK9 (lane 2). Aside from the weak catalase activity of *E. coli* HPII, strain UM202/pSmK9 also expresses a second catalase activity with the same migration rate as the additional band occurring in the aforementioned peroxidase-staining experiments. Together with the sequence analyses, these experiments indicate that the EHEC KatP protein acts as a bifunctional catalase-peroxidase.

Visualization of KatP expressed by wild-type strain EDL 933

When peroxidase activity stainings were performed with crude extracts of wild-type EHEC O157:H7 strain EDL 933 and its cured derivative EDL 933-cu, the KatP band was detected exclusively in the periplasmic fraction of EDL 933 (Fig. 4c, lane 1). This band was lacking in both fractions of EDL 933-cu (Fig. 4c, lanes 3 and 4). Unexpectedly, both strains showed a further band with a migration rate different from that of HPI or KatP (Fig. 4c, lanes 2 and 4). This band occurred strictly in the cytoplasmic fraction and was not detected in any of the other strains tested. Therefore, this indicates a third protein with peroxidase activity, which presumably is encoded by the chromosome or the 3.3 kb plasmid of EDL 933. To get more information about the origin of this additional band, we tested *E. coli* O157:H7 strain A8993/CS2, which lacks the 3.3 kb plasmid, by peroxidase activity staining. The same band with different migration rates from HPI and KatP as in EDL 933 was found in this strain.

Survival experiments

To assess the biological function of the KatP enzyme, H₂O₂-challenge experiments were performed with EDL 933 and its cured derivative EDL933-cu using three different methods. (i) Viable cell counts were determined after treatment of mid-exponential-phase cultures with different concentrations of H₂O₂ (Storz & Toledano, 1994). (ii) Overnight cultures of the test strains were

Table 2. Detection of the *katP* catalase-peroxidase gene in diarrhoeagenic *E. coli* by PCR with primers wkat-B and wkat-F

Strains were defined as being positive when the expected 2125 bp PCR product was detected and could be restricted with *Bam*HI into a 1.3 kb and a 0.85 kb fragment. Haemolytic activity was screened on blood-agar plates as described by Schmidt *et al.* (1994a). Detection of Shiga-like toxin genes was performed as described previously (Schmidt *et al.*, 1994b).

Pathogroup	No. of strains	<i>katP</i> PCR*	Haemolysis	<i>slt</i>
EHEC (O157)	7	7	7 (7)†	7
SLTEC (non-O157)	12	2	10 (8)†	12
EPEC	15	0	0	0
ETEC	5	0	0	0
EIEC	4	0	0	0
EAggEC	20	0	2 (0)†	0

* All *katP*-positive strains show also the enterohaemolytic phenotype.

† The numbers of enterohaemolytic strains are given in brackets, the haemolytic phenotype of the other strains resembles alpha-haemolysin.

plated on an agar plate in 3 ml soft agar and a disc impregnated with 5 µl of different H₂O₂ solutions (up to a concentration of 30 % v/v) was placed in the centre of the agar plate. After overnight incubation at 37 °C, the zones of inhibition were determined (zone of inhibition assay) (Storz & Toledano, 1994). (iii) The bacteria were spread across a H₂O₂-gradient agar plate using sterile cotton swabs. Resistance was scored as the length of growth into the gradient (Brown *et al.*, 1995). No significant differences in H₂O₂ resistance were observed between EDL 933 and EDL 933-cu by any of the methods used. However, both strains were more H₂O₂ resistant than the laboratory strain DH5α.

Screening of diarrhoeagenic *E. coli* for the presence of the *katP* gene by PCR

To investigate the distribution of the *katP* gene in diarrhoeagenic *E. coli*, a PCR assay was developed. Primers wkat-B (nt 145–167, sense strand) and wkat-F (nt 2251–2271, antisense strand) were designed to amplify a 2125 bp fragment of the EHEC-*katP* gene (see Fig. 2). The primer-binding sites were selected from regions of low similarity after comparison of the *katP* sequence with catalase-peroxidase sequences available from the EMBL/GenBank database libraries. The specificity of the PCR assay was tested with *katG*-positive *E. coli* strains HB101, DH5α and EDL 933-cu, these strains being negative by PCR. Therefore, none of the chromosomal catalase genes *katG* and *katE* were amplified by the PCR assay used here. Strains DH5α/pSmK9 and EDL 933 were positive by PCR. All PCR products were verified by restriction enzyme digestion with *Bam*HI, creating 1.3 kb and 0.85 kb

restriction fragments, in accordance with the nucleotide sequence of *katP*.

Following this, representative strains of the major groups of diarrhoeagenic *E. coli*, including EPEC, EIEC, ETEC, EAggEC, EHEC and SLTEC of other serogroups than O157 were subjected to PCR analysis using primers wkat-B and wkat-F, followed by restriction analysis with *Bam*HI. The results of these experiments (Table 2) indicate that the *katP* gene is prevalent in EHEC strains of serogroup O157 that also harbour the EHEC-haemolysin operon. All *katP*⁺ O157 strains have also been shown to express a functional KatP protein by activity tests. By testing non-O157 SLTEC we found 1/3 *E. coli* O26 strains and 1/3 *E. coli* O111 strains to be *katP*-positive. These strains were also enterohaemolytic. The EPEC, ETEC, EIEC and EAggEC strains tested here were all negative by the *katP*-specific PCR assay.

DISCUSSION

We have detected and characterized a new catalase-peroxidase (KatP) of enterohaemorrhagic *E. coli* O157, which is produced in addition to the known *E. coli* catalases HPI and HP11. The results of sequence analyses and activity tests categorize KatP, like *E. coli* HPI, as a member of the bacterial bifunctional catalase-peroxidase family. Although KatP and HPI share high sequence similarity and a similar molecular mass, their electrophoretic mobilities on non-denaturing gels differ markedly. One explanation for this phenomenon could be that the active forms of HPI and KatP consist of different subunit numbers.

Amino acid analyses of the N termini of KatP and other members of the bifunctional catalase-peroxidase family revealed a typical signal sequence only for KatP. However, this was not demonstrated for the other members. Such signal sequences are required for Sec-dependent transport of bacterial proteins through the cytoplasmic membrane. In Gram-negative bacteria, those proteins may then be located in the periplasm or be translocated through the outer membrane by the action of additional transport systems (Salmond & Reeves, 1993). Therefore, catalase and peroxidase activities of KatP were investigated in cytoplasmic and periplasmic crude extracts as well as in culture supernatants. Culture supernatants of test strains did not reveal any catalase or peroxidase activity. Furthermore, supernatants, which were precipitated with TCA and separated on denaturing polyacrylamide gels, did not reveal the expected 80 kDa band of KatP. However, we found good evidence that KatP is located primarily in the periplasm in wild-type *E. coli* EDL 933. Comparison of this strain with its plasmid-cured derivative, EDL 933-cu, showed that crude extracts of EDL 933 had higher periplasmic peroxidase activity, whereas both strains shared similar cytoplasmic peroxidase activity levels. In addition, non-denaturing gel electrophoresis of crude extracts of EDL 933 revealed the presence of the KatP band in the periplasmic fraction but not in the cytoplasmic fraction.

The HPI catalase-peroxidase, reportedly a periplasmic enzyme (Heimberger & Eisenstark, 1988) was primarily found in the cytoplasmic fraction with the preparation method used here. Only trace amounts of HPI were detected in the periplasmic fractions of EDL 933 and DH5 α . These findings are in contrast to that of Heimberger & Eisenstark (1988), who found HPI in periplasmic and membrane fractions. This may be the result of different preparation methods.

Periplasmic and cytoplasmic peroxidase activities were very high in strains DH5 α and UM202, which harbour plasmid pSmK9. High levels of enzymic activity in these strains may be due to a gene-dose effect caused by the high copy number of vector pBluescript IKS.

Since UM202 is HPI negative, peroxidase activity of UM202/pSmK9 must be caused by KatP alone. The high cytoplasmic peroxidase activity of UM202/pSmK9 was unexpected, since KatP was thought to function primarily in the periplasm in wild-type EDL 933. A possible explanation for this phenomenon may be that the export rate could not keep up with the high cytoplasmic expression of the KatP protein. As a result, there would be an accumulation of KatP in the cytoplasm.

Members of the bifunctional catalase-peroxidase family have been described in *E. coli* (Triggs-Raine *et al.*, 1988), *Mycobacterium* spp. (Morris *et al.*, 1992; Heym *et al.*, 1993), *Salmonella typhimurium* (Loewen & Stauffer, 1990), *Bacillus stearothermophilus* (Loprasert *et al.*, 1989), and *Rhodobacter capsulatus* (Forkl *et al.*, 1993). In all the aforementioned examples, the catalase-peroxidases are encoded on the chromosome. This is the first description of a periplasmic catalase-peroxidase encoded by a plasmid of an obligate bacterial pathogen. Periplasmic catalases have been described in phytopathogenic strains of *Pseudomonas syringae*. These bacteria contain multiple catalases, which are found in the cytoplasm, periplasm and the membrane fraction. The production of multiple catalases is highly correlated with the ability of *P. syringae* to colonize plant tissues (Klotz & Hutcheson, 1992).

Upon physical contact with bacteria or bacterial structures, macrophages and neutrophils produce cytotoxic oxidants during the so-called oxidative burst. It has been suggested that bacterial catalases and superoxide dismutases detoxify these agents, therefore preventing the infecting bacteria from being damaged. Catalase-mediated defence against reactive oxygen molecules represents a possible virulence factor for pathogens that have the ability to invade host cells, i.e. macrophages. This has been investigated in *Listeria monocytogenes* (Goebel *et al.*, 1993; Myers *et al.*, 1993), *Salmonella typhimurium* (Kagaya *et al.*, 1992), *Shigella flexneri* (Franzon *et al.*, 1990) and *Mycobacterium tuberculosis* (Beaman & Beaman, 1984).

To assess a function of the KatP enzyme for EHEC O157:H7 strains, H₂O₂-challenge experiments were performed. No significant differences in H₂O₂ resistance were observed between EDL 933 and EDL 933-cu. However, both strains were more H₂O₂ resistant than the laboratory strain DH5 α . Therefore, only minor effects of the KatP enzyme on EDL 933 could be observed *in vitro*.

At present, two determinants of the large plasmid pO157 have been molecularly characterized, the EHEC-haemolysin and the EHEC KatP catalase-peroxidase. The occurrence of both determinants seems to be restricted to Shiga-like-toxin-producing *E. coli*, as shown in this and in previous studies. Both proteins may have the potential to enhance the virulence of EHEC O157 strains. However, *in vitro* and, in particular, *in vivo* tests need to be conducted to address the functions of EHEC-haemolysin and KatP catalase-peroxidase to EHEC virulence.

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