KatP contributes to OxyR-regulated hydrogen peroxide resistance in *Escherichia coli* serotype O157:H7

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Escherichia coli K-12 defends itself against peroxide-mediated oxidative damage using two catalases, KatG and KatE, and the peroxiredoxin, alkyl hydroperoxide reductase, encoded by *ahpC*. In *E. coli* O157:H7 strain ATCC 43895 (EDL933), plasmid pO157 carries an additional catalase-peroxidase gene, *katP*. KatP has been shown to be a functional catalase-peroxidase. However, deletion of pO157 does not alter the peroxide resistance of strain EDL933, leaving the physiological role of *katP* unclear. To examine the individual roles of peroxide-resistance genes in *E. coli* O157:H7, mutant strains of ATCC 43895 were constructed bearing individual deletions of *katG*, *katE*, *katP* and *ahpC*, as well as double, triple and quadruple deletions encompassing all possible gene combinations thereof. The wild-type and all 15 mutant strains were compared for differences in aerobic growth, ability to scavenge exogenous H₂O₂ and resistance to exogenous peroxides. Although KatG scavenged the most exogenous H₂O₂, KatP scavenged statistically greater amounts than either KatE or AhpC during exponential growth. However, *katG* and *ahpC* together were sufficient for full peroxide resistance in disc diffusion assays. Strains with only *katG* or *ahpC* were the only triple deletion strains with significantly shorter generation times than the quadruple deletion strain. *ahpC* was the only gene that could allow rapid transition from lag phase to exponential phase in a triple deletion strain. Gene expression studies revealed that *katP* is an OxyR-regulated gene, but its expression is suppressed in stationary phase by RpoS. These studies indicate that pO157-borne *katP* contributes to the complex gene network protecting strain 43895 from peroxide-mediated oxidative damage in an OxyR-dependent manner.

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INTRODUCTION

Enteric organisms encounter peroxides from various sources, including those derived from metabolism, environmental sources and host immune responses. *Escherichia coli* has developed various defences against peroxide-mediated oxidative damage, including catalases and peroxiredoxins (Imlay, 2008; Poole, 2005). The most important system for scavenging hydrogen peroxide (H₂O₂) and organic hydroperoxides in *E. coli* is composed of the alkyl hydroperoxide reductase proteins (Ahp) encoded by *ahpCF* (Imlay, 2008; Seaver & Imlay, 2001). AhpC is a non-haem peroxidase that breaks down both H₂O₂ and organic peroxides using a cysteine-based reduction (Christman *et al.*, 1985; Poole, 2005). AhpF is a flavoprotein that reduces AhpC. *E. coli* *ahpCF* is regulated by both the peroxide-inducible transcriptional regulator OxyR (Morgan *et al.*, 1986) and RpoS (Jung & Kim, 2003).

Two chromosomally encoded catalases, KatG (Loewen *et al.*, 1985) and KatE (Loewen, 1984), also scavenge H₂O₂ in *E. coli*, and KatG becomes the major protective enzyme when high doses of H₂O₂ saturate Ahp (Seaver & Imlay, 2001). KatG is a bifunctional catalase-peroxidase regulated by OxyR (Morgan *et al.*, 1986). KatG is also induced by RpoS upon entry into stationary phase, in an OxyR-independent manner (Mukhopadhyay & Schellhorn, 1994). KatE is a catalase that is not controlled by OxyR, but is induced by RpoS during the switch from exponential to stationary growth (Schellhorn & Hassan, 1988).

OxyR is a member of the LysR family of transcriptional regulators that binds as a tetramer near the –35 region of target promoters and activates transcription by direct interaction with RNA polymerase (Imlay, 2008). Activated OxyR binds to specific sequences with four ATAGNT elements spaced at 10 base intervals (Toledano *et al.*, 1994). It is believed that specific binding is mediated by four contacts of intermediate affinity, which explains the divergence from the consensus of the binding sites in the promoters of various regulated genes (Toledano *et al.*, 1994). When the predicted binding sites in the promoters

Abbreviations: Ahp, alkyl hydroperoxide proteins; AR, Amplex Red; CH, cumene hydroperoxide; CI, 95% confidence interval; GT, generation time; HRP, horseradish peroxidase; LPD, lag phase duration.

of seven members of the OxyR regulon were compared to the consensus in that study, at least 12 of the 20 specific consensus nucleotides were matched by each member.

A plasmid-borne catalase-peroxidase gene, *katP*, was identified on the large virulence plasmid pO157 of *E. coli* serotype O157:H7 strain EDL933 and has since been shown to be widespread among Shiga toxin-producing *E. coli* (STEC) (Brunner *et al.*, 1996; Schmidt *et al.*, 1999). Although KatP is a functional catalase-peroxidase, a plasmid-cured derivative of EDL933 showed no difference in resistance to exogenous peroxides compared to the parent strain expressing KatP, suggesting that *katP* may not have a functional role in H₂O₂ resistance (Brunner *et al.*, 1996). KatP was shown to have an N-terminal signal peptide and accumulated primarily in the periplasm (Brunner *et al.*, 1996). However, little else is known about the expression, regulation and/or functional role of KatP in STEC. The large plasmid of STEC carries genes such as *katP*, *espP* (serine protease) and the *ehx* (enterohaemolysin) operon, which are considered virulence-associated genes, although a direct relationship with virulence has yet to be established (Law, 2000). The enterohaemolysin encoded by the *ehxCABD* operon of pO157 was shown to be regulated by the *grlA* gene, located in the locus of enterocyte effacement (LEE) (Saitoh *et al.*, 2008). GrlA positively regulates LEE gene expression by effects on the LEE transcriptional activator, Ler (Deng *et al.*, 2004). Overexpression of *grlA* induced the pO157-encoded haemolysin and suppressed flagellar expression (Iyoda *et al.*, 2006; Saitoh *et al.*, 2008). However, the regulation of *katP* has not been thoroughly investigated.

In a previous study, using a biofilm-forming variant of *E. coli* O157:H7 strain ATCC 43895, we showed that biofilm cells formed on glass were more resistant to H₂O₂ than their planktonic counterparts (Uhlich *et al.*, 2006a). We also determined that the non-biofilm-forming strain ATCC 43895 could attach to glass, but did so in scattered, patchy monolayers without forming a stainable, three-dimensional biofilm. Surprisingly, the patchy cells were more resistant to H₂O₂ than cells in the robust biofilm, suggesting that the resistance of attached cells may rely more on the expression of peroxide resistance genes than on physical occlusion of H₂O₂ by the biofilm. To compare the resistance mechanisms used by planktonic and biofilm cells of *E. coli* O157:H7 strain 43895, it was necessary to first investigate the role of *katP* and its relationship with the other peroxide-resistance genes of serotype O157:H7. In this study mutant strains of 43895 were constructed bearing individual deletions of *katG*, *katE*, *katP* and *ahpC*, and double, triple and quadruple deletions encompassing all possible gene combinations, to compare the role of each gene in the peroxide resistance of *E. coli* ATCC 43895.

METHODS

Bacterial strains, media and growth assays. *E. coli* strain ATCC 43895 [Centers for Disease Control and Prevention (CDC) EDL933]

was obtained from the American Type Culture Collection (ATCC). It is referred to hereafter as strain 43895. *E. coli* strain 43895OR is a phase-variable, curli-producing strain isolated from a freezer stock of the non-curliated strain 43895 that switches back to strain 43895 by a one-way phase variation involving a single nucleotide transversion in the *csgD* promoter (Uhlich *et al.*, 2001). Mutations reported in this study were generated first in strain 43895OR for use in a separate study. Strains of 43895OR bearing each gene deletion(s) were allowed to phase-switch to the non-curli-expressing parent 43895 (Uhlich *et al.*, 2009). The mutant strains of 43895 were frozen in brain heart infusion broth (BHI) (Becton Dickinson) with 15 % glycerol and used for characterization of peroxide resistance in serotype O157:H7. For growth studies in broth, 24 h cultures of strain 43895 and the mutant strains, grown in BHI at 37 °C, were passed 1:100 into fresh BHI, grown for an additional 18 h, and diluted 1:100 into 20 ml BHI in 250 ml flasks. Cultures were grown at 37 °C in a shaking water bath and optical density (600 nm) measurements were taken every 30 min for 4 h. Growth curves from the results of three independent trials from each strain were generated by the Gompertz function, substituting absorbance data for log(c.f.u. ml⁻¹) (Buchanan & Phillips, 1990). The calculated lag phase duration (LPD) and generation times (GT) were subjected to analysis of variance and Bonferroni means separations (Miller, 1981). For growth studies on agar, strains were streaked from a single colony onto LB (Becton Dickinson) agar containing 100 µg ampicillin ml⁻¹ and 1 mM IPTG, and incubated at 37 °C for 18 h. Swimming motility assays were performed as previously described (Uhlich *et al.*, 2009).

DNA cloning and construction of recombinant plasmids.

TransforMax EC100 (Epicentre Biotechnologies) was used as the host for all cloning steps unless otherwise specified, and was propagated in BHI or LB broth. The entire *katP* and *grlA* genes were PCR amplified using primers agagaatttccttaacccgagctattcg/agactc-gagtttctcctcatgagggaggtg and cgaaattccctcggtgctaaa/agaaagcttcga-gaaaaggcttaccctgg, respectively, and cloned into pCR2.1 TOPO (Invitrogen). *katP* and *grlA* were excised at the *EcoRI/XhoI* and *EcoRI/HindIII* sites, respectively, and moved into corresponding sites behind the IPTG-inducible *trc* promoter on plasmid pSE380 (Invitrogen) to produce plasmids pSE380-KatP and pSE380-GrlA.

Construction of mutant strains. Strains with the target gene deletions are shown in Table 1. Mutations in the *katG*, *katE*, *katP*, *ahpC*, *rpoS* and *oxyR* genes were constructed using the Quick and Easy Gene Deletion kit (Gene Bridges). Forward and reverse primers were designed from the available EDL933 genome sequence (GenBank accession no. AE005174) to provide 60 bp of upstream and downstream sequence homology that terminated with the start and stop codons of the target genes. The 3' ends of each primer included sequence complementary to kit DNA template, which contained a neomycin-resistance cassette flanked by FRT recombination sites. The primers were used to generate PCR products consisting of the target genes and flanking sequences where the entire sequence between start and stop codons was replaced by the neomycin-resistance cassette. Linear PCR templates of the mutated genes were integrated into strains by Red/ET recombination directed from plasmid pRedETtet and the neomycin-resistance cassettes were resolved from all chromosomal integrations generating non-polar mutations by the manufacturer's protocols (Gene Bridges). The neomycin-resistance cassette was not resolved from the *oxyR*-deleted strain as the growth impairments under aerobic conditions made screening for resolved strains difficult. However, the *oxyR* gene of strain 43895 is on the opposite strand of the downstream gene and would be unlikely to create polar effects. The correct integration of all interrupted genes was verified by PCR using primers (not shown) that were designed from sequences outside the chromosomal regions that provided homology for recombination.

Table 1. *E. coli* serotype O157:H7 strain ATCC 43895 (43895) and mutant derivatives containing deletions of specified genes

Strain (abbreviation)	Deleted gene(s)
43895	
43895-G (G)	<i>katG</i>
43895-E (E)	<i>katE</i>
43895-C (C)	<i>ahpC</i>
43895-P (P)	<i>katP</i>
43895-GE (GE)	<i>katG, katE</i>
43895-GC (GC)	<i>katG, ahpC</i>
43895-GP (GP)	<i>katG, katP</i>
43895-CE (CE)	<i>ahpC, katE</i>
43895-EP (EP)	<i>katE, katP</i>
43895-CP (CP)	<i>ahpC, katP</i>
43895-GCP (GCP)	<i>katG, ahpC, katP</i>
43895-GCE (GCE)	<i>katG, ahpC, katE</i>
43895-GEP (GEP)	<i>katG, katE, katP</i>
43895-CEP (CEP)	<i>ahpC, katE, katP</i>
43895-GCEP (GCEP)	<i>katG, ahpC, katE, katP</i>
43895-RpoS	<i>rpoS</i>
43895-OxyR	<i>oxyR</i>

H₂O₂ detection. H₂O₂ was quantified using Amplex Red (AR) (Invitrogen). In the presence of H₂O₂, horseradish peroxidase (HRP) converts AR to the fluorescent oxidation product resorufin, which can be detected spectrophotometrically or fluorometrically. Using the H₂O₂ detection method described by Seaver & Imlay (2001), we generated a standard curve to convert fluorescence data to H₂O₂ concentration. Eight serial 1:2 dilutions of a 2.5 µM H₂O₂ (Sigma-Aldrich) solution were prepared in phosphate buffer (50 mM KH₂PO₄; pH 7.8), loaded in quadruplicate wells of a 96-well black polystyrene plate (Denville Scientific) and measured on a Safire² (Tekan US) microplate reader at wavelength settings of 530 nm (excitation) and 590 nm (emission); the fluorescence data were plotted against H₂O₂ concentration.

H₂O₂-scavenging assay. The ability of various strains to break down H₂O₂ was measured by a previously described assay (Seaver & Imlay, 2001) with the following modifications. For exponential phase studies at 37 °C, overnight cultures of each strain in BHI broth were inoculated 1:100 into 10 ml fresh BHI broth and grown to OD₆₀₀ 0.3. A 5 ml aliquot of each strain was washed twice in PBS and resuspended in PBS to OD₆₀₀ 0.1. For stationary phase experiments at 37 °C, 24 h cultures of strains in BHI broth were subcultured (1:100) in 10 ml fresh BHI broth and grown for an additional 18 h. Cultures were washed in PBS, resuspended to OD₆₀₀ 0.1, and washed a second time with PBS. For *katP* complementation of scavenging in stationary phase, strain 43895-GCEP/pSE380-KatP was incubated for 17 h in the presence of 100 µg ampicillin ml⁻¹ and protein expression was induced with 1 mM IPTG for 1 h. H₂O₂ was added to all samples to a concentration of 1.5 µM and samples were assayed for H₂O₂ as described above after 30 min incubation at 25 °C. Eight replicates from each of three independent samples from each strain were tested in a 96-well format. The results were analysed by an analysis of variance and the significance of the means was tested using the Bonferroni technique.

H₂O₂ disc diffusion assay. The resistance of strains to peroxide challenge was measured using a disc diffusion assay as previously described (Uhlich *et al.*, 2006b). Discs were saturated with either H₂O₂ diluted in sterile water or cumene hydroperoxide (CH) (Sigma-

Aldrich) diluted in DMSO, and the diameters of the induced zones of inhibition were recorded after 18 h at 37 °C. The results of eight replicates from two independent samples from each strain were analysed using an analysis of variance with means separation by the Bonferroni technique. Comparisons of strains 43895-GCEP/pSE380 and 43895-GCEP/pSE380-KatP were performed on agar containing 100 µg ampicillin ml⁻¹ and 1 mM IPTG. Eight replicates of three independent samples of each strain were compared using Student's *t* tests.

Quantitative real-time reverse-transcriptase PCR (qRT-PCR).

The transcription of *katG*, *katE*, *katP* and *ahpC* was compared under various conditions by qRT-PCR using primer pairs aaactgacgcacagg-gatatgg/cagatcgataatgtctgctcgc, cacaatcagtcgtcttggtgg/atcgttgatcgagtt-cggttcg, tgcctgcacccgttgatgatg/tggatcattcaggaaacgggtg and aagcagca-cagtacgtagcttc/tttaccaaccaggtccagagac, respectively. Strains were prepared for RNA extraction by inoculating LB (1:100) with 24 h cultures and growing to OD₆₀₀ 0.3 (exponential phase) or for 24 h (stationary phase) at 37 °C. Aliquots of 20 ml (exponential samples) or 3 ml (stationary samples) were exposed to 1 mM H₂O₂ (induced) or equal volumes of H₂O (non-induced) for 10 min at 37 °C and RNA was extracted using TRI Reagent (Molecular Research Center). Contaminating DNA was removed by DNase I digestion using Turbo DNA-free (Ambion) and cDNA was generated from 1 µg total RNA using the SuperScript III First Strand Synthesis System (Invitrogen). qRT-PCR was performed on a 7900HT Fast Real-time PCR System (Applied Biosystems) using 20 µl reactions containing 1.2 ng cDNA (or 1.2 ng RNA as a negative control), 10 pmol of each primer (Integrated DNA Technologies) and 10 µl Fast Syber Green Master Mix (Applied Biosystems). Amplification of the *gnd* gene (Rashid *et al.*, 2006), using primer pair catcggcaccaataggtgaac/gggcagaagaagcc-tatgaac, was used as a control to normalize the results. The *gnd* gene amplified with similar efficiency to the target genes and had constant expression under the conditions tested. The reactions were subjected to a 20 s hold at 95 °C followed by 40 cycles of 1 s at 95 °C and 20 s at 64 °C. Real-time PCR data were analysed using the 2^{-ΔΔC_T} method (Livak & Schmittgen, 2001). Expression values from the mean of triplicate measurements of PCR product amplified from cDNA generated from four independent cultures of each strain were converted to fold-change in expression compared to the reference strain. The 95 % confidence interval (CI) of the fold changes were estimated by performing the 2^{-x} transformation on the confidence limits of ΔΔC_T. Increases or decreases in expression were considered significant if the CI of the fold change excluded 1.

RESULTS

katG and *katP* are the major H₂O₂ scavengers in strain 43895 when challenged with high concentrations of H₂O₂

When exponential-phase cultures were compared, strain 43895-GCEP scavenged less (*P*<0.05) exogenous H₂O₂ than strain 43895 (Fig. 1). Triple deletion strains 43895-GCP and 43895-GEP, which carry only *katE* and *katC*, respectively, of the major peroxide resistance genes, and the double deletion strain 43895-GP, which carries both the *katE* and *ahpC* genes, scavenged no more (*P*>0.05) H₂O₂ than strain 43895-GCEP. Strains 43895-G and 43895-GE broke down more (*P*<0.05) H₂O₂ than the quadruple mutant, but less (*P*<0.05) than the wild-type strain. All other strains were not significantly different (*P*>0.05) in their scavenging ability from the wild-type strain, including

Fig. 1. Comparison of the H₂O₂-scavenging abilities of strain 43895 and 15 mutant strains with deletions of peroxide resistance genes as shown in Table 1. Strains grown to exponential (shaded bars) or stationary (white bars) phase at 37 °C were exposed to 1.5 μM H₂O₂, incubated for 30 min at 25 °C in the presence of AR and HRP, and the captured fluorescence data were converted to μM H₂O₂ using a standard curve. Each bar represents the mean concentration (±SE) of H₂O₂ remaining in eight replicates each of three independent samples of each strain. Italicized letters represent the results of a Bonferroni means separation for exponential phase (upper case) or stationary phase (lower case) comparisons, where strains with the same letter are not significantly different from each other.

the triple deletion strains 43895-CEP and 43895-GCE, which carried only the resistance genes *katG* and *katP*. Collectively, these data indicate that under the tested conditions, *katG* and *katP* encode proteins with the greatest exogenous H₂O₂-scavenging ability in strain 43895, with *katG* being more important than *katP*. While *ahpC* scavenged small amounts of H₂O₂, *katE* showed no significant scavenging activity. During exponential growth, deletion strains in which *katG* was one of the deleted genes showed significantly less ($P < 0.05$) scavenging ability than the wild-type strain, except for strains 43895-GC and 43895-GCE, which were not different ($P > 0.05$) from strain 43895. This indicates that double or certain triple deletion strains missing both *katG* and *ahpC* had a greater ability to scavenge H₂O₂ than mutants missing *katG* alone.

When stationary-phase cultures of the 15 mutant strains were compared to the wild-type strain (Fig. 1), strain 43895-GCEP had significantly less ($P < 0.05$) H₂O₂-scavenging ability than strain 43895. The triple deletion strain with only *katE* remaining (strain 43895-GCP) had no more ($P > 0.05$) scavenging ability than strain 43895-GCEP, while strains 43895-CEP, 43895-GCE and 43895-GEP, containing only *katG*, *katP* or *ahpC*, respectively, had scavenging abilities comparable ($P > 0.05$) to the wild-type strain 43895. In general, single and double deletion strains tested in stationary phase showed little loss of scavenging compared to the wild-type strain, suggesting that there is considerable functional overlap of the peroxide resistance

genes during the stationary phase. Strain 43895-GP was the only double deletion strain that scavenged significantly less ($P < 0.05$) H₂O₂ than strain 43895, indicating that KatG and KatP were the most important scavengers during the stationary phase. When strain 43895-GCEP containing pSE380-KatP was tested for scavenging ability in stationary phase we could detect no H₂O₂ in the samples (mean H₂O₂ concentration -0.32 μM, SD 0.12 μM), indicating that a *trans*-copy of *katP* complemented the scavenging deficiencies of the 43895-GCEP.

The H₂O₂ concentrations greater than 1.5 μM detected in some samples likely resulted from H₂O₂ derived from the medium or generated by the dye/HRP detection system (Seaver & Imlay, 2001). However, as all the strains were tested under identical conditions, this additional H₂O₂ would not affect the relative comparisons between strains in this study.

***katG* and *ahpC*, but not *katP*, are important for protection against exogenous organic and inorganic peroxide challenge**

The results of peroxide challenge assays using either H₂O₂ or CH-impregnated discs are shown in Fig. 2. When challenged with H₂O₂, strain 43895-G was the only strain bearing a single gene deletion that showed a zone of inhibition significantly greater ($P < 0.05$) than strain 43895. When challenged with CH, strain 43895-C had a larger

Fig. 2. Comparison of the H₂O₂ sensitivities of strain 43895 and the 15 mutant strains with deletions as shown in Table 1. Strains were plated on BHI agar and exposed to discs saturated with 125 µg H₂O₂ (shaded bars) or CH (white bars). Means were calculated from eight discs incubated on each of two independent samples for each strain. Bars represent the mean zones of inhibition ($\pm$ SE) formed on each strain minus the mean inhibitory zone formed on the wild-type strain, 43895. Italicized letters represent the results of a Bonferroni means separation for H₂O₂- (upper case) or cumene hydroperoxide- (lower case) challenged strains, where strains with the same letter are not significantly different from each other.

($P < 0.05$) inhibition zone than 43895 and was the only strain bearing a single gene deletion that showed a significant difference in inhibition zone size compared to strain 43895. When strains bearing triple deletions were compared to strain 43895-GCEP, which would be expected to have minimal resistance to peroxides, resulting in the largest zone of inhibition, strains containing only *katP* (43895-GCE) or only *katE* (43895-GCP) had inhibition zones that were not significantly different ($P > 0.05$) from strain 43895-GCEP following challenge with either organic or inorganic peroxide. When strain 43895-GEP, with only *ahpC*, was challenged with H₂O₂, a zone of inhibition greater ($P < 0.05$) than that of 43895 but less ($P < 0.05$) than that of 43895-GCEP was produced. When challenged with CH, the zone of inhibition was not different ($P > 0.05$) from 43895. When strain 43895-CEP, containing only *katG*, was challenged with H₂O₂ or CH, the zone of inhibition was not different ($P > 0.05$) from that shown by strain 43895. These results indicate that in challenges using peroxide-saturated discs, *katG* and *ahpC* provided all of the peroxide resistance, with *katG* providing more resistance against H₂O₂ and *ahpC* providing more resistance against organic peroxide.

When strains 43895-GCEP/pSE380 and 43895-GCEP/pSE380-KatP were compared in disc diffusion assays, the mean inhibitory zone (MIZ, mm) surrounding H₂O₂-impregnated discs was not significantly different ($P > 0.05$) between the two strains (MIZ 20.46, SD 0.51 for 43895-

GCEP/pSE380; MIZ 20.27, SD 0.68 for 43895-GCEP/pSE380-KatP). When challenged with CH, there was no difference ($P > 0.05$) between strains 43895-GCEP/pSE380 (MIZ 23.10, SD 0.46) and 43895-GCEP/pSE380-KatP (MIZ 23.40, SD 0.37). Overexpression of *katP* from an inducible promoter in strain 43895-GCEP did not provide protection against organic or inorganic peroxides.

***ahpC* and *katG* contribute to aerobic growth of strain 43895 in broth**

The results of aerobic growth studies comparing strain 43895 and the 15 mutant strains in BHI broth are shown in Table 2. For both LPD and GT, strain 43895-GCEP showed significant ($P < 0.05$) increases compared to the wild-type strain 43895. Strain 43895-GEP was the only triple deletion strain with a LPD not different ($P > 0.05$) from strain 43895 and different ($P < 0.05$) from strain 43895-GCEP, indicating that *ahpC* was the most important gene for shortening lag phase. Strain 43895-CP was the only double deletion strain that was different ($P < 0.05$) from strain 43895, indicating that *katP* may also be important in preparing strain 43895 for exponential growth. Strains 43895-CEP and 43895-GEP were the only triple deletion strains with GT significantly shorter ($P < 0.05$) than strain 43895-GCEP, indicating that *katG* and *ahpC* play a greater role in maintaining maximum growth in strain 43895 than *katE* or *katP*.

Table 2. Comparison of growth curves generated from strains 43895 and the 15 deletion mutants by plotting the OD₆₀₀ of cultures against time

The growth curves were analysed by fitting a Gompertz model to each trial and gene deletion combination. The calculated lag phase duration (LPD) and generation time (GT) for each strain were subjected to an analysis of variance and the means were separated using the Bonferroni technique. Strains with the same letter are not significantly different from each other.

Strain	LPD (min)	Bonf.	GT (min)	Bonf.
GCEP	84.65	ab	35.22	a
CEP	80.40	abc	28.01	cd
GEP	65.51	e	27.51	cd
GCP	91.77	a	33.34	ab
GCE	79.87	abcd	30.36	abc
EP	67.23	cde	26.56	cd
CP	85.49	ab	26.76	cd
CE	73.69	bcde	26.52	cd
GP	65.96	de	28.25	cd
GE	66.84	cde	26.29	cd
GC	75.57	bcde	30.11	bcd
P	75.07	bcde	26.86	cd
E	65.55	e	26.49	cd
C	65.55	e	25.58	cd
G	73.28	bcde	25.43	d
43895	65.16	e	28.82	bcd

katP* supports growth of strain 43895 on agar in the absence of *katG*, *ahpC* and *katE

When single colonies of strains 43895-GCE/pSE380, 43895-GCEP/pSE380 and 43895-GCEP/pSE380-KatP were streaked onto LB agar containing IPTG, strain 43895-GCEP/pSE380 showed reduced growth compared to strain 43895-GCE/pSE380 (Fig. 3). The growth of strain 43895-

GCEP carrying the IPTG-inducible, recombinant *katP* plasmid was similar to that of strain 43895-GCE.

katP*, *katG* and *ahpC* are regulated by *oxyR* and *katE* is regulated by *rpoS

When the expression of peroxide-resistance genes in H₂O₂-induced and non-induced strains of 43895 was compared (Table 3), *katG*, *ahpC* and *katP*, but not *katE*, were found to be significantly ($P < 0.05$) upregulated following peroxide induction in exponential-phase cells. In stationary phase, there was also a significant upregulation of *katG* and *ahpC*, although the average fold-change increase was less than that observed in exponential-phase cells. There were no significant differences in the expression of either *katP* or *katE* in the induced compared to the non-induced strains in stationary phase. To determine if the H₂O₂-induced expression changes were mediated by OxyR, an *oxyR*-deleted strain of 43895 was compared under H₂O₂-induced and non-induced conditions (Table 3). There was no significant induction ($P > 0.05$) of any of the four genes during either exponential or stationary phase, indicating that the changes in *katG*, *ahpC* and *katP* expression were *oxyR* dependent. The expression of *katP* was also compared between H₂O₂-induced cultures of 43895 and 43895-OxyR. There was 5.10-fold (CI 2.10–12.49) lower ($P < 0.05$) expression of *katP* in strain 43895-OxyR compared to strain 43895 after both strains were challenged with H₂O₂ during exponential growth. In stationary-phase cells, there was a slight (1.02-fold, CI 0.13–7.66) but insignificant ($P > 0.05$) increase in *katP* expression in the H₂O₂-induced 43895-OxyR compared to the induced 43895. The induction of *katP* in exponential phase was lost in the *oxyR* mutant.

Using the consensus DNA motif proposed as the DNA recognition site for interaction with OxyR (Toledano *et al.*,

Fig. 3. Growth of strains 43895-GCE/pSE380 (a), 43895-GCEP/pSE380 (b) and 43895-GCEP/pSE380-KatP (c) at 37 °C on LB agar supplemented with 100 µg ampicillin ml⁻¹ and 1 mM IPTG.

Table 3. Relative fold increase (+) or decrease (–) in the expression of *katG*, *ahpC*, *katE* and *katP* in the indicated target strains as compared to reference strains

RNA was harvested from strains grown in LB broth at 37 °C to an OD₆₀₀ of 0.3 (exponential phase) or after 24 h incubation (stationary phase). Gene expression was determined by qRT-PCR and normalized to the expression of the *gnd* gene. The RNA collected from four independent samples was reverse transcribed and used to prime triplicate reactions that were analysed by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001) and converted to fold-change in expression compared to the reference strain.

Reference strain	Target strain	Exponential phase				Stationary phase			
		<i>katG</i>	<i>ahpC</i>	<i>katE</i>	<i>katP</i>	<i>katG</i>	<i>ahpC</i>	<i>katE</i>	<i>katP</i>
43895 non-induced	43895 H ₂ O ₂ -induced	+30.06*	+6.68*	+1.97	+5.24*	+16.00*	+2.71*	+4.38	+2.25
43895ΔoxyR non-induced	43895ΔoxyR H ₂ O ₂ -induced	+1.49	+1.52	+2.27	+1.22	+1.00	–1.37	–1.52	–1.35
43895 non-induced	43895ΔrpoS non-induced	–1.49	–2.45	–1.51	–1.04	+1.60	+1.64	–16.67*	+4.47*
43895 H ₂ O ₂ -induced	43895ΔrpoS H ₂ O ₂ -induced	–1.52	–1.81	–2.65	+1.13	+2.32	+1.90*	–20.00*	+2.31

*Values marked by an asterisk represent expression increases or decreases where the 95 % confidence interval of the fold-change excluded 1.

1994), the promoter region of strain 43895 *katP* was scanned for similar sites. In the 92 bp region between *katP* and the predicted upstream gene L7016, there was a 37 bp sequence, starting 78 nucleotides upstream of the *katP* start codon, that conformed to the consensus and matched 14 of the 20 specific nucleotides in the predicted contact regions. The last nine bases of the site overlapped the predicted σ^{70} *katP* promoter region. Examination of the *ahpC* and *katG* promoters of strain 43895 revealed consensus OxyR-binding sites that matched 12 and 14 specific bases of the consensus, respectively, and terminated 1 bp upstream in each predicted –35 promoter region.

To determine the effect of *rpoS* on gene expression, an *rpoS*-deficient strain was compared with the wild-type 43895 under both H₂O₂-induced and non-induced conditions (Table 3). When non-induced strains were compared, there was no difference in expression levels of any of the tested peroxide-resistance genes in exponential phase. However, in stationary phase there was a significant decrease ($P<0.05$) in the level of *katE* and an increase ($P<0.05$) in *katP* in strain 43895-RpoS compared to 43895. When induced cultures of 43895-RpoS were compared with induced cultures of 43895, there were no significant ($P>0.05$) changes in expression of any gene in exponential phase, but in stationary phase there was significant decrease ($P<0.05$) in *katE* expression and a slight but significant ($P<0.05$) increase in *ahpC* expression in the *rpoS*-deleted strain compared to the wild-type. The increase in *katP* expression in the *rpoS*-deleted strain seen during stationary phase was not present when the strains were exposed to H₂O₂.

GrlA does not affect *katP* expression

When strain 43895 carrying either plasmid pSE380 or pSE380-GrlA was tested in a swimming motility assay in the presence of IPTG, the presence of pSE380-GrlA

resulted in a >50 % reduction in the migration zone, indicating that the recombinant GrlA was functional and suppressed flagellar motility as described by Iyoda *et al.* (2006) (results not shown). When the expression of *katP* and *katE* was tested in strain 43895 carrying either pSE380-GrlA or pSE380 there was a slight, but non-significant ($P>0.05$), reduction in the transcription of both *katP* [mean fold change (MFC) 2.55] and *katE* (MFC 2.11) in the presence of recombinant GrlA, indicating that GrlA is not required for *katP* or *katE* expression under the tested conditions. Although GrlA regulates the pO157-encoded haemolysin (Saitoh *et al.*, 2008), this study found no evidence that it regulates *katP*.

DISCUSSION

The results of the scavenging studies indicated that KatG was the major H₂O₂ scavenger in strain 43895, but that KatP could also scavenge significant amounts of exogenous H₂O₂. The lower scavenging ability of AhpC was not unexpected as it has been shown that while Ahp is the major scavenger of low levels of H₂O₂, it becomes saturated at higher levels and KatG becomes the major scavenger (Seaver & Imlay, 2001). This study indicates that *katG* and *katP* could both encode important scavengers of high H₂O₂ levels in strain 43895. KatE failed to demonstrate scavenging ability in any of the strains, including the triple deletion strain where *katE* was the only remaining major resistance gene. Although lack of KatE activity in exponential-phase cells would be expected, it is worth noting the inactivity in stationary phase, where transcription levels calculated from H₂O₂-exposed cells collected at the same time point indicated that RpoS had induced a 20-fold increase in *katE* transcripts. Apparently the increases in *katE* could not be used for protection against exogenous H₂O₂.

In general the triple deletion strains were the most informative in this study as they allowed examination of the individual phenotypes without overlapping effects from the other three resistance genes. Strains 43895-GCE and 43895-CEP both had scavenging abilities comparable to strain 43895. As a result, successive deletion of additional resistance genes in strains containing an intact *katG* or *katP* would be expected to show no additional alterations in H₂O₂ scavenging. This proved true except for strains 43895-GC and 43895-GCE, which had more scavenging ability during exponential growth than strain 43895-G. It is unclear why deletion of *ahpC* or *ahpC/katE* in strain 43895-G would increase scavenging in exponential phase. One possibility might be that *ahpC* expression repressed another peroxide-scavenging gene in the *katG* mutant strain, which was derepressed by deletion of *ahpC*. Clearly, more studies will be needed to explain these findings. In contrast to the scavenging assays, strains 43895-GC and 43895-GCE provided less protection against exogenous H₂O₂ than 43895-G in disc diffusion assays. This is a more expected outcome with the progressive deletion of resistance genes. It is unclear why a greater scavenging ability would not allow increased protection against exogenous peroxides in the disc diffusion assays for those strains.

When the triple deletion strain (43895-GCE) bearing only *katP* was tested in disc inhibition assays, *katP* generated no more protection against either organic or inorganic peroxide than that seen in the strain missing all four potential major peroxide resistance genes. It is unknown why KatP, in strain 43895-GCE, was able to scavenge exogenous H₂O₂ but not provide protection against the same peroxide in a disc diffusion assay. One possibility may be that there was a failure to activate or a suppression of *katP* transcription under the conditions used in the disc assay. However, when *katP* was placed behind an inducible promoter on a high-copy-number plasmid, it did not provide protection against organic or inorganic peroxide in strain 43895-GCEP, suggesting that a lack of *katP* expression in strain 43895-GCE is not responsible for the observed peroxide protection failure in disc assays. The inducible plasmid-borne *katP* was able to complement the growth deficiencies of 43895-GCEP on agar plates and increase H₂O₂ scavenging, indicating that a functional protein was being produced in strain 43895-GCEP. Brunder *et al.* (1996) showed that KatP is predominantly located in the periplasm and that KatG was located primarily in the cytoplasm of strain EDL 933. Whether the periplasmic KatP can protect the cell from H₂O₂ that rapidly diffuses across membranes (Seaver & Imlay, 2001) when cytoplasmic sources of protection have been eliminated is unknown. Additional studies to determine the kinetic efficiency of KatP are needed to answer that question.

The disc assays showed that *katG* is the most important gene for protection against high levels of exogenous H₂O₂ and that *ahpC* is the most important for resistance to

exogenous organic peroxides in strain 43895, although both are required for full resistance to either peroxide. This agrees with previous published results using K12 strains (Seaver & Imlay, 2001; Storz *et al.*, 1989). KatE, which has catalase but not alkyl peroxidase activity (Claiborne *et al.*, 1979), would not be expected to protect against CH. The lack of protection against H₂O₂ is also not surprising in light of its inability to scavenge exogenous H₂O₂ in the AR assays. Moreover, the disc assay challenges growing cells, in which the RpoS-dependent *katE* could be inactive.

Under the conditions tested in this study, all of the single, double and triple gene deletion strains achieved growth comparable to the wild-type. However, deletion of *katG* and *ahpC* significantly shortened the GT compared to strain 438950-GCEP, indicating that *katG* and *ahpC* do influence growth. The importance of these genes in maintenance of 43895 growth may be more evident under different conditions. The contributions of *katP* to growth of strain 43895 did become more obvious on the surface of LB agar. Strain 43895-GCE and strain 43895-GCEP carrying an inducible *katP* both showed heavier growth than strain 43895-GCEP.

The qRT-PCR expression data indicated that H₂O₂ exposure resulted in the induction of *katP* in exponential phase. Moreover, these experiments also confirmed that *katG* and *ahpC*, but not *katE*, were also induced in both exponential and stationary phases. In general, the induction of genes was greater in exponential phase compared to stationary phase. This is in agreement with González-Flecha & Demple (1995), who reported higher expression from H₂O₂-induced *katG*:: β -galactosidase fusions in exponential than in stationary phase. The induction deficiencies of the OxyR mutant strain add *katP* to the OxyR regulon.

The 16- to 20-fold drop in *katE* expression in the *rpoS* deleted strain compared to the wild-type strain in stationary, but not in exponential phase, confirms that strain 43895 *katE* is an RpoS-dependent, stationary-phase-induced gene as described for *E. coli* K-12 (Schellhorn & Hassan, 1988). A greater than fourfold induction of *katP* in stationary phase following deletion of *rpoS* was observed in comparisons of non-induced strains but not in H₂O₂-induced strains. This suggests that RpoS could have a suppressive effect on *katP* in strain 43895, which is lifted during peroxide stress. This is interesting, as studies have shown that *rpoS* has a positive effect on the OxyR-regulated *katG* and *ahpC* in *E. coli* K-12 strains (Jung & Kim, 2003; Mukhopadhyay & Schellhorn, 1994). Using promoter fusions measured over time, Mukhopadhyay & Schellhorn (1994) found that an *rpoS* deletion resulted in a twofold drop in the baseline expression. We did not observe a positive regulation of *katG* and *ahpC* by RpoS in this study. However, while qRT-PCR comparisons at a single time point allowed screening of larger numbers of genes under more conditions, they may have missed modest variations in expression. Moreover, the analysis

using pooled estimates of standard error, although appropriate for large datasets, may not have been stringent enough to detect small differences in expression. Individual comparisons using promoter fusions may be needed to fully define the effect of RpoS on the expression of *katG*, *ahpC* and *katP*. However, if basal *katG* and *ahpC* expression is maintained by RpoS, simultaneous suppression of *katP* by RpoS may provide a mechanism to minimize unnecessary KatP expression when oxidative stresses are minimal. KatP would be expressed only when higher peroxide levels activated OxyR. When H₂O₂-induced strains were compared, there was also a small increase in *ahpC* expression in strain 43895Δ*rpoS* relative to strain 43895 in stationary phase. However, the increase was less than twofold and was not seen with comparisons of non-induced strains.

This study has shown that the peroxide-resistance genes *katG*, *ahpC* and *katE* of *E. coli* serotype O157:H7 strain 43895 are similar in regulation and function to those of *E. coli* K-12. It has also demonstrated that the plasmid-encoded KatP is an OxyR-regulated peroxide scavenger that could protect strain 43895 during aerobic growth. Clearly, KatP can function as an H₂O₂ scavenger in serotype O157:H7 strains, but the conditions under which it operates remain elusive. Future studies contrasting the function and regulation of the major catalase/peroxidase genes under different growth conditions, in biofilms, or in challenges with host immune cells may reveal an environment where *katP* assumes a more important role in oxidative stress resistance.

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