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3-phenylpropionate catabolism and the *Escherichia coli* oxidative stress response

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Abstract

Cells have devised a variety of protection systems against the toxic effects of dioxygen. Dioxygenases are part of this defence mechanism. In *Escherichia coli*, the positive regulator HcaR, a member of the LysR family of regulators, controls expression of the neighbouring genes, *hcaA1*, *hcaA2*, *hcaC*, *hcaB* and *hcaD*, coding for the 3-phenylpropionate dioxygenase complex and 3-phenylpropionate-2',3'-dihydrodiol dehydrogenase, that oxidizes 3-phenylpropionate to 3-(2,3-dihydroxyphenyl) propionate. Differences between expression of *hcaR* and expression of its target, *hcaA*, suggest that HcaR is involved in control of other cellular processes or that other regulatory proteins modulate *hcaA* expression. Protein expression profiling was used to identify other HcaR targets. Two-dimensional gel electrophoresis was used to compare the proteomes of wild-type *E. coli* and strains in which *hcaR* was disrupted. Several polypeptides whose production was up- or downregulated in the *hcaR* mutant were involved in the oxidative stress response. Subsequent experiments demonstrated that *hcaR* disruption was involved in regulation of genes involved in the oxidative stress response. Modification of the stress response also occurred in an *hcaA1A2CD* mutant strain. Using gel retardation, the HcaR binding site was estimated to be located about –70 to –55 bp upstream of the *hcaA* transcription start site. The expression of *hcaR* was repressed in the absence of oxygen by the ArcA/ArcB two-component system. © 2004 Elsevier SAS. All rights reserved.

Keywords: Oxidative stress; LysR; Proteome comparison; ArcA/ArcB

1. Introduction

Despite its many roles in electron transfer reactions providing energy to the cell, dioxygen is a very toxic compound, generating numerous kinds of reduced forms of oxygen species (RFO) [32]. Dioxygenases, that directly scavenge dioxygen, may therefore play an important role in protecting the cell against the deleterious effects of oxygen.

In *Escherichia coli*, HcaA is a dioxygenase which affects the metabolism of 3-phenyl propionic acid. HcaR, a member of the LysR family of regulatory proteins [34], was initially identified as a positive regulator of the *hcaA1*, *A2*, *C*, *B* and *D* operon [6]. Its disruption abolishes growth on 3-phenyl propionic acid [6,38]. The production of HcaR, the *hcaR* gene product, increases during stationary phase in an *rpoS*-independent manner [38] and is also subject to glucose-dependent repression [38]. A comparative study of regulation of the first step in 3-phenylpropionate catabolism and regulation of *hcaR* revealed that HcaR may play a pleiotropic role [38]. Sequence compar-

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isons with bacterial polypeptides contained in databases (<http://www.genome.wisc.edu/>, <http://www.ncbi.nlm.nih.gov/>, and <http://www.genomesonline.org/>) revealed that HcaR authentic homologs are present only in some enterobacteria, such as *E. coli* O157:H7, *Shigella flexneri*, and *Photobacterium luminescens* (<http://genolist.pasteur.fr/PhotoList/>). The presence of *hcaR* may be related to the specific biotope encountered by these bacterial species. This conjecture prompted us to try and identify other putative targets of HcaR. In this report, using 2D gel electrophoresis to analyse the *E. coli* proteome, we showed that the disruption of *hcaR* up- or downregulated the production of 30 proteins involved in various processes, 22 of which were identified. We subsequently showed that the absence of HcaR generates significant changes in control of the stress response. The relationship between *hcaR* expression levels and the presence of oxygen was also investigated.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated aerobically at 37 °C in LB medium or minimal media M63 [16] supplemented with gluconate (0.4%) or succinate (0.2%) as carbon sources. When specified, sodium nitrite NaNO₃ was added at a final concentration of 40 mM. Anaerobic cultures were grown in Erlenmeyer flasks with rubber stoppers and with shaking. Anoxia was achieved by bubbling with N₂ for

Table 1
Strains, plasmids, and phages used in this study

Strains	Relevant characteristics	Source or reference
BL21 DE3	<i>gal ompT</i> lysogen of DE3 carrying T7 polymerase gene	[35]
FB8	F ⁻	[3]
FB85	F ⁻ , <i>hcaR::cat</i>	[38]
FB80	F ⁻ , Δ <i>lac</i> X74	[21]
FB82	F ⁻ , Δ <i>lac</i> X74, <i>hcaR::lacZ</i>	[38]
FB825	F ⁻ , Δ <i>lac</i> X74, <i>hcaR::lacZ</i> , <i>oxyR::Kan</i>	This study
FB826	F ⁻ , Δ <i>lac</i> X74, <i>hcaR::lacZ</i> , <i>soxR::cat</i>	This study
FB827	F ⁻ , Δ <i>lac</i> X74, <i>hcaR::lacZ</i> , <i>arcA::Kan</i>	This study
FB82Δ1	F ⁻ , Δ <i>lac</i> X74, <i>hcaRΔ1::lacZ</i>	This study
FB82Δ2	F ⁻ , Δ <i>lac</i> X74, <i>hcaRΔ2::lacZ</i>	This study
Plasmids		
pRS415	Ap ^R	[30]
pET22b	Ap ^R	Novagen cat. no. 69744-3
PDIA10120	4.6-kb fragment of <i>E. coli</i> inserted into pUC18 (c37.11)	[38]
pDIA10122	<i>hcaR</i> inserted into pET22b	This study
Phage		
λRS45		[30]

2 min. Antibiotics were added to the following final concentrations (μg/ml) as required: chloramphenicol (Cm), 20; kanamycin (Km), 25; ampicillin (Amp) 50. Solid media contained 1.5% Difco agar.

2.2. Genetic techniques

P1 lysates and transductions were done as described by Miller [16]. Cells were transformed by the calcium chloride method [14] or electroporation [7].

2.3. Construction of chromosomal operon fusions

The 304-bp *EcoRI*–*Bam*HI and 258-bp *EcoRI*–*Bam*HI DNA fragments, containing the Δ1 and Δ2 promoter regions of *hcaR* (Fig. 2), were amplified using pDIA 10120 as template, with 5'-AAGCCGACCGTTCGAATTCACCAACG-3' and 5'-ATCGCGGATCCGCGCCGTAGTTCCATCACCA-3', or with 5'-CCGAATTCCAAATCTGAGGGTGTGGTC-3' and 5'-AAGATCGCGGATCCGGCTGC-3' as primers. After restriction cutting and gel separation, these fragments were purified and inserted into the multiple cloning site of pRS415, in front of the *lac* operon, and transferred by homologous recombination into λRS45. This phage was then used to lysogenise the FB80 strain as previously described [30] thus giving strains FB82Δ1, and FB82Δ2. Single lambda prophage integration was confirmed by PCR [22].

2.4. DNA manipulations and molecular cloning

Chromosomal DNA was isolated according to the Mar-mur method [15]. Large-scale plasmid DNA preparations were carried out using the Plasmid Maxi kit (Qiagen Inc., Germany) as recommended by the manufacturer. Small-scale plasmid DNA preparations were done as described by Birnboim and Doly [1]. Restriction, modification and ligation were carried out according to the manufacturer's recommendations. After gel separation, DNA fragments of interest were isolated by use of the QIAquick gel extraction kit (Qiagen Inc., Germany). DNA fragments were amplified in a Hybaid PCR thermocycler, using the Expand High Fidelity PCR system (Boehringer, Germany).

2.5. Primer extension

Total RNA was isolated from *E. coli* FB8 grown in minimal medium supplemented with 0.2% succinate. Exponentially growing cells were harvested at an OD_{600 nm} of 0.8 and collected by centrifugation. Total RNA was extracted by use of the High Pure RNA Isolation kit from Roche. The primers, R1 (5'-TAGTTCCATCACCTTCCCCTTGTTATCG-3') and A1 (5'-AATCAGTTGGTAAATGTTCAAA-TCTGAGGGTGTGG-3') (see Fig. 2 for positions of the primers), were labelled with α [³²P]-ATP and hybridised with 10 μg of RNA. The same primers were used to generate

Table 2
Sequences of the PCR primers

Gene	Forward	Reverse
<i>sodA</i>	5'-TATGCTTACGATGCCCTGGAACCGCACTTCG-3'	5'-AATGTAGTCCGGACGGCGTTCTGGAATTTA-3'
<i>sodB</i>	5'-ATGCTCTGGCACCGCACATTTCTGC-3'	5'-TTCACCAGCGCCAGAAAGTGCTCC-3'
<i>sodC</i>	5'-TAGTCTGGCTATTCTGGCGCTGG-3'	5'-TTCACCGCCACCGCCAGCGGTTTAGG-3'
<i>katE</i>	5'-AACTCACTGCGTGCCGGTAGCCGTGG-3'	5'-TAGCGGCTATAGCAACACCGTACCG-3'
<i>katG</i>	5'-ATCTCAACCGTGGTGGCCAGCCG-3'	5'-AAGTTGGCACCCAGTACACGCATGCC-3'
<i>ahpC</i>	5'-ATACCGAAGGCCGCTGGAGCG-3'	5'-ATTGCCTGGATGATACCCTGCG-3'
<i>ahpF</i>	5'-TACCTTGAGAAATTGACCAAGCC-3'	5'-TACGTTCGACTGCGCCTTCGAGCC-3'
<i>mrsA</i>	5'-ATGCCCTGCCTGGACGTAACACC-3'	5'-TTCCGCCAATTCCACAGTAACCATAACG-3'
<i>gor</i>	5'-TATCGCCTCCATCAACCGCGCGGC-3'	5'-ATGCGGCACGGCTGGCGGTGAGTGG-3'
<i>trxB</i>	5'-AACACAGTAAACTGCTTATCCTGGG-3'	5'-AAGTGCTGCCATGCAGCCTGTACCG-3'

a sequence ladder using the Sanger method [26]. Annealing was performed at 80 °C. Elongation of the DNA primer using reverse transcriptase and analysis of the products was performed as described previously [25].

2.6. Slot blot analyses

Digoxigenin-labelled probes were obtained by use of the PCR DIG probe synthesis kit from Roche. The primers used are listed in Table 2. Total RNA was extracted from exponentially growing cells by the High Pure RNA Isolation kit from Roche. Increasing amounts (50, 100, 200, and 400 ng) of total RNA were transferred to a positively charged nylon membrane and hybridised with the DIG-labelled probes. Chemiluminescent detection was performed with anti-DIG-AP and CDP-Star (Roche). The chemiluminescent signal was detected on X-ray film after 1–5 min exposure time. The slot blots were analysed and quantified by Image Master 1D software from Pharmacia.

2.7. Gel shift assay

Gel shift assays were performed according to methods described by Parsek et al. [20]. An end-labelled 274-bp fragment was generated by standard PCR using the ³³P-primers *hcaR8* (5'-AAG ATC GCG GAT CCG GCT GC-3') and *hcaR31* (5'-CCG AAT TCC AAA TCT GAG GGT GTG GTC-3'). Different amounts of purified HcaR protein were incubated with a fixed low DNA concentration (5000 cpm corresponding to 1–5 fmol of the labelled DNA fragment) in 10 µl of incubation buffer (KCl 50 mM, MgCl₂ 10 mM, glycerol 10%, Tris-HCl 50 mM, pH 7.9, Nonidet P-40 0.1%) containing 50 ng/ml poly dIdC and 0.3 mg/ml bovine serum albumin. After 30 min at room temperature, bromphenol blue-glycerol loading dye as added and the samples were immediately loaded onto a vertical non-denaturing 5% polyacrylamide gel and separated at +4 °C in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.2) at 12 V/cm. The dried gel was exposed to PhosphorImager scanning.

2.8. Production of HcaR

To overproduce and to purify HcaR protein, the *hcaR* gene was amplified by PCR using (5'-AAT TCC ATA TGG AAC TAC GGC ATT TAC GC-3') and (5'-CCC CTC GAG CGG TGC CGT TAC GCT TGC CAA ACG-3') as forward and reverse primers. The PCR product was digested with *NdeI* and *XhoI* and then cloned into the pET22b expression vector under control of the inducible T7lac promoter (Novagen cat. no. 69744-3) yielding pDIA10122. A 1.5-l culture of *E. coli* BL21 (DE3) [35] containing pDIA10122 was grown aerobically at 30 °C in LB medium supplemented with Ap (100 µg/ml) and Cm (20 µg/ml). When the OD_{600 nm} reached 2, IPTG (0.5 mM) was added to the medium to induce *hcaR* expression. One hour after induction, bacteria were harvested by centrifugation. The pellet was resuspended in 75 ml of buffer A (NaCl 300 mM, NaH₂PO₄ 50 mM, imidazole 20 mM, pH 8). Lysozyme was added at a final concentration of 1.5 mg/ml and the mixture was incubated for 30 min at 4 °C. The cells were disrupted by sonication and the bacterial extract was centrifuged at 12 000 g for 30 min at 4 °C. The supernatant was centrifuged again in the same conditions and kept for further purification.

2.9. Purification of HcaR in native conditions

The purification procedure was adapted from the QIAexpress Protein Purification system from Qiagen (<http://www.qiagen.com>). All steps were performed at +4 °C. The supernatant (75 ml), obtained as above, was mixed with 4 ml of Ni-NTA superflow resin (Qiagen, cat. no. 30410) and packed in a 5 ml polypropylene column (Qiagen, cat. no. 34964) by gravity flow. After two washes with 8 ml buffer A, the protein was recovered by elution with increasing concentrations of imidazole (100, 200 and 300 mM) in buffer A. The fractions with the best purification profiles on SDS-PAGE gel were applied to a PD-10 column Sephadex G-25M (Pharmacia Biotech, cat. 17-0851-01) and eluted directly with one volume of buffer B (NaCl 500 mM, NaH₂PO₄ 40 mM, pH 8). The fractions obtained at each step were analysed by SDS-PAGE. Most of the HcaR protein was recovered in the 100 and 200 mM imidazole fractions. The purified protein does not support freezing but was stable at +4 °C in buffer

B complemented with sodium azide 0.01% for a few weeks. The protein was estimated to be ca. 95% pure on SDS–PAGE gel.

2.10. Analytical 2D gel electrophoresis

Strains FB8 and FB85 were grown in minimal medium with succinate. Exponentially growing cells (100 ml of $OD_{600\text{ nm}} = 0.4$) were harvested by centrifugation. The pellet was then resuspended in 1 ml of distilled water and 15 μl of a DNase (1 mg/ml)/RNase (0.5 mg/ml) solution was added. Cells were disrupted at 4 °C by a Branson sonifier 250 (3 \times 20 s) and the cell debris was removed by ultracentrifugation (60 min, 4 °C, 90 000 g). Approximately 100 μg of proteins were resuspended in sample buffer (Pharmalyte 3–10 0.5%, urea 8 M, DTT 100 mM, Nonidet P40 2%) (ratio 1/5). Immobiline DryStrips (pH 4–7) and the Multiphor II Isoelectric system from Pharmacia were used for isoelectric focusing. Strips were rehydrated for 16 h at 20 °C and then focused for 45 000 V h^{-1} . The second dimension was performed with 11.5% polyacrylamide gels in the presence of SDS, using the Protean II 2D Multi-Cell system from BioRad. The gels were then fixed in 40% ethanol/10% acetic acid and silver-stained. The gels were digitised by a JX-330 scanner (Sharp, Hamburg, Germany). Spot detection and quantification were performed on a SPARC station 5 microcomputer (Sun Microsystems, Mountain View, CA) using the PDQUEST software package (BioRad, Ivry, France).

2.11. MALDI-TOF spectrometry

Protein spots were excised and the gel slices were digested with trypsin (Roche) as described previously [29]. The matrix used for the desalted digestions was a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in TFA 0.1%. MALDI-TOF spectra were obtained with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (PE Biosystems Inc., Framingham, MA). The analysis was performed in positive ion reflector mode. The trypsin autolysis products were used as internal standards. Data mining was performed by the ProFound and MS-FIT software against non-redundant databases. A mass deviation of 0.1–0.3 Da was allowed in the database searches.

2.12. Protein determination

Protein concentrations were determined as described by Bradford [2] using bovine serum albumin as the standard.

2.13. Enzyme assays

β -Galactosidase was assayed by the method described by Pardee et al. [19]. The activities of HPI and HPII catalases were measured as described previously [39].

3. Results

3.1. Proteome modifications generated by disruption of the *hcaR* gene

To identify putative targets of HcaR, we studied the proteome modifications generated by the disruption of its structural gene *hcaR*. In a first set of experiments, strains FB8 and its *hcaR::cat* derivative (FB85) were grown in M63 minimal medium with succinate as the carbon source (*hcaR* is highly expressed in bacteria grown with this carbon source [38]). After cell disruption, proteins were separated on a 2D SDS–PAGE gel, silver-stained, scanned and matched with a reference gel as described in Section 2. The amount of 51 polypeptides was affected by *hcaR* inactivation. The amount of 25 polypeptides increased and that of 26 polypeptides decreased. The comparison of spots of interest with our database, which contained 170 identified polypeptides from *E. coli*, combined with mass spectrometry, enabled 22 of these to be identified (Table 3). Three proteins (enolase, fructose-biphosphate aldolase and triosephosphate isomerase), the expression of which was decreased in the *hcaR* mutant, were involved in glycolysis. In contrast, the amount of phosphoenolpyruvate synthase, involved in gluconeogenesis, increased twofold in the mutant and may have compensated for the decrease in the three glycolysis enzymes for phosphoenolpyruvate biosynthesis. The amount of two tricarboxylic acid cycle enzymes (aconitate hydratase 2 and malate dehydrogenase) also decreased in the *hcaR* mutant. In contrast, the expression level of the β chain of ATP synthase was strongly increased in the mutant and may have compensated for the decrease in the two enzymes of the TCA cycle. Disruption of *hcaR* decreased the expression level of proteins involved in S-adenosylmethionine (AdoMet) metabolism (methionine adenosyltransferase). This might consequently modify the level of polyamine synthesis. This was not the case, however, because the amount of the different polyamines synthesized in the *hcaR* mutant did not vary when compared to the wild type (data not shown). The increase in PotD, a membrane protein involved in the transport of spermidine from the periplasm to the cytoplasm [10], could compensate for the decrease in spermidine that is formed from decarboxylated AdoMet and putrescine.

Several observations prompted us to explore the effect of *hcaR* disruption on the expression level of genes involved in the oxidative stress response: (i) the amount of thioredoxin reductase increased (this enzyme reduces thioredoxin used by the cell to reduce the disulphur bonds generated by protein oxidation) [37]; (ii) the amount of the stress response protein, DnaK, which accumulates in *E. coli* after exposure to hydrogen peroxide [17], increased; (iii) the amount of Mn-dependent superoxide dismutase (MnSOD) [36] decreased in the *hcaR* mutant.

Table 3

Identification of polypeptides up or downregulated in the *hcaR* mutant FB85. Bacteria were grown in M63 minimal medium with succinate as carbon source

Gene	Protein	Swissprot accession number	FB85/FB8	Metabolic process
<i>eno</i>	Enolase	P08324	R/2	Glycolysis
<i>fbaA</i>	Fructose-biphosphate aldolase	P11604	R/2	Glycolysis
<i>tpiA</i>	Triosephosphate isomerase	P04790	R/2	Glycolysis
<i>glpK</i>	Glycerol kinase	P08859	R/2	Glycerol catabolism
<i>aceF</i>	Pyruvate dehydrogenase	P06959	R/2	Pyruvate dehydrogenase
<i>acnB</i>	Aconitate hydratase 2	P36683	R/5	Tricarboxylic acid cycle
<i>mdh</i>	Malate dehydrogenase	P06994	R/3	Tricarboxylic acid cycle
<i>ppsA</i>	PEP synthase	P23538	I × 2	Gluconeogenesis
<i>atpB</i>	ATP synthase beta chain	P00824	I × 4	ATP synthesis
<i>trpA</i>	Tryptophan synthase subunit A	P00928	R/2	Tryptophan biosynthesis
<i>gdhA</i>	Glutamate dehydrogenase	P00370	R/2	Glutamate biosynthesis
<i>argS</i>	Arginyl-tRNA synthetase	P11875	R/2	tRNA charging pathway
<i>serS</i>	Seryl-tRNA synthetase	P09156	R/2	tRNA charging pathway
<i>metK</i>	Methionine adenosyltransferase	P04384	R/5	S-adenosyl-L-methionine synthesis
<i>potD</i>	Subunit of putrescine/spermidine ABC transporter	P23861	I × 4	Polyamine transport
<i>upp</i>	Uracil phosphoribosyltransferase	P25532	R/4	Pyrimidine ribonucleotides salvage pathway
<i>purA</i>	Adenylosuccinate synthetase	P12283	R/5	Purine nucleotide biosynthesis
<i>sodA</i>	Superoxidedismutase (Mn)	P00448	R/2	Oxidative stress
<i>trxB</i>	Thioredoxin reductase	P09625	I × 2	Thioredoxin pathway
<i>dnaK</i>	HSP-70-type molecular chaperone	P04475	I × 4	Stress response
<i>rpsA</i>	Ribosomal protein S1	P02349	R/2	Translation
<i>pnp</i>	Polyribonucleotide nucleotidyltransferase	P05055	R/2	RNA degradation

Exponentially growing cells (100 ml of OD_{600 nm} = 0.4) were harvested by centrifugation. After 2D electrophoresis, spot quantification induction (I) and repression (R) factors were calculated as described in Section 2.

3.2. Effects of *hcaR* disruption on expression of genes involved in the oxidative stress response

The oxidative stress response involves mechanisms that lower the intracellular concentration of oxygen and its reduced forms (RFO) and repair damage generated by the interaction of these compounds with DNA, amino acids, proteins and phospholipids. We found that HcaR controls the production of two proteins involved respectively in the enzymatic interception of RFO (MnSOD) and in repair of their toxic activity for proteins (thioredoxin reductase). To determine whether HcaR also modulates production levels of other proteins involved in control of RFO and their effects, slot blot experiments were performed with RNA extracted from strains FB8 and FB85 harvested either during the exponential or during the stationary growth phase. The probes used in these slot blot experiments were derived from genes involved in control of RFO and/or their effects. Disruption of *hcaR* modified the expression levels of genes involved in interception of RFO (Table 4). The expression levels of all three genes encoding superoxide dismutases (involved in the dismutation of superoxide ions) *sodA*, *sodB*, and *sodC* decreased both during the exponential growth and during the stationary phase in an *hcaR* mutant. The expression level of the *katG* and *katE* genes, encoding respectively the two distinct catalases HPI and HPII involved in hydrogen peroxide dismutation [8], was the same in the wild-type strain and in its *hcaR* derivative during the exponential growth phase. In contrast, during the stationary phase, the expres-

Table 4

Effect of *hcaR* disruption on expression levels of genes involved in the oxidative stress response

Gene	Exponential growth	Stationary phase
<i>sodA</i>	/1.2	/1.5
<i>sodB</i>	/1.3	/1.8
<i>sodC</i>	/1.6	/2.1
<i>KatE</i>	=	=
<i>katG</i>	=	/2
<i>ahpC</i>	=	/2
<i>ahpF</i>	=	/5
<i>gor</i>	/3	/3
<i>msrA</i>	×3.2	=
<i>trxB</i>	×1.6	=

Bacteria were grown in M63 minimal medium with succinate as carbon source. Samples were harvested during exponential growth or stationary phase and RNA were extracted and hybridised to the probes as described in Section 2.

sion of *katG* was significantly decreased in the *hcaR* mutant (Table 4). HPI and HPII catalase activities were assayed in crude extracts of FB8 and FB85 strains harvested during the stationary phase. HPI activity was sevenfold lower in the *hcaR* mutant (Table 5). Disruption of *hcaR* decreased expression of *ahpC* and *ahpF* which encode the two subunits of alkyl hydroperoxide reductase which functions as a primary scavenger of endogenous H₂O₂ [28]. The expression level of genes involved in repair of damage generated by the interaction of RFO compounds with amino acids and proteins was also altered by *hcaR* disruption. Indeed, the expression level of *gor*, coding for glutathione reductase that reduces

Table 5
Effect of *hcaR* inactivation on HPI (*katG*) and HPII (*katF*) catalase activities

	FB8	FB85
HPI + HPII	55 ± 6	24 ± 2
HP II	27 ± 3	20 ± 1
HP I	28 ± 4	4 ± 0.5

Bacteria were grown in M63 minimal medium with succinate as carbon source. Catalase activities in extracts of FB8 and FB85 grown to stationary phase were measured by the spectrometric method as cited in Section 2.

oxidized glutathione and reduces the disulphur bonds generated by protein oxidation [11], was threefold lower in the *hcaR* mutant during both the exponential growth phase and the stationary phase (Table 4). In contrast, the expression level of *trxB*, the structural gene of thioredoxin reductase which participates in various redox reactions through reversible oxidation of the thioredoxin-active dithiol centre to a disulphide, was increased by disruption of *hcaR* during exponential growth (Table 4). The dithiol-disulphide exchange reaction reduces the disulphur bonds generated by protein oxidation [24]. Also, the expression level of *msrA*, encoding the methionine sulphoxide reductase that catalyses the reduction to methionine of methionine sulphoxide present in proteins [24], was increased threefold in the *hcaR* mutant only during exponential growth (Table 4).

3.3. Effects of *hcaA* disruption on expression of the genes involved in the oxidative stress response

The effect of HcaR disruption on the expression level of genes involved in the oxidative stress response may be direct or indirect. An indirect effect could be the consequence of the strong decrease in *hcaA1*, *A2*, *C*, *B*, *D* operon expression in the absence of HcaR [38]. The effect of *hcaA1*, *A2*, *C*, *B*, *D* operon disruption upon the expression level of genes involved in the oxidative cell response was investigated. Disruption of the *hcaA1*, *A2*, *C*, *B*, *D* operon also significantly decreased *katG* (twofold), *sodA* (threefold), and *sodC* (sixfold) expression during the stationary growth phase. Thus, the effect of *hcaR* disruption on genes involved in the oxidative stress response was related to the decrease in expression of genes coding for enzymes implicated in the early steps of 3-phenyl propionic acid utilisation.

3.4. The expression level of *hcaR* is related to aerobic metabolism

As shown above, the function of *hcaR* is related to the oxidative stress response during the exponential and stationary phases. We thus searched for a relationship between the *hcaR* expression level and oxidative metabolism. In a first set of experiments, expression of *hcaR* was measured in bacteria grown anaerobically or aerobically in the presence or absence of compounds known to induce oxidative stress responses. *hcaR::lacZ* transcriptional fusion was not induced after incubation with sublethal doses of H₂O₂, paraquat or

Table 6
Expression of the *hcaR::lacZ* transcriptional fusion for bacteria grown in the absence or in the presence of oxygen

β-galactosidase specific activity (nmoles substrate converted/min mg dry weight bacteria)		
M63 B1 + gln + O ₂	M63 B1 + gln - O ₂	M63 B1 + gln - O ₂ + NaNO ₃
200 ± 6	45 ± 1.5	45 ± 1.5

Bacteria were grown aerobically or anaerobically in M63 minimal medium with gluconate as carbon source in the absence or in the presence of sodium nitrite NaNO₃ (40 mM) as an electron acceptor. For each experiment, β-galactosidase activity was assayed four or five times during exponential growth. The β-galactosidase specific activities are the average of at least three experiments; the error range represents one standard deviation.

cumene hydrogen peroxide (data not shown), known to induce superoxide dismutase [36], catalase [39] and cumene hydrogen peroxidase [33]. When bacteria were grown in the absence of oxygen, the expression of *hcaR* was decreased threefold compared to growth in aerobic conditions (Table 6). This decrease was not alleviated by adding nitrate, which can serve as an electron acceptor and consequently allow the respiratory chain to work in the absence of oxygen (Table 6). Thus, the expression level of *hcaR* is either related to the presence of the dioxygen molecule or to some other feature of aerobic metabolism. The restoration of the full expression level of *hcaR* required about two generations following the addition of oxygen during the mid-growth exponential phase in bacteria previously grown under anaerobic conditions (data not shown).

3.5. Study of the intergenic region between *hcaA* and *hcaR*

In order to gain insight into regulatory features that might be involved in this regulation we analysed the *hcaR hcaA* intergenic region. HcaR positively regulates the expression of its neighbouring genes, *hcaA1*, *A2*, *C*, *B*, *D*, and negatively regulates the expression of its own gene [38]. As a consequence, one or two HcaR binding sites could be present in the intergenic region between *hcaA* and *hcaR*. The two mRNA 5' ends of *hcaA* and *hcaR* were mapped using primer extension experiments as described in Section 2 (Fig. 1). A poor -10 region (AAGGAT) and a good -35 region (TTGACC) were found upstream of the transcription start site of *hcaR* (Fig. 1). For *hcaA*, a weak -10 region (CAACAT) and a more typical -35 region (TTCACA) were found upstream of its transcription start site (Fig. 1). Generally, the DNA sites for LysR binding are located 35–80 bases upstream of the transcription start site of the target gene [27]. They are always extremely difficult to identify and no clear-cut consensus binding sites have been proposed for the LysR family of regulators [27]. We used three *hcaR::lacZ* transcriptional fusions to attempt to locate the DNA binding site for negative autoregulation of *hcaR*. The deletion of 153 bases from 237 to 86 bases upstream of the *hcaR* transcription start site increased its expression threefold (Fig. 2) (that is, to a level similar to that measured in a strain disrupted



Fig. 1. The intergenic region between *hcaR* and the *hca* A1, A2, C, B, D operon. Vertical arrows indicate the transcription start sites (+1). The -35 and -10 regions and the proposed ArcA binding site are boxed. Sequences of primers used to amplify the fragment tested for gel retardation experiments (oligo 31 and oligo 8) are indicated by plain arrows. Mapping of the transcription starts at *hcaR* and the *hca* A1, A2, C, B, D operon. Total RNA was extracted from *E. coli* strain FB8 grown in minimal medium in the presence of succinate. Primer extension experiments were performed using A1 oligonucleotide R1 (dashed arrow), for *hcaR* and B oligonucleotide A1 (dashed arrow) for *hcaA*. Sequencing reactions (lane ACGT) were performed with the (R1) or (A1) oligonucleotides and pDIA 10120 as template. An asterisk indicates the 5' end of mRNA.

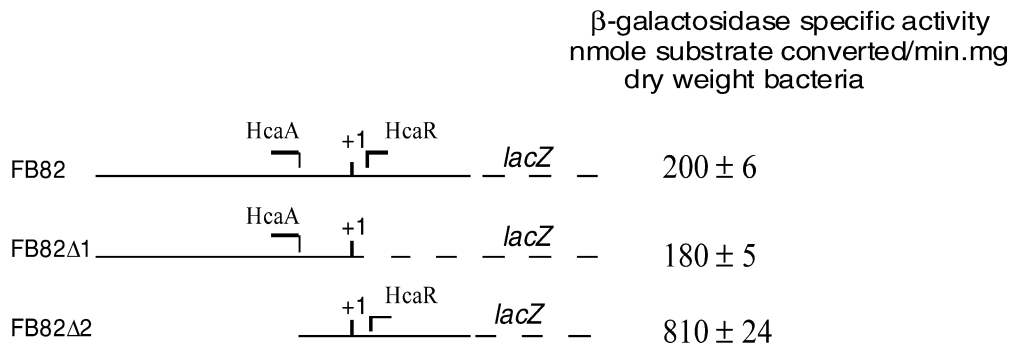


Fig. 2. Localization of the region required for negative autoregulation of *hcaR*. Bacteria were grown aerobically or anaerobically in M63 minimal medium with gluconate as carbon source. For each experiment, β-galactosidase activity was assayed four or five times during exponential growth. The β-galactosidase specific activities are the average of at least three experiments; the error range represents one standard deviation.

for *hcaR* [38]). In contrast, deletion of 107 bases from 67 to 172 bases downstream from the *hcaR* transcription start site had no effect on its negative autoregulation. Furthermore, we found that this 274-base fragment containing both *hcaA* and *hcaR* promoters was required for the induction of *hcaA* in the presence of 3-phenylpropionate (data not shown).

To demonstrate that HcaR binds this DNA fragment, gel shift assays were performed with the pure HcaR protein isolated as described in Section 2. Addition of various amounts (0.1–50 μg) of purified HcaR to the 274-bp fragment resulted

in the formation of a DNA–protein complex with a mobility of 0.65 as compared to that of free DNA (Fig. 3). The complex was well detected with 0.5 μM of HcaR. However, the addition of 3-phenylpropionate, which, when added to the growth medium, is the inducer of HcaR for the induction of *hcaA*, did not enhance formation of the DNA–protein complex (data not shown). The use of DNase I protection experiments did not enable us to identify the bases involved in binding of HcaR to DNA. This may be due to the weak stability of the complex.

Table 7

Expression of the *hcaR::lacZ* transcriptional fusion in a wild-type strain (FB82) and its *oxyR* (FB825), *soxR* (FB826), and *arcA* (FB827) derivatives

	β-galactosidase specific activity (nmoles substrate converted/min mg dry weight bacteria)			
	Control	<i>oxyR</i>	<i>soxR</i>	<i>arcA</i>
M63 B1 + gln + O ₂	200 ± 6	230 ± 7	250 ± 7.5	350 ± 10
M63 B1 + gln – O ₂	45 ± 1.5	50 ± 1.5	40 ± 1.2	400 ± 12

Bacteria were grown aerobically or anaerobically in M63 minimal medium with gluconate as carbon source. For each experiment, β-galactosidase activity was assayed four or five times during exponential growth. β-Galactosidase specific activities are the average of at least three experiments; the error range represents one standard deviation.

3.6. Expression of *hcaR* is regulated by the two-component system *arcA/arcB*

Activators often act in concert with other regulators. It was therefore of interest to see whether we could find, upstream of *hcaR*, the signature of known regulators involved in monitoring the presence of oxygen. A putative ArcA binding site (TGTTCAAATC) was found 79 base pairs upstream of the *hcaR* transcription start site. This sequence matched 6 of the 10 bases of the consensus ArcA sequence (5' [A/T]GTTAATTA[A/T]) [12]. This prompted us to explore whether the ArcA/ArcB two-component system was involved in regulation of *hcaR* expression. The expression level of *hcaR* was also measured in *soxR* and *oxyR* mutants because SoxR [5] and OxyR [31] regulators modulate expression of many genes including *sodA*, *gor*, *katG*, *ahpC* and *ahpF*. Disruption of *soxR* or *oxyR* had no effect on *hcaR* expression (Table 7). This result is consistent with the fact that the addition of paraquat or hydrogen peroxide did not induce expression of *hcaR*. In contrast, disruption of *arcA* abolished the decrease in *hcaR* expression when bacteria were grown in the absence of oxygen. Thus, *hcaR* can be considered a target of the ArcA/ArcB two-component system, but not of SoxR or OxyR. Nevertheless, this regulatory effect of the ArcA/ArcB two-component system seemed to be indirect, since deletion of the four proximal bases (TGTT) of the putative ArcA binding site had no effect on *hcaR* repression under anaerobic growth conditions (Fig. 4).

4. Discussion

Proteome comparison of an *hcaR* disruptant with its wild-type parent uncovered an unexpected relationship between HcaR activity and expression of genes involved in the oxidative stress response. The involvement of oxidative stress was further substantiated by slot blot hybridisation experiments using ten genes involved in the oxidative stress response as probes. The effect of *hcaR* inactivation on the oxidative stress response was shown to be related to its regulatory effects on *hcaA*. This effect occurs independently from *rpoS* that is normally expressed in *hcaR*

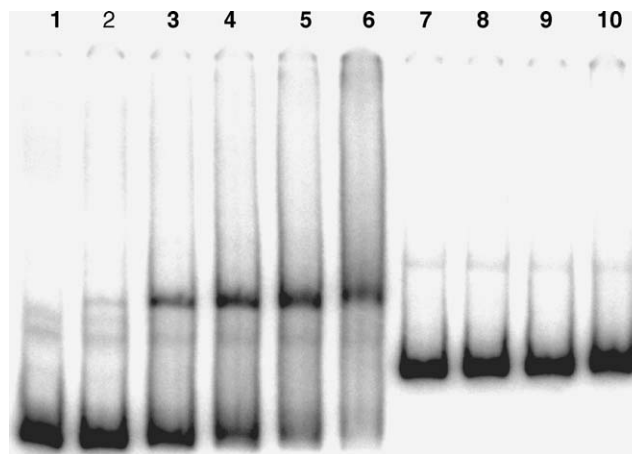


Fig. 3. Gel shift assay. Labelled DNA (1–5 fmol) was incubated with various amounts of purified HcaR for 30 min at room temperature and separated in a 5% polyacrylamide gel as described in Section 2. A 274-bp fragment containing the *hcaA* and *hcaR* promoter regions was used in lanes 1–6; a 335-bp fragment that does not contain the *hcaA* and *hcaR* promoter regions was used in lanes 7–10 as a control for non-specific binding. Final protein concentrations were: lanes 1 and 7: no protein; lane 2: 0.1 μM; lanes 3 and 8: 0.5 μM; lanes 4 and 9: 1 μM; lanes 6 and 10: 5 μM.

and *hcaA* disruptants (data not shown). The catalytic activity of the *hcaA* gene product could be related to oxidative stress if we consider that one O₂ molecule is incorporated into the 3-phenylpropionate molecule during its oxidation into *cis*-3-(carboxyethyl)-3,5-cyclohexadiene-1,2-diol by the 3-phenylpropionate dioxygenase system. The absence of this catalytic activity for *E. coli* strains grown with succinate as carbon source decreased the expression level of members of the OxyR regulon (*ahpC*, *ahpF*, *gor*, *katG*) [41] and the SoxR-regulated gene (*sodA*) [13]. Thus, impairment of 3-phenylpropionate dioxygenase activity could decrease production of reduced forms of oxygen species that were shown to induce *oxyR* and *soxR* regulons, without any effect on the ability to survive oxidative stress generated by paraquat or H₂O₂ (data not shown). This absence of effects on oxidative stress responses can be explained by a concomitant increase in the expression level of genes like *msrA* [18] or *trxB* [23] that were shown to be involved in the repair of damage related to oxidative stress. Expression of HcaR was repressed in the absence of oxygen and our results imply that this regulation is the consequence of anaerobic metabolism. This is consistent with results showing that repression of *hcaR* in the absence of oxygen is due to the ArcA/ArcB two-component system [12], known to sense the redox state of the cell by detecting an electron transport component in its reduced form [9]. Our results also suggest that ArcA/ArcB-mediated control of *hcaR* expression is indirect. Thus, other regulatory events could interfere with ArcA control of *hcaR* expression that was shown to increase at the end of exponential growth [38]. The decrease in or absence of non-induced expression of the *hcaA*, *A2*, *C*, *B*, *D* operon modifies the number of polypeptides involved in oxidative catabolism and the stress response. This shows that,

	β -galactosidase specific activity (nmoles substrate converted/min.mg dry weight bacteria)	
	M63 B1 + gln + O2	M63 B1 + gln -O2
FB 82	200 ± 6	40 ± 2
FB82 Δ 2	220 ± 11	35 ± 2

FB82

FB82 Δ 2

```

/accgcaccattttgggtgtcaatcagttggtaaatggttcaaactgagggtgtgggtcatatttt
                                ^
                                |
                                v
                                ArcA
                                |
                                v
                                +1
ttaatcctgaaagataagatgtattgaccttctatntttgtgccataaggatgttctgtgttggt
-35          -10          +1
tgctaatatgtgaaatagtttaatttctaccagctgagacgttttttcgataacaaggggaaggtg
atggaactacggcatttacgctatttcgtcgcagtgccaggcactgaacttaccgggcggcg hcaR
  
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Fig. 4. Expression of *hcaR::lacZ* transcriptional fusion in a strain (FB82) and its derivative (FB82 Δ 2) deleted for the four proximal bases of the putative ArcA binding site located upstream of *hcaR*. Bacteria were grown aerobically or anaerobically in M63 minimal medium with gluconate as carbon source. For each experiment, the β -galactosidase activity was assayed four or five times during exponential growth. The β -galactosidase specific activities are the average of at least three experiments; the error range represents one standard deviation.

even in the absence of its cognate substrate, 3-phenyl propionic acid dioxygenase activity can play a role in modulation of the *E. coli* cell response to oxidative stress, mainly during the stationary phase. The presence of an active 3-phenyl propionic acid dioxygenase in a wild-type strains leads induction of members of *oxyR* and *soxR* regulons as compared with the *hcaR* and *hcaA* derivatives. Induction of such proteins enhances the bacterial cell response to oxidative stress. Expression of the *hcaA*, *A2*, *C*, *B*, *D* operon is not affected by *soxR* or *oxyR* disruption (data not shown) and thus cannot be considered as a target of OxyR or SorR.

Sequence comparison with polypeptides contained in databases of the partial or complete bacterial genome sequences (<http://www.genomesonline.org/>) only identified an HcaR homolog in pathogenic *E. coli* like O157:H7 (100% identical) and *Shigella flexneri* (100% identical). Among six pathogenic *E. coli* strains tested, only O157:H7 and the enterotoxinogen strain EDL 1493 were able to use 3-phenylpropionate as a carbon source (unpublished data). Also, HcaR was found in the *Photobacterium luminescens* genome (47% identical) (<http://genolist.pasteur.fr/PhotoList/index.html>). This suggests that the presence of HcaR is related to environments specifically encountered by some *E. coli* strains and *P. luminescens*. *E. coli* strains are present in human and animal intestines. *P. luminescens* is present in the gut of nematodes [40]. In human and animal intestines and in the gut of nematodes, 3-phenylpropionate

is formed by the action of the microflora on plant constituents, tyrosine and phenylalanine [4]. The presence of the 3-phenylpropionate catabolic pathway may be advantageous in these environments for defence against oxidative stress. Bacterial cells have evolved mechanisms that allow them to grow in favourable conditions and to survive in unfavourable conditions. In *E. coli*, groups of genes are involved in specific cell responses to heat, osmotic, and oxidative stress. In most of these cases, bacteria encounter less favourable environments than under the usual laboratory growth conditions. Consequently, a significant number of unknown genes, identified by complete genome sequencing programs, may be involved in responses to unfavourable environments. These cellular responses to unfavourable environments involve proteins specifically involved in stress responses and some enzymes and regulators of catabolic or anabolic pathways.

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