Global effects of homocysteine on transcription in *Escherichia coli*: induction of the gene for the major cold-shock protein, CspA

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Homocysteine (Hcy) is a thiol-containing amino acid that is considered to be medically important because it is linked to the development of several life-threatening diseases in humans, including cardiovascular disease and stroke. It inhibits the growth of *Escherichia coli* when supplied in the growth medium. Growth inhibition is believed to arise as a result of partial starvation for isoleucine, which occurs because Hcy perturbs the biosynthesis of this amino acid. This study attempted to further elucidate the inhibitory mode of action of Hcy by examining the impact of exogenously supplied Hcy on the transcriptome. Using gene macroarrays the transcript levels corresponding to 68 genes were found to be reproducibly altered in the presence of 0.5 mM Hcy. Of these genes, the biggest functional groups affected were those involved in translation (25 genes) and in amino acid metabolism (19 genes). Genes involved in protection against oxidative stress were repressed in Hcy-treated cells and this correlated with a decrease in catalase activity. The gene showing the strongest induction by Hcy was *cspA*, which encodes the major cold-shock protein CspA. RT-PCR and reporter fusion experiments confirmed that *cspA* was induced by Hcy. Induction of *cspA* by Hcy was not caused by nutritional upshift, a stimulus known to induce CspA expression, nor was it dependent on the presence of a functional CspA protein. The induction of *cspA* by Hcy was suppressed when isoleucine was included in the growth medium. These data suggest that the induction of CspA expression in the presence of Hcy occurs because of a limitation for isoleucine. The possibility that Hcy-induced *cspA* expression is triggered by translational stalling that occurs when the cells are limited for isoleucine is discussed.

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INTRODUCTION

The sulphur-containing amino acid homocysteine (Hcy) is the last intermediate in the methionine biosynthetic pathway in *Escherichia coli*. It has become a focus of much research in recent years because of reports showing that elevated levels of Hcy in human serum are associated with a number of life-threatening diseases. We have shown that exogenous supplementation of glucose-based media with concentrations of Hcy as low as 0.2 mM can inhibit the growth of *E. coli* (Roe *et al.*, 2002; Tuite *et al.*, 2005). Growth inhibition by Hcy appears to be caused by a perturbation of branched-chain amino acid (BCAA) biosynthesis that gives rise to a partial starvation for isoleucine (Tuite *et al.*, 2005). Supplementation of the growth medium with isoleucine fully reverses the inhibitory effects of 0.5 mM Hcy. Hcy

inhibits threonine deaminase, the enzyme that catalyses the first committed step of isoleucine biosynthesis, and this inhibition is the likely cause of the isoleucine starvation and growth inhibition that occur in Hcy-treated *E. coli* cells (Tuite *et al.*, 2005).

High levels of serum Hcy are associated with serious diseases in humans, including cardiovascular disease (Anderson *et al.*, 2000; Vollset *et al.*, 2000), birth defects and pregnancy complications (van der Put *et al.*, 2001; Vollset *et al.*, 2000), as well as neurodegenerative diseases such as Alzheimer's disease (Seshadri *et al.*, 2002). More recently it has been shown that there is a causal link between elevated serum-Hcy levels and stroke (Casas *et al.*, 2005). The underlying mechanisms linking high-serum Hcy levels and disease in humans remain to be elucidated. However, a number of different theories have been proposed and these have been reviewed recently (Jakubowski, 2004). Hcy can induce cellular damage through modification of cellular proteins.

Abbreviations: BCAA, branched-chain amino acid; Hcy, homocysteine; THF, tetrahydrofolate.

Proteins can become homocysteinylated by the formation of an amide bond between Hcy and the ϵ -amino group of lysine residues (Jakubowski, 1999, 2002). Proteins modified in this way are known to elicit an immune response and this is thought to contribute to the pathology of disease (Jakubowski, 2004). In addition, S-nitrosylated Hcy, a methionine analogue that is formed when Hcy reacts with nitric oxide, can be misincorporated into proteins during translation (Jakubowski, 2000). An alternative model accounting for Hcy toxicity suggests that Hcy might trigger oxidative stress in human cells (Loscalzo, 1996; Lynch & Frei, 1997; Starkebaum & Harlan, 1986; Upchurch *et al.*, 1997). In *E. coli*, cysteine, an amino acid that is closely related to Hcy, is known to trigger oxidative stress by enhancing the Fenton reaction (Berglin *et al.*, 1982; Park & Imlay, 2003). However, at present there is no evidence that Hcy induces oxidative stress in *E. coli*.

In *E. coli* Hcy is produced by two routes: the majority of the Hcy in the cell results from the cleavage of cystathionine by cystathionine β -lyase; a smaller, undefined amount is generated by the action of LuxS on S-ribosylhomocysteine during the production of the intracellular signalling molecule autoinducer-2 (AI-2) (Greene, 1996; Winzer *et al.*, 2002). Hcy is converted to methionine in a methylation reaction that is catalysed by either of two methionine synthases, MetE or MetH. The methyl donor in this reaction is 5-methyltetrahydrofolate (CH₃-THF). The synthesis of CH₃-THF requires two enzymes, serine hydroxymethyltransferase (GlyA) and methylenetetrahydrofolate reductase (MetF), whose function is to transfer a single carbon atom from serine to tetrahydrofolate to produce methylenetetrahydrofolate (methylene-THF) and then to reduce methylene-THF to CH₃-THF (Greene, 1996). The same biochemical steps are employed in human cells to convert Hcy to methionine. Indeed MetH, GlyA and MetF all share significant homology with their human counterparts. The similarities in the metabolism of Hcy between bacteria and humans have prompted us to use *E. coli* as a simple model system for investigating the toxic effects of this thiol-containing amino acid.

In the present study we sought to further elucidate the molecular basis for the inhibitory effects of Hcy on the growth of *E. coli* cells by using DNA microarray technology to investigate the global effects of Hcy on transcription. We found that two principal classes of genes exhibited reproducible changes in expression in the presence of Hcy: those involved in translation and those involved in amino acid biosynthesis. Genes involved in the oxidative stress response were found to be repressed. We also found that *cspA*, the gene encoding the major cold-shock protein CspA, was induced strongly during growth in the presence of Hcy and this induction was prevented when isoleucine was present in the growth medium. Taken together with our previous work (Tuite *et al.*, 2005), these data suggest that in *E. coli* growth in the presence of Hcy results in perturbation of amino acid biosynthesis and translation.

METHODS

Bacterial strains, growth media and chemicals. *Escherichia coli* strain Frag1 (F⁻ *thi rha lac gal*) was from our frozen stocks. Frag1(pMM016) was generated by transformation of Frag1 with plasmid pMM016, which carries a *cspA::lacZ* translational fusion (Bae *et al.*, 1997). All growth experiments were carried out at 37 °C with aeration in a minimal medium (MM) that was adjusted to pH 6.0. MM contained 20 mM citric acid, 56 mM Na₂HPO₄, 5 mM K₂HPO₄, 0.4 mM MgSO₄·7H₂O, 7.6 mM (NH₄)₂SO₄, 6 μ M ammonium ferrous sulphate and 3 μ M thiamine. Cells were grown overnight in 25 ml MM supplemented with limiting glucose (0.04%, w/v) in a 250 ml flask at 37 °C with vigorous shaking. The next day glucose (0.4%, w/v) was added to this culture, and growth was continued to an OD₆₀₀ of 0.8. This culture was then used to inoculate 25 ml MM (0.4%, w/v, glucose) to an OD₆₀₀ of 0.05. Cell growth was measured spectrophotometrically (Jenway 6305 spectrophotometer) by measuring the OD₆₀₀ of 1 ml samples at regular intervals during growth. Hcy was added to growth media at a concentration of 0.5 mM when required. Unless specified, all chemicals used in this study were supplied by Sigma and BDH.

RNA preparation for gene microarray analysis. Cells were grown in 25 ml MM (in 250 ml flasks) to an OD₆₀₀ of 0.2, before the addition of Hcy (0.5 mM) to half of the samples. The flasks were returned to the incubator for 1 h, before 20 ml cells was removed and added to a sterile centrifuge tube containing 3.3 ml RNA stabilization reagent RSR-3 [95% ethanol, 5% phenol (v/v)] (Bhagwat *et al.*, 2003). Cells were then harvested by centrifugation (15 min, 4 °C, 8200 g), the supernatant decanted and the pellets stored at -20 °C until required. RNA was prepared using a modified version of the protocol described in the Panorama *E. coli* Gene Arrays Manual (Sigma-Genosys). The cell pellet was resuspended in 250 μ l resuspension buffer [0.3 M sucrose, 10 mM sodium acetate (pH 4.2)] and 37.5 μ l 0.5 M EDTA. Resuspended cells were then transferred to a fresh microcentrifuge tube before the addition of 375 μ l lysis buffer [2% SDS, 10 mM sodium acetate (pH 4.2)], mixed well by vortexing then incubated on ice for 3 min. RNA was then prepared by phenol/chloroform extraction. After the first phenol step, the RNA/DNA mix was ethanol precipitated and the nucleic acid pellet was resuspended in 50 μ l TE buffer containing 5 U RNase-free DNase I (Roche), and incubated at room temperature for 30 min. This was followed by a further phenol extraction step, then a phenol/chloroform/isoamyl alcohol (25:24:1) step and finally a chloroform/isoamyl alcohol (24:1) step. The RNA was then concentrated by ethanol precipitation, before being resuspended in 50 μ l deionized H₂O and stored at -70 °C until required.

Probe synthesis and hybridization of gene arrays. Hybridization probes were generated using *E. coli* gene-specific primers covering the entire genome (Sigma-Genosys) and following the protocol provided by the manufacturer, which was suitable for achieving >50% incorporation of the [α -³³P]dCTP. Panorama *E. coli* gene arrays were obtained from Sigma-Genosys and prehybridized in 5 ml hybridization buffer [5 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA, pH 7.7), 2% SDS, 1 $\times$ Denhardt's reagent and 100 μ g denatured, sonicated salmon sperm DNA ml⁻¹]. After incubation for 1 h at 65 °C, the prehybridization solution was replaced with the denatured radioactively labelled cDNA probe. The probes were denatured in 3 ml hybridization buffer and incubated at 95 °C for 4 min immediately before use. After hybridization for 16 h at 65 °C in a hybridization incubator (Robbins Scientific), the filters were washed briefly three times at room temperature with 50 ml wash solution (0.5 $\times$ SSPE, 0.2% SDS) and then washed three times at 65 °C in 100 ml wash solution. The washed filters were blotted on Whatman 3MM paper for 5 min and then placed in

a plastic wrap. The filters were exposed to a phosphorimager screen for 24–72 h and the screen was scanned at 50 μm resolution using a Fujix BAS1800-II phosphorimager.

Data acquisition and analysis. The Fujix BAS image files were analysed using Array-Vision software running on a Microsoft Windows 2000 workstation. The signal intensity for each spot was determined using the integrated intensity function, which calculates the volume of each spot by summing the value of each pixel within the boundaries of the spot (minus the local background). The pixel density values were exported to a tab-delimited text file. Each open reading frame (ORF) on the array is represented by duplicate spots of 10 ng PCR product. To compare the signal intensities between filters, a relative intensity for each ORF was calculated by dividing the mean intensity for a given ORF by the total signal intensity on the filter and multiplying by 100. The total signal intensity on the filter was calculated by summing the intensities of all ORFs on the array ($n=4290$).

Two macroarray experiments were performed for each condition, using independently isolated RNA preparations. Thus the data were derived from a total of four macroarrays (two untreated controls and two plus Hcy). Genes that showed a mean expression ratio of twofold or greater (between treated and untreated) were short-listed for further analysis. Genes were eliminated from this list if the expression ratio of one replicate was less than ± 1.5 -fold. A statistical analysis of this subset of genes was performed using SigmaStat 3.0 (Systat Software). This subset of genes consisted of two subgroups; one up-regulated in response to Hcy and one down-regulated. The median expression ratios for each of these subgroups were compared to the median expression ratio for the remainder of the genes on the macroarray using a Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks. In both cases the median expression ratios were significantly different with $P \leq 0.001$.

RT-PCR. Cells were grown as described above for gene macroarray analysis, and then 20 ml cells was transferred to a sterile centrifuge tube containing RSR-3 (see above), pre-incubated on ice. Cells were then harvested by centrifugation (15 min, 4 °C, 8200 g). RNA was prepared using the GenElute Mammalian Total RNA Miniprep kit (Sigma), following the manufacturer's instructions. The RNA was then treated to remove DNA contamination using the DNA-free kit (Ambion) following the manufacturer's protocol. cDNA was synthesized from 20 μl diluted RNA by using Expand reverse transcriptase with random primer p(dN)₆ (both supplied by Roche). Aliquots (2 μl) of the resulting cDNA were subjected to 18, 24 and 30 cycles of PCR and run on agarose gels. Primers for the gene encoding polymerase A (*polA*) were used as controls to detect contaminating DNA and allow normalization of cDNA template concentration. Non-reverse-transcribed RNA was used as template for PCRs to ensure complete removal of genomic DNA. Specific primers for *cspA*, *argF*, *argH*, *cysN* and *gatZ* (Table 1) were used in conjunction with cDNA generated from *E. coli* Frag1 grown in the presence or absence of 0.5 mM Hcy. RNA and cDNA were prepared twice, from independent cultures, and the RT-PCRs for each set of primers were repeated at least twice from each cDNA preparation.

Transduction of *cspA::cat* gene deletion into *E. coli* Frag1. Transductions were carried out following the method of Miller (1992). The *E. coli* strain JM83 *cspA::cat* (Mitta *et al.*, 1997) was used as the donor. The P1vir phage was a gift from Charles Dorman (Trinity College, Dublin). Disruption of the *cspA* gene was confirmed by PCR. The resultant strain was designated COB229.

Measurement of β -galactosidase activity. β -Galactosidase activity was measured as described by Miller (1972). Briefly, cells were grown in MM (see above) to the desired sampling point and the OD₆₀₀ measured. Then 75 μl cells was aliquoted to a sterile centrifuge

Table 1. Primers

Gene	Primer sequence (5'–3')
<i>argF</i>	CGCGCGTTACCTATTTAG GGTATAGATAAAGTCCGC
<i>argH</i>	GCTGCATACCGGGCGTAG CAGCGTCATCATCATGCC
<i>cspA</i>	GTCCGGTAAAATGACTGG CAGGCTGGTTACGTTACC
<i>cysN</i>	GGCATCACCATTGACGTG GGAGAAAGCGGCTGTTCC
<i>gatZ</i>	GGCAGCAAGAAAATGCGG GATCCGACAGGCTGTAAC
<i>polA</i>	GAAGAGTTGTTGGGGCTG CAGACGAAGGGTGAGCTC

tube containing 675 μl Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM β -mercaptoethanol), 50 μl chloroform and 25 μl 0.1% (w/v) SDS stock. The tube was mixed thoroughly and incubated at 28 °C for 5 min. Then 150 μl of a 4 mg ml⁻¹ ONPG stock was added to the tube, and incubation continued until the contents turned yellow. The reaction was then stopped by the addition of 375 μl 1 M Na₂CO₃ and the time noted. Cell debris was removed by centrifugation at 19800 g for 4 min, and 1 ml of supernatant was used to measure OD₄₂₀. β -Galactosidase activity, in Miller units, was then calculated using the following formula: Miller units = $[\text{OD}_{420}/(t \times 0.075 \times \text{OD}_{600})] \times (1000/1)$, where t is the reaction time in minutes.

Cell extract preparation and measurement of catalase activity.

Crude cell extracts were prepared according to the method of Harris (1981) with some modifications. At an OD₆₀₀ $\approx$ 0.28, 200 ml cultures were harvested by centrifugation at 12000 g for 10 min and washed once with MM. The resulting pellets were resuspended in 4 ml buffer containing 0.05 M Tris/HCl buffer (pH 7.6), 0.05 M MgCl₂, 0.025 M KCl, 1 mM 2-mercaptoethanol and 5% (v/v) glycerol. The cell suspension was disrupted by sonication using an MSE Soniprep 150 set at ~ 16 μm amplitude for six 30 s pulses, with a 30 s interval between pulses. Sonication was carried out on ice. The extracts were clarified by centrifugation at 14000 g for 20 min, and the supernatant was stored at -80 °C. The amount of protein in samples was determined using the Bio-Rad RC DC kit, which is a version of the well-documented Lowry method, using BSA as a standard. Catalase activity measurements were subsequently carried out using the method of Beers & Sizer (1952). It is based on the principle that the breakdown of substrate, H₂O₂, which has an absorbance peak at 240 nm, can be followed spectrophotometrically. A 4 ml quartz cuvette was soaked overnight in concentrated HCl, then rinsed thoroughly with 100% ethanol and allowed to air dry. Then 250 μl cell extract [2 mg (ml protein)⁻¹] and 2.25 ml phosphate buffer [144 mM K₂HPO₄, 56 mM KH₂PO₄ (pH 7.2)] were added to the cuvette and used to zero the spectrophotometer at 240 nm. A 750 μl volume of substrate solution (a 10⁻² dilution of a 30%, v/v, H₂O₂ stock in phosphate buffer) was added and this was taken to be zero time point. Spectrophotometric readings were taken every 10 s for the first 400 s; after this time, gas bubbles accumulated within the cuvette, leading to erroneous measurements. The relative levels of catalase activity could be determined by plotting the decrease in absorbance versus time. The relative rates of catalase activity were then determined by calculating the slope of these lines.

RESULTS

Global effects of Hcy on gene expression

We have shown previously that the growth of *E. coli* cells is inhibited in the presence of Hcy (Roe *et al.*, 2002; Tuite *et al.*, 2005), with ~50 % inhibition occurring in the presence of 0.5 mM Hcy (Tuite *et al.*, 2005). Hcy has been shown to cause a perturbation of isoleucine biosynthesis, through inhibition of threonine deaminase, the enzyme responsible for catalysing the first dedicated step in the biosynthesis of isoleucine (Tuite *et al.*, 2005). In the present study we sought to investigate the effect of Hcy on gene expression in *E. coli* using gene macroarrays.

Four cultures were grown to early exponential phase ($OD_{600} \sim 0.2$) in MM. Two of these were used as untreated controls and 0.5 mM Hcy was added to the other two. RNA was prepared from all four cultures 1 h after the addition of Hcy (See Methods). Four Panorama genome macroarrays (Sigma-Genosys) were probed with labelled cDNA prepared from these RNA extracts, as described in Methods. Genes exhibiting reproducible changes in expression greater than twofold, in the presence of Hcy, are presented in Table 2 grouped by function. A total of 68 genes fulfilled these criteria, showing a reproducible alteration in expression in the presence of 0.5 mM Hcy. This group comprised 38 genes that were up-regulated in response to Hcy and 30 genes whose expression decreased in the presence of Hcy. Using the Kruskal–Wallis one-way ANOVA, the median expression ratio for the 38 up-regulated genes was found to be significantly different from the median expression ratio for the remainder of the genes ($n=4169$), with $P \leq 0.001$. Likewise, the median expression ratio for the 30 down-regulated genes was also significantly different ($P \leq 0.001$) from the median expression ratio for the remainder of the genes ($n=4169$).

Hcy leads to induction of genes involved in translation

The largest functional group of genes that showed altered transcription in the presence of Hcy consisted of genes with translation-related functions. Twenty-one genes whose protein products are constituents of the 50S or 30S ribosomal subunits were up-regulated in response to Hcy. Whether rRNA genes are also influenced by Hcy remains unknown because the gene macroarrays used in these experiments did not include DNA corresponding to rRNA genes. A gene thought to be involved in the maturation of the 30S subunit or in the initiation of translation, *yfiA* (Maki *et al.*, 2000), was also induced. In the same operon as *yfiA* is *trmD*, a gene encoding a tRNA methyltransferase (Bystrom & Bjork, 1982), and this was also found to be induced in response to Hcy. These results suggest that translation may be perturbed in *E. coli* cells exposed to high levels of Hcy. These results are unlikely to arise simply because of the effect of Hcy on growth rate (~50 % reduction in the presence of Hcy); a reduced growth rate would usually be expected to reduce,

not increase, the expression of translation-related functions in the cell. This observation also gives us confidence that many of the changes in expression observed represent specific responses to the presence of Hcy rather than a general response to reduced growth rate per se. However, it is accepted that some of the changes in expression detected may simply arise as a consequence of reduced growth rate.

Amino acid metabolism is disrupted by Hcy

Several genes encoding proteins involved in amino acid metabolism were expressed at significantly altered levels in Hcy-treated cells. The most striking finding was the up-regulation of a large number of genes involved in arginine biosynthesis. The up-regulation of the *argF* and *argH* genes, which are located in separate operons, was confirmed using RT-PCR (Fig. 1). In addition, several genes encoding components of the *artPIMQJ* arginine transporter also showed some degree of up-regulation (>1.5-fold) (data not shown). Of the genes showing down-regulation in the presence of Hcy, the largest effects were observed in genes encoding enzymes involved in the biosynthesis of cysteine, isoleucine and methionine. In the case of cysteine biosynthesis, the largest changes were seen for the *cysD*, *cysK*, *cysM* and *cysN* genes (Table 2). The down-regulation of *cysN* was confirmed by RT-PCR (Fig. 1). We also observed a degree of down-regulation (>1.5-fold) of many of the other genes required for the conversion of sulphate to cysteine, including down-regulation of the sulphate ABC transport

Fig. 1. RT-PCR analysis of the transcription of *cspA*, *argF*, *argH*, *cysN* and *gatZ*. cDNA concentrations were normalized using primers against *polA*. RNA was prepared from cells grown in the presence (+ Hcy) or absence (-Hcy) of 0.5 mM Hcy.

Table 2. Genes showing ≥ 2.0 -fold change in expression in the presence of 0.5 mM Hcy

Gene and functional class*	Fold change†	Function‡
Amino acid metabolism		
<i>argA</i>	2.5 (0.7)	<i>N</i> -Acetylglutamate synthetase
<i>argB</i>	2.4 (0.8)	Acetylglutamate kinase
<i>argF</i>	4.3 (1.4)	Ornithine transcarbamylase
<i>argG</i>	3.0 (0.4)	Argininosuccinate synthetase
<i>argH</i>	3.1 (0.7)	Argininosuccinate lyase
<i>argI</i>	3.1 (1.2)	Ornithine transcarbamylase
<i>asnA</i>	2.6 (0.7)	Asparagine synthetase A
<i>lysP</i>	2.5 (0.5)	Lysine permease
<i>asd</i>	-2.2 (0.01)	Aspartate semialdehyde dehydrogenase
<i>cysD</i>	-2.5 (0.8)	Sulphate adenylyltransferase
<i>cysK</i>	-2.3 (0.1)	Cysteine synthase A
<i>cysM</i>	-2.4 (0.1)	O-Acetylserine sulphhydrylase B
<i>cysN</i>	-3.9 (2.2)	ATP sulphurylase subunit
<i>gltB</i>	-2.3 (0.2)	Glutamate synthase (NADPH) large subunit
<i>ilvC</i>	-2.0 (0.01)	Ketol-acid reductoisomerase
<i>ilvI</i>	-2.3 (0.3)	Acetohydroxy acid synthase III
<i>livH</i>	-3.8 (0.1)	BCAA uptake permease
<i>metE</i>	-2.4 (0.1)	Homocysteine transmethylation
<i>yaeC</i>	-2.3 (0.2)	Methionine transport binding protein
Translation		
<i>lepA</i>	2.0 (0.02)	GTP-binding protein LepA
<i>obgE</i>	2.9 (0.3)	GTPase – chromosomal partitioning
<i>rplA</i>	2.1 (0.3)	50S ribosomal subunit protein L1
<i>rplE</i>	2.1 (0.5)	50S ribosomal subunit protein L5
<i>rplF</i>	2.1 (0.8)	50S ribosomal subunit protein L6
<i>rplJ</i>	2.0 (0.1)	50S ribosomal subunit protein L10
<i>rplL</i>	2.1 (0.7)	50S ribosomal subunit protein L7/L12
<i>rplN</i>	2.3 (0.04)	50S ribosomal subunit protein L14
<i>rplO</i>	2.2 (0.1)	50S ribosomal subunit protein L15
<i>rplP</i>	2.3 (0.6)	50S ribosomal subunit protein L16
<i>rplQ</i>	2.3 (0.5)	50S ribosomal subunit protein L17
<i>rplS</i>	2.2 (0.1)	50S ribosomal subunit protein L19
<i>rplU</i>	2.0 (0.1)	50S ribosomal subunit protein L21
<i>rplV</i>	2.0 (0.4)	50S ribosomal subunit protein L22
<i>rplX</i>	2.2 (0.3)	50S ribosomal subunit protein L24
<i>rplY</i>	2.0 (0.3)	50S ribosomal subunit protein L25
<i>rpmA</i>	2.1 (0.3)	50S ribosomal subunit protein L27
<i>rpmE</i>	2.3 (0.8)	50S ribosomal subunit protein L31
<i>rpsD</i>	2.1 (0.3)	30S ribosomal subunit protein S4
<i>rpsE</i>	2.2 (0.4)	30S ribosomal subunit protein S5
<i>rpsF</i>	2.0 (0.2)	30S ribosomal subunit protein S6
<i>rpsP</i>	2.1 (0.1)	30S ribosomal subunit protein S16
<i>rpsR</i>	2.1 (0.2)	30S ribosomal subunit protein S18
<i>trmD</i>	2.3 (0.04)	tRNA (guanine-7)-methyltransferase
<i>yfiA</i>	2.3 (0.1)	Translation initiation
Carbon utilization/metabolism		
<i>aldA</i>	-2.5 (0.3)	Aldehyde dehydrogenase
<i>gatB</i>	-3.1 (0.4)	Galacticol-specific enzyme IIB of PTS
<i>gatC</i>	-7.0 (0.9)	Galacticol-specific enzyme IIC of PTS
<i>gatZ</i>	-5.8 (0.9)	Putative tagatose 6-phosphate kinase
<i>gltA</i>	-2.1 (0.9)	Citrate synthase
<i>malE</i>	-2.1 (0.5)	Periplasmic maltose-binding protein
<i>mdh</i>	-2.4 (0.4)	Malate dehydrogenase

Table 2. cont.

Gene and functional class*	Fold change†	Function‡
<i>pgk</i>	−2.3 (0.4)	Phosphoglycerate kinase
<i>sucA</i>	−2.5 (0.6)	α-2-Oxoglutarate dehydrogenase
<i>sucD</i>	−2.1 (0.2)	Succinyl-CoA synthetase α subunit
<i>tpiA</i>	−2.0 (0.1)	Triosephosphate isomerase
Stress responses/adaptation		
<i>cspA</i>	5.5 (0.2)	Major cold-shock protein CspA
<i>hscA</i>	2.0 (0.6)	Heat-shock protein HscA
<i>fldB</i>	−2.0 (0.3)	Flavodoxin II
<i>hdeA</i>	−3.3 (0.8)	HdeA precursor
<i>sodA</i>	−2.9 (0.1)	Manganese superoxide dismutase
Miscellaneous		
<i>hycI</i>	2.0 (0.6)	Maturation protease for Ni-containing Hyd-3
<i>rpoA</i>	2.0 (0.03)	RNA polymerase, alpha subunit
<i>copA</i>	−2.5 (0.6)	P-type ATPase Cu ²⁺ transporter
<i>pntA</i>	−2.6 (0.2)	Pyridine nucleotide transhydrogenase
Function unknown		
<i>ypjD</i>	2.0 (0.1)	Putative membrane protein
<i>yhcB</i>	−2.1 (0.3)	Conserved
<i>yjell</i>	−2.0 (0.5)	Conserved

*Functional classes are those defined by the EcoCyc website (www.ecocyc.org).

†A minus sign indicates repressed transcript levels in the Hcy-treated culture compared to the untreated control. Mean values are shown with standard deviations from the mean given in parentheses. The median expression ratios for the 38 up-regulated genes and the 30 down-regulated genes were significantly different ($P \leq 0.001$) from the median expression ratio of the rest of the genes ($n = 4169$), as determined using a Kruskal–Wallis one-way ANOVA.

‡Function was assigned using the Colibri *E. coli* genome website (<http://genolist.pasteur.fr/colibri>).

system encoding genes (data not shown). Two isoleucine biosynthetic genes showed reduced expression (Table 2): *ilvI*, which encodes a subunit of the AHAS III enzyme responsible for the conversion of α-ketobutyrate to aceto-hydrobutyrate (Umbarger, 1996), and *ilvC*, which encodes the enzyme required for the subsequent conversion of aceto-hydrobutyrate to dihydromethylvalerate (Umbarger, 1996). The *livH* gene, which encodes the permease subunit of the BCAA high-affinity transport system (Adams *et al.*, 1990), also showed reduced expression. Two genes involved in methionine metabolism were also down-regulated: the first was the *metE* gene, which encodes the vitamin-B₁₂-independent homocysteine transmethylase, responsible for the transfer of a methyl group from CH₃-THF to Hcy to generate methionine (Greene, 1996); the second was the *yaeC* gene, encoding the binding protein for the MetD DL-methionine transporter (Merlin *et al.*, 2002).

Genes involved in oxidative stress are repressed

A number of reports have indicated that Hcy toxicity in some eukaryotic cells is caused by oxidative stress. It was therefore surprising to find that a number of genes known to be involved in the oxidative stress response of *E. coli* were expressed at reduced levels in the presence of 0.5 mM Hcy. Two genes belonging to the SoxRS regulon, *sodA*, which

encodes superoxide dismutase, and *fldB*, which encodes flavodoxin II, were repressed by Hcy. The gene encoding catalase hydroperoxidase I, *katG*, was also down-regulated to a degree (> 1.5-fold) in the presence of Hcy (data not shown). To establish if this comparatively small degree of repression was significant, we measured catalase activity in crude cell extracts prepared from cells grown in MM in the presence or absence of Hcy (0.5 mM). Catalase activity was found to be reduced by approximately 60 % in extracts prepared from Hcy-treated cells (0.009 ± 0.002 versus 0.022 ± 0.002 OD units min^{−1}, $n = 4$). These data suggest that Hcy does not induce a classic oxidative stress response in *E. coli*; rather, it appears that Hcy treatment might lead to a more reduced cytoplasmic environment.

Repression of genes involved in carbon utilization

There was a down-regulation of many genes required for central metabolism, including six genes encoding enzymes involved in glycolysis and the TCA cycle (*gltA*, *mdh*, *pgk*, *sucA*, *sucD* and *tpiA*) (Table 2). In addition, a further five genes encoding enzymes for these pathways, *gpmA*, *eno*, *icd*, *sucB* and *sucC*, showed a degree of down-regulation (> 1.7-fold; data not shown). Genes required for utilization of alternative carbon sources also exhibited

down-regulation, in particular the galacticol utilization genes *gatB*, *gatZ* and *gatC*, which showed the largest degree of down-regulation of all the genes tested (Table 2). The repression of *gatZ* by 0.5 mM Hcy was confirmed using RT-PCR (Fig. 1).

The major cold-shock protein CspA is induced strongly by Hcy

The gene showing the largest degree of up-regulation (5.5-fold) in response to Hcy was *cspA*, which encodes the major cold-shock protein CspA. This result was surprising since increased levels of *cspA* mRNA are mainly thought to occur in *E. coli* when cells have been subjected to a cold shock (Goldstein *et al.*, 1990). The function of CspA remains uncertain but it is thought to act as an RNA chaperone and may act to facilitate translation at low temperatures by preventing the formation (or destabilization) of secondary structures in mRNA (Phadtare *et al.*, 2000). RT-PCR was used to confirm that *cspA* mRNA is present at elevated levels in Hcy-treated cells (Fig. 1). Although the RT-PCR data were qualitatively similar to the macroarray data, there appeared to be a difference in the relative induction levels of *cspA* and *argH* using these two experimental approaches. The *argH* gene appeared to give the largest induction using RT-PCR, whereas *cspA* gave the largest induction in the macroarray experiments. The reason for this difference is not clear but it may be due to handling differences in the preparation of cDNA for the two approaches.

Next we used a *cspA::lacZ* translational fusion, carried on plasmid pMM016 (Bae *et al.*, 1997), to measure the expression of *cspA* in response to Hcy. β -Galactosidase activity was measured in control and Hcy-treated (0.5 mM) cultures of Frag1(pMM016) at suitable time intervals before and for 3 h after the addition of Hcy. The growth rate of the Hcy-treated culture was reduced by 31 % compared to the untreated control (Fig. 2a). β -Galactosidase activity was found to drop during growth in the control cultures (Fig. 2a), consistent with a previous study showing a rapid drop in *cspA* mRNA as cell density increases (Brandi *et al.*, 1999). In contrast, there was a marked increase in *cspA* expression in the Hcy-treated cultures; after 3 h expression was approximately fivefold higher in Hcy-treated cultures than in the control cultures (Fig. 2a). This increase in *cspA* expression was not simply due to a decrease in the growth rate, since cultures whose growth rates were reduced by the addition of 0.25 M NaCl (growth rate approximately 35 % slower than untreated controls) showed no increase in *cspA* expression (Fig. 2b).

CspA has been shown to be induced by nutrient up-shift (Yamanaka & Inouye, 2001). Since Hcy can be used by the cell to synthesize methionine, we tested whether the addition of this amino acid was sufficient to account for the observed induction of *cspA* expression. Methionine did give rise to a small increase in *cspA* expression (0.5-fold induction 3 h after addition) but this was not sufficient to

Fig. 2. Growth in presence of Hcy leads to increased *cspA* expression. β -Galactosidase activity was measured in *E. coli* Frag1(pMM016) (*cspA::lacZ*) in the presence of (a) 0.5 mM Hcy, (b) 0.25 M NaCl and (c) 2 mM methionine. Growth (solid symbols) and β -galactosidase activity (open symbols) were measured in the absence of any addition (Δ , $\blacktriangle$) and in the presence of additions ($\square$, $\blacksquare$). Additions were made at the time point indicated by the arrow. Mean values are shown $\pm$ SD ($n=4$).

account for the large induction observed in the presence of Hcy (Fig. 2c).

High levels of *cspA* expression in the presence of Hcy suggested the possibility that growth under these conditions may require a functional CspA protein. To investigate this,

an *E. coli* Frag1 strain harbouring a *cspA::cat* disruption was generated by P1 transduction and designated COB229. When grown in the presence of a range of Hcy concentrations, COB229 exhibited the same pattern of growth inhibition as the wild-type parent (data not shown), suggesting that CspA is not essential for survival in the presence of Hcy.

Induction of *cspA* by Hcy does not require a functional CspA protein

In a mutant strain carrying a truncated allele of *cspA* the *cspA::lacZ* translational fusion present on pMM016 is expressed at higher levels than in a wild-type background in response to cold shock (Bae *et al.*, 1997). This finding suggests that *cspA* is subject to negative autoregulation. Other reports also indicate that CspA acts to negatively regulate *cspA* expression (Brandi *et al.*, 1999; Graumann *et al.*, 1997). It was possible therefore that Hcy-induced expression of *cspA* could result if Hcy interfered with the activity of the CspA protein. We investigated this possibility by examining the expression of a *cspA::lacZ* translational fusion in a background deficient for *cspA*. Plasmid pMM016 was transformed into COB229 (*cspA::cat*), generating strain COB230. Hcy was found to stimulate *cspA* expression in this genetic background by approximately 3.5-fold (Fig. 3). Furthermore, levels of *cspA* expression were found to be higher in the *cspA::cat* background than in the wild-type (Fig. 3). These data suggest that Hcy-induced

expression of *cspA* does not require the CspA protein and indicate that, consistent with reports in the literature, *cspA* expression is subject to negative autoregulation.

Hcy-induced *cspA* expression is reduced by isoleucine

The growth inhibition that arises in Hcy-treated *E. coli* cells is caused by a partial auxotrophy for isoleucine; when included in the growth medium in millimolar quantities this amino acid can fully relieve the growth inhibition caused by 0.5 mM Hcy (Tuite *et al.*, 2005). This finding is explained by the fact that threonine deaminase, the first enzyme on the isoleucine biosynthetic pathway, is inhibited by Hcy, thereby depleting the cell of isoleucine (Tuite *et al.*, 2005). We investigated whether the induction of *cspA* by Hcy was related to the partial auxotrophy for isoleucine caused by Hcy treatment. β -Galactosidase assays were performed on Frag1 (pMM016) growing in the presence and absence 0.5 mM Hcy, both with and without added isoleucine (0.15 mM). As expected, isoleucine supplementation alone had no significant effect on *cspA* expression. However, the addition of isoleucine to Hcy-treated cultures almost fully prevented the induction of *cspA* (Fig. 4). This result indicates that *cspA* induction is likely to be caused, directly or indirectly, by the depletion of isoleucine that arises in Hcy-treated cells.

Fig. 3. The CspA protein is not required for Hcy-mediated induction of *cspA* transcription. β -Galactosidase activity was measured in Frag1(pMM016) (*cspA::lacZ*) (Δ , $\square$) and COB230(pMM016) (*cspA::cat*; $\diamond$, $\circ$). Growth (solid symbols) and β -galactosidase activity (open symbols) were measured in the absence of any addition ($\diamond$, Δ) and in the presence of 0.5 mM Hcy ($\square$, $\circ$). Hcy was added at the time point indicated by the arrow. Mean values are shown $\pm$ SD ($n=4$).

Fig. 4. *cspA* induction by Hcy is reduced in the presence of isoleucine. Growth (solid symbols) and β -galactosidase activity (open symbols) were measured in the absence of any addition (Δ , $\blacktriangle$), in the presence of 0.5 mM Hcy ($\square$, $\blacksquare$), in the presence of 0.15 mM isoleucine ($\circ$, $\bullet$) and in the presence of both 0.5 mM Hcy and 0.15 mM isoleucine ($\diamond$, $\blacklozenge$). Additions were made at the time point indicated by the arrow. Mean values are shown $\pm$ SD ($n=4$).

DISCUSSION

Homocysteine (Hcy) has emerged as a molecule of significant medical importance in recent years. High levels of Hcy in serum are associated with a number of life-threatening diseases, including cardiovascular disease, stroke, developmental defects and Alzheimer's disease. Despite much research into this molecule, little consensus has emerged regarding its mode of action. We have sought to address the question of Hcy toxicity using *E. coli* as an amenable model system. In this study we examined the effect of exogenously added Hcy on the transcriptome of *E. coli* Frag1. Our data indicate that the transcription of two principal groups of genes is perturbed in the presence of Hcy: those involved in amino acid biosynthesis and those involved in translation.

Genes involved in the biosynthesis of cysteine are repressed. In minimal medium *E. coli* synthesizes cysteine by combining sulphide with *O*-acetyl-L-serine in a reaction catalysed by *O*-acetylserine lyase, which is encoded by the *cysK* and *cysM* genes. Both *cysK* and *cysM* are repressed in the presence of Hcy. Sulphide is produced from sulphate in a series of four biochemical reactions. Several of the genes involved in these reactions (*cysD*, *N*, *H*, and *J*) are also repressed by Hcy. All of these genes belong to the CysB regulon. CysB is a LysR-like transcriptional activator that up-regulates the transcription of the cysteine biosynthetic genes in the presence of the inducer *N*-acetyl-L-serine, which is produced spontaneously from *O*-acetyl-L-serine (Kredich, 1996). Reduced expression of the cysteine biosynthetic genes occurs when cysteine is present in the medium. Cysteine causes feedback inhibition of serine acetyltransferase, the enzyme required for *O*-acetyl-L-serine synthesis, and this leads to a depletion of the CysB inducer (Kredich, 1996). One plausible explanation for the decreased expression of the cysteine biosynthetic genes in the presence of Hcy is that serine acetyltransferase may be inhibited by Hcy, which is chemically very similar to cysteine, giving rise to inappropriately low levels of the inducer, *N*-acetyl-L-serine. Apart from the *cys* genes indicated here, none of the other genes detected in the macroarray analyses were found to have CysB-binding sites (data not shown), making it unlikely that those changes were influenced by CysB.

The *metE* gene, which encodes methionine synthase, is also repressed in the presence of Hcy (Table 2). This is probably accounted for by an increased availability of methionine within the cell, which arises because of an increase in the availability of the MetE substrate, Hcy. Increased intracellular methionine would then trigger a repressive effect through the MetJ repressor together with its corepressor *S*-adenosylmethionine (SAM). Interestingly, increased Hcy levels have been reported to stimulate *metE* transcription through the activation of MetR, a positive transcriptional regulator of *metE*. However, these studies were conducted using *metJ* mutant strains of *E. coli* (Byerly *et al.*, 1990; Urbanowski & Stauffer, 1989; Wu *et al.*, 1995), making it difficult to predict what impact the increase in methionine levels might have on *metE* transcription. It appears that

under the growth conditions tested in the present study the repressive effect of MetJ-SAM on *metE* transcription outweighs the inducing effect of MetR-Hcy. Indeed, an *in vitro* study of *metE* transcription has shown that MetR-Hcy fails to induce *metE* in the presence of MetJ-SAM (Cai *et al.*, 1989).

Genes involved in isoleucine biosynthesis (*ilvC*, *I*) or transport (*livH*) were also found to be repressed in Hcy-treated cells (Table 2). We have shown elsewhere (Tuite *et al.*, 2005) that Hcy-treated cells become depleted for isoleucine. Hcy inhibits threonine deaminase, the first enzyme on the isoleucine biosynthetic pathway, leading to a partial auxotrophy for this amino acid (Tuite *et al.*, 2005). Here *ilvC* and *ilvI* are shown to be repressed in the presence of Hcy, although it is not clear at present why this is the case. The transcription of *ilvC* is positively regulated by IlvY in the presence of substrate for the IlvC enzyme (acetohydroxy acid isomeroreductase), either acetolactate or acetohydroxybutyrate (Umbarger, 1996). Acetohydroxybutyrate levels are likely to be reduced in Hcy-treated cells since the activity of threonine deaminase is impaired by Hcy, thereby reducing the metabolite flux through the isoleucine branch of the pathway. Reduced levels of acetohydroxybutyrate could account for the reduced expression of *ilvC* observed in Hcy-treated cells.

A number of studies of Hcy toxicity in eukaryotic systems suggest that exogenous Hcy treatment triggers oxidative stress through the increased production of hydrogen peroxide (Loscalzo, 1996; Stamler *et al.*, 1993; Starkebaum & Harlan, 1986). In bacteria there is evidence of a link between cysteine and oxidative stress (Berglin *et al.*, 1982; Park & Imlay, 2003). In the present study we show that catalase activity is actually reduced in Hcy-treated cells and a number of genes (*sodA*, *fldB*, *katG*) involved in protecting *E. coli* cells against oxidative stress are repressed (Table 2). These results suggest that under these growth conditions Hcy does not exert its toxic effect via oxidative stress. Furthermore, the reduced expression of these genes may indicate that the cells experience a reductive stress when exposed to high levels of extracellular Hcy. Interestingly, Hcy triggers a decrease in glutathione peroxidase in human endothelial cells derived from umbilical veins and also acts to block the H₂O₂-mediated activation of HSP70 expression (Outinen *et al.*, 1998). In *Salmonella typhimurium*, Hcy acts to counteract the inhibitory effects of reactive nitrogen species (De Groote *et al.*, 1996). The thiol-containing compound DTT can prevent induction of a heat-shock response in HeLa cells and this is attributed to it reducing activity (Huang *et al.*, 1994). It seems likely that in *E. coli* high levels of intracellular Hcy act to reduce the levels of reactive oxygen species that act, via OxyR and SoxRS, to stimulate the expression of the catalases and superoxide dismutase.

The finding that Hcy triggers the induction of the major cold-shock protein CspA in *E. coli* was surprising since the expression of this protein is mainly thought to be induced in response to cold shock (Goldstein *et al.*, 1990) or nutritional up-shift (Brandi *et al.*, 1999; Yamanaka & Inouye, 2001). As

discussed above, Hcy treatment leads to a partial auxotrophy for isoleucine; growth inhibition by Hcy is fully reversed by supplementing the medium with isoleucine (Tuite *et al.*, 2005). We show that the induction of *cspA* triggered by Hcy is essentially blocked by the inclusion of isoleucine in the growth medium (Fig. 4), suggesting that *cspA* induction arises in Hcy-treated cells as a result of isoleucine starvation. Cells that are starved for isoleucine will also have reduced levels of the corresponding charged tRNA, in this case isoleucyl-tRNA^{Ile}. This will have consequences for translation in the cell; ribosomes will stall on the mRNA at Ile codons. It is possible that this ribosomal stalling acts as the signal leading to induction of *cspA*, since it is known that inhibitors of translational elongation, such as chloramphenicol and tetracycline, stimulate the expression of CspA (Bianchi & Baneyx, 1999; Jiang *et al.*, 1993; VanBogelen & Neidhardt, 1990). Furthermore, these antibiotics, as well as cold shock itself, lead to an increased expression of ribosomal proteins (VanBogelen & Neidhardt, 1990). It seems possible therefore that the increased expression of ribosomal proteins observed in the presence of Hcy may also be accounted for by ribosomal stalling that occurs because of reduced charged isoleucyl-tRNA^{Ile} availability. Further work will be required to clarify this point.

This study has provided us with a greater insight into the effects of the thiol-containing amino acid Hcy on the physiology of *E. coli* cells. It is clear that Hcy interferes with amino acid metabolism and perturbs the process of translation. Together these effects account for the toxicity of exogenous Hcy to *E. coli*. In contrast to the situation in some human cells, Hcy does not induce oxidative stress in *E. coli*. Future studies should address the possibility that eukaryotic translation might be perturbed by Hcy, perhaps contributing to the toxicity of this amino acid in humans.

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