The response of *Escherichia coli* to exposure to the biocide polyhexamethylene biguanide

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Received 31 October 2005
Revised 4 January 2006
Accepted 10 January 2006

The global response of *Escherichia coli* to the broad-spectrum biocide polyhexamethylene biguanide (PHMB) was investigated using transcriptional profiling. The transcriptional analyses were validated by direct determination of the PHMB-tolerance phenotypes of derivatives of *E. coli* MG1655 carrying either insertionally inactivated genes and/or plasmids expressing the cognate open reading frames from a heterologous promoter in the corresponding chromosomally inactivated strains. The results showed that a wide range of genes was altered in transcriptional activity and that all of the corresponding knockout strains subsequently challenged with biocide were altered in tolerance. Of particular interest was the induction of the *rhs* genes and the implication of enzymes involved in the repair/binding of nucleic acids in the generation of tolerance, suggesting a novel dimension in the mechanism of action of PHMB based on its interaction with nucleic acids.

INTRODUCTION

Polyhexamethylene biguanide (PHMB) is a broad-spectrum antibacterial agent that has been widely used for many years as an antiseptic in medicine and the food industry, and its current applications also include impregnation of fabrics to inhibit microbial growth (Cazzaniga *et al.*, 2002; Payne & Kudner, 1996); water treatment (Kusnetsov *et al.*, 1997); disinfection of a variety of solid surfaces such as contact lenses (Hiti *et al.*, 2002); as a mouthwash (Rosin *et al.*, 2001, 2002); treatment of hatching eggs to prevent *Salmonella* infection (Cox *et al.*, 1998, 1999); and as a treatment against fungi (Messick *et al.*, 1999) and *Acanthamoeba* (Donoso *et al.*, 2002; Gray *et al.*, 1994; Narasimhan *et al.*, 2002) in infective keratitis. Its preparations are mixtures of polymeric biguanides of structure $[-(\text{CH}_2)_6\text{NH.C(=NH).NH.C(=NH).NH}]_n$ where $n=2\text{--}40$, with a mean size of $n=11$, giving a molecular mass range of approximately 400–8000, with various combinations of amino ($-\text{NH}_2$), guanide [$-\text{NH.C(=NH).NH}_2$] or cyanoguanide [$-\text{NH.C(=NH).NH.CN}$] as end-groups.

PHMB is bacteriostatic at low concentrations (typically $1\text{--}10\text{ mg l}^{-1}$), but bactericidal at higher concentrations, and inhibition of growth and bactericidal activity both increase with increased polymerization (Broxton *et al.*, 1983; Gilbert

et al., 1990a). The lethal action is considered to involve interaction at the cytoplasmic membrane to cause non-specific alterations in membrane integrity. The proposed basis for the polymer-size effect is that PHMB interacts with acidic membrane-lipids to cause phase separation and domain formation; larger PHMB molecules produce larger domains and therefore more disruption (Broxton *et al.*, 1984; Ikeda *et al.*, 1984). This view was refined by Gilbert *et al.* (1990b) who showed that although the activity increased with increasing length of the polymer, the effect of polymer length was much reduced above $n=6$. Comparison of whole cells and spheroplasts showed that the cell envelope, while not providing complete protection, provides a significant exclusion barrier. Removal of lipopolysaccharides from the outer envelope markedly increased the activity of high- but not low-molecular-mass fractions. These observations, and the discovery of a strong synergy between low- and high-molecular-mass components in biocidal activity, led Gilbert *et al.* (1990b) to conclude that the low-activity, low-molecular-mass components enable larger homologues to gain access to sites of action in the cytoplasmic membrane.

Acanthamoeba castellanii treated with high concentrations of PHMB contained clusters of densely stained precipitates (Khunkitti *et al.*, 1998). Moreover, PHMB treatment produced increased amounts of phosphorus inside the cells compared with untreated controls, and these accumulations were often confined to cell walls and nuclei (Khunkitti *et al.*, 1999). Reduced membrane permeability causing retention of phosphorus, coagulation of proteins and aggregation of phospholipids have been considered as possible causes of elevated phosphorus but the possibility of association between PHMB and nucleic acids has not been

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Abbreviation: PHMB, polyhexamethylene biguanide.

Original macroarray data have been deposited at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi>), accession number GSE2827.

considered hitherto, despite rapid growth in the literature in the last 20 years (Wallace, 2003) on the interaction of DNA with the natural polyamines, putrescine, spermine and spermidine (for a review, see Cohen, 1998), and with a variety of other synthetic polycationic compounds that are currently being developed as vehicles for non-viral transfection of DNA into cells for therapeutic purposes (Vijayanathan *et al.*, 2002).

Recently, we showed that PHMB interacts strongly and cooperatively with nucleic acids *in vitro* and that the structural status of the nucleic acid affects the nature of its initial interactions with PHMB (Allen *et al.*, 2004). Here we show that the interaction of PHMB with nucleic acids *in vitro* has a parallel *in vivo*, giving a novel facet to the action of PHMB. Using whole-genome transcriptional profiling, we show that the transcriptional rate of a wide range of genes in *Escherichia coli* strain MG1655 is altered by exposure of cells to PHMB, and that enzymes involved, *inter alia*, in the metabolism/repair of nucleic acids contribute to the tolerance of *E. coli* to bacteriostatic concentrations of PHMB. Phenotypic analyses in deletion mutants of strain MG1655 and in complemented deletions were used to confirm the biological significance of the results derived from transcriptional profiling.

METHODS

Bacterial strains and deletion mutants. Strains and plasmids are listed in Tables 1 and 2. *E. coli* K-12, strain MG1655 (F⁻, λ⁻, *rph-1*) (Blattner *et al.*, 1997) and its deletion mutants in which the genes *aspA*, *flgJ*, *hdeA*, *hns*, *osmB*, *recA*, *rhsE* and *yebG* had been interrupted by insertion of a kanamycin-resistance cassette, were obtained from the University of Wisconsin *E. coli* Genome Project (www.genome.wisc.edu). In addition, a *cpxP*-deletion mutant in *E. coli* W3110 [F⁻, λ⁻, IN(*rrnD*-*rrnE*) *rph-1*] (*E. coli* Genetic Stock Center, Yale University, CT, USA) bearing the pKD4/pKD46 plasmids (Table 1) was created in our laboratory using the method of Datsenko & Wanner (2000) with the forward primer CATGACTTTA CGTTGTTTCA CACCCCTGA CGCATGTTTG TGTAGGCTGG AGCTGC and reverse primer CTGACGCTGA TGTTCGGTTA AACTTATGCC GTCGAACATA TGAATATCCT CCTTAGTTC. Three PCR screens using locus-specific primers and the respective common test primer were used to verify the presence of both new junction fragments. A fourth PCR was carried out with both flanking locus-specific primers to verify the loss of the parental

(non-mutant) fragment and gain of the new mutant fragment. DNA oligonucleotides were purchased from Sigma-Genosys or Gibco-BRL. To allow phenotypic comparison with wild-type MG1655 and the Wisconsin mutants, the *cpxP* gene deletion was transferred from strain W3110 to strain WG1655 by ultrasonic partial fragmentation of genomic DNA from the mutant strain, electroporation into MG1655 and screening for kanamycin resistance, using standard methods. The resulting MG1655 Δ*cpxP* mutant was verified using the PCR reactions described above. Parallel attempts to create knockout mutants in either *frmB* or *ycgW* were unsuccessful, consistent with a report that these are essential genes (Gerdes *et al.*, 2003). PHMB-resistance phenotypes of all deletion mutants were determined by measurement of minimum inhibitory concentration (MIC).

Overexpression plasmids. The Genome Analysis Project Japan (<http://ecoli.aist-nara.ac.jp/>) provides clones of each ORF predicted from the genome sequence of *E. coli* W3110 (Mori *et al.*, 2000). Every ORF has been cloned into a plasmid (known as pCA24N) containing the IPTG-inducible promoter pT5/*lac*, an N-terminus histidine tag of the target ORF and an in-frame fusion of green fluorescent protein (GFP) at the C-terminus of the target. A *cis*-coded *lacI*^q is present to allow strict repression of the expression from the pT5/*lac* promoter (<http://ecoli.aist-nara.ac.jp/gb5/Resources/archive/archive.html>). The fusion of GFP to a protein can have adverse effects upon protein folding, stability and function. Therefore, the GFP-encoding portion of the original pCA24N-based expression plasmid was removed using a *NotI* digest. The overexpression plasmids, previously known as pCA24N-xxxX [The Genome Analysis Project Japan website (<http://ecoli.aist-nara.ac.jp/>)] with the GFP portion removed, are here referred to as pMJA-xxxX (Table 1). The plasmids were expressed in *E. coli* strain MG1655, and PHMB-resistance phenotypes were determined by measurement of MIC.

Culturing conditions. Liquid cultures were grown in LB medium at 37 °C with constant shaking at 200 r.p.m. unless stated otherwise. Growth was monitored by measuring optical attenuation at 600 nm (*D*₆₀₀) in a Genequant Pro spectrometer (Amersham). For long-term storage, liquid cultures were mixed 1:1 (v/v) with sterile glycerol, mixed thoroughly and stored at -80 °C. To minimize PHMB adsorption to glass and thus to ensure reproducibility, all flasks used during bacterial growth experiments involving PHMB were washed in concentrated nitric acid, rinsed twice in distilled water, air-dried, rinsed with 2% dimethyldichlorosilane in 1,1,1-trichloroethane (BDH), dried, baked at 130 °C and then rinsed three times in distilled water.

PHMB. PHMB, kindly provided by Avecia (Manchester, UK), was a mixture of homologues with *n* ranging from 2 to 15 and with a mean value of 5.5.

Table 1. *E. coli* K-12 derivatives used in this study

Strain	Genotype	Source
W3110	F ⁻ λ ⁻ IN(<i>rrnD</i> - <i>rrnE</i>), <i>rph-1</i>	CGSC no. 4474
MG1655	F ⁻ λ ⁻ <i>rph-1</i>	Blattner <i>et al.</i> (1997)
MG1655 Δ <i>aspA</i>	F ⁻ λ ⁻ <i>rph-1</i> Δ <i>aspA</i> ::Km	U. W. <i>E. coli</i> Genome Project
MG1655 Δ <i>flgJ</i>	F ⁻ λ ⁻ <i>rph-1</i> Δ <i>flgJ</i> ::Km	U. W. <i>E. coli</i> Genome Project
MG1655 Δ <i>hns</i>	F ⁻ λ ⁻ <i>rph-1</i> Δ <i>hns</i> ::Km	U. W. <i>E. coli</i> Genome Project
MG1655 Δ <i>rhsE</i>	F ⁻ λ ⁻ <i>rph-1</i> Δ <i>rhsE</i> ::Km	U. W. <i>E. coli</i> Genome Project
MG1655 Δ <i>osmB</i>	F ⁻ λ ⁻ <i>rph-1</i> Δ <i>osmB</i> ::Km	U. W. <i>E. coli</i> Genome Project
MG1655 Δ <i>recA</i>	F ⁻ λ ⁻ <i>rph-1</i> Δ <i>recA</i> ::Km	U. W. <i>E. coli</i> Genome Project

Table 2. Plasmids used in this study

pMJA-based plasmids are IPTG inducible, containing His-tagged ORFs; see text for details.

Plasmid	Relevant characteristics	Source
pKD46	Recombination plasmid	Datsenko & Wanner (2000)
pKD4	Amplification of Km resistance	Datsenko & Wanner (2000)
pCR-Blunt	Blunt-ended cloning vector	Invitrogen
pMJA	Overexpression plasmid (ORFless)	This study
pMJA- <i>aspA</i>	<i>aspA</i> overexpression plasmid	This study
pMJA- <i>flgJ</i>	<i>flgJ</i> overexpression plasmid	This study
pMJA- <i>hns</i>	<i>hns</i> overexpression plasmid	This study
pMJA- <i>osmB</i>	<i>osmB</i> overexpression plasmid	This study
pMJA- <i>recA</i>	<i>recA</i> overexpression plasmid	This study
pMJA- <i>rhsB</i>	<i>rhsB</i> overexpression plasmid	This study
pMJA- <i>rhsD</i>	<i>rhsD</i> overexpression plasmid	This study
pMJA- <i>rhsE</i>	<i>rhsE</i> overexpression plasmid	This study
pMJA- <i>yebG</i>	<i>yebG</i> overexpression plasmid	This study
pMJA- <i>yhaB</i>	<i>yhaB</i> overexpression plasmid	This study

Enzymes. Restriction endonucleases, DNA ligase, Vent and *Taq* polymerase supplied with their appropriate buffers were obtained from New England Biolabs. Bovine serum albumin (BSA), where required, was supplied with the enzyme. Deoxyribonuclease, free from ribonuclease, was obtained from Qiagen. M-MLV reverse transcriptase and reaction buffer were obtained from Promega.

Transcriptional profiling of *E. coli* using macroarrays.

Samples of cell culture (1 ml) were used to isolate total RNA using an RNeasy miniprep kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in RNase-free water (Sigma). The RNA concentration was determined from A_{260} . The C-terminal primer set comprising 4290 ORF-specific C-terminal primers (Sigma-Genosys) was used to generate hybridization probes in a standard first-strand cDNA synthesis. The Sigma-Genosys protocol was used to achieve >60% incorporation of ^{33}P from [α - ^{33}P]dCTP (74–111 TBq mmol $^{-1}$, NEN Life Science Products). Unincorporated nucleotides were removed by gel filtration through a MicroSpin G-25 Sephadex column (Amersham-Pharmacia) according to the manufacturer's instructions.

Hybridization of cDNAs to Panorama *E. coli* gene arrays (Sigma-Genosys) and subsequent washing steps were carried out according to the manufacturer's instructions. Images representing the localization of hybridized probes were captured on a Personal Imager FX (Bio-Rad) using the PC-based Quantity One software. Arrays were stripped for reuse by washing at 100 °C with stripping solution as specified by the manufacturer.

Spot intensities on a given array were normalized by calculation of the intensity of each as a fraction of the total intensity of all spots taken together. The normalized intensities for each ORF in test (PHMB-exposed) and control (unexposed) arrays were compared and the induction ratios (normalized intensity of test relative to that of the control) were calculated. Genes were considered to exhibit significantly changed expression if the $\log_{10}$ (induction ratio) was greater than 2 SD from the mean of the $\log_{10}$ (induction ratio) for all spots, in three separate experiments. Data are presented as fold-change of test and control (higher value divided by the lower) with positive and negative signs indicating induction and repression by PHMB, respectively. Original data have been deposited at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi>), accession number GSE2827.

MIC. *E. coli* strains were assessed for tolerance to PHMB by growth in the presence of increasing concentration of PHMB to determine the MIC. They were grown for 18 h in LB broth (5 ml, containing additions where appropriate, e.g. IPTG). Each culture was diluted in fresh LB broth (containing additions where appropriate) to an optical attenuation of precisely 0.100, then 145 μl aliquots were dispensed into 96-well micro-titre plates containing PHMB (5 μl per well) to give final concentrations ranging from 1.25 to 6.75 mg l $^{-1}$ in increments of 0.25 mg l $^{-1}$. Plates were incubated at 37 °C, 200 r.p.m. for 48 h and growth monitored by measuring D_{600} . MIC was taken as the lowest concentration showing retarded growth. In all cases, triplicate assays were always in complete agreement (i.e. the same well in triplicate plates). The precision of the MIC values was therefore limited by the increment size and was taken as ± 0.125 mg l $^{-1}$.

RESULTS

Effect of PHMB on the growth of *E. coli*

At all concentrations tested (2.5–15 mg l $^{-1}$), addition of PHMB altered the growth characteristics of *E. coli* (Fig. 1). Concentrations ≥ 10 mg l $^{-1}$ were bactericidal. The addition of 7.5 mg PHMB l $^{-1}$ at $D_{600} \sim 0.3$ caused a temporary cessation in growth lasting approximately 4–5 h, after which growth resumed. The effect of the addition of 7.5 mg PHMB l $^{-1}$ to rapidly growing early exponential *E. coli* cultures on the shape of the growth curve was reproducible (see Figs 1 and 2) and this system was used as the basis for further experiments.

DNA macroarray-based global transcription profiling of *E. coli* in response to PHMB treatment

The comparison between point A and point B (Fig. 2) was used to examine the initial response to PHMB exposure. RNA yields from the cultures at these points were approximately 1 mg (ml culture) $^{-1}$. Genes of known function

Fig. 1. Effect of addition of PHMB on growth of *E. coli*. PHMB was added at early exponential phase (2.5 h, as indicated by the arrow) to give final concentrations of: ■, 0 mg l⁻¹; ◆, 2.5 mg l⁻¹; ●, 5 mg l⁻¹; ▲, 7.5 mg l⁻¹; △, 10 mg l⁻¹; ◇, 12.5 mg l⁻¹; □, 15 mg l⁻¹.

with significantly altered expression profiles are listed in Table 3(a), classified according to the latest known functional assignments (<http://genprotec.mbl.edu/> and Serres *et al.*, 2004). Genes in known operons or possible operons are grouped together regardless of functional category. Genes of no known function (Table 3b) accounted for approximately half of those whose expression was altered immediately upon exposure to PHMB. The overwhelming majority of these were elevated in expression, and putative functions assigned to their protein products include transcriptional regulators and membrane proteins. Overall, upon

Fig. 2. Sampling for differential-expression profiling between PHMB-exposed (▲) and unexposed (■) *E. coli*. PHMB (final concentration 7.5 mg l⁻¹) was added to the test culture at point P (2.5 h). RNA samples were isolated from unexposed exponentially growing *E. coli* (point A) and from PHMB-exposed *E. coli* (point B) at the same culture age (3.75 h). It should be noted that error bars are included (2 SD, *n*=3 culture vessels); however, most errors are too small to see.

exposure to PHMB, 71 genes were induced and 12 repressed significantly (Table 3).

PHMB-phenotype of knockout mutants and overexpression strains

Where possible, knockout strains were obtained for genes showing significant changes in transcript abundance and the deletion mutants tested for PHMB tolerance by determination of MIC. MICs were determined for the knockout strain itself (i.e. MG1655 Δ xxxX), an overexpression strain (i.e. MG1655 pMJA-xxxX) and a complement of the knockout strain (i.e. MG1655 Δ xxxX pMJA-xxxX). The results of these MIC measurements are shown in Table 4.

DISCUSSION

Outer membrane

In the outer membrane PHMB was hitherto thought to interact primarily with LPS and to have a negligible interaction with proteins (Gilbert *et al.*, 1990a). However, several genes associated with outer-membrane components (O-antigen, Rhs elements, flagella, fimbriae) were induced by exposure to PHMB, as now described.

Five genes that are either definitely (*rfaL*, *yefI*, *rfc*, *rfbX*, Table 3a) or putatively (*htrL*, Table 3b) involved in the synthesis and processing of the O-antigen, were induced immediately after exposure to PHMB. These five genes are part of the *rfb* (O-antigen) gene cluster (b2032, b2035, b2037) and *rfa* (LPS core) gene cluster (b3622, b3618), respectively, and it is possible that other members of the clusters were induced, but missed in this analysis. Since *rfbX* and *htrL* are involved in O-antigen biosynthesis and *E. coli* does not express a functional O-antigen, the biological significance of these results remains unclear.

The genes encoding two lipid-anchored outer-membrane proteins, OsmB and VacJ, were also induced on exposure to PHMB (Table 3a). The *osmB* knockout strain was slightly more resistant to PHMB than the wild-type strain. However, complementation of *osmB* in this knockout strain, and overexpression in the wild-type, led to strong resistance (Table 4). Indeed, overexpression of *osmB* in the wild-type gave an MIC value more than twice that of the wild-type. OsmB is a multi-stress-responsive lipoprotein, being both osmotically inducible and regulated by RpoS to appear in stationary phase (Jung *et al.*, 1990), and also under the control of a second independent regulatory system RcsCDB (Boulanger *et al.*, 2005). This system is activated by environmental conditions that have the common consequence that they alter the envelope composition and/or topology, leading to the suggestion that perturbations in the cell envelope might be the inducing signal recognized by the RcsC sensor (Gottesman, 1995). Interestingly, cells express specific RcsCB-regulated genes in order to cope with the stress induced by chlorpromazine (Conter *et al.*, 2002), which, like PHMB, is a cationic amphipathic molecule that

Table 3. Genes of (a) known and (b) unknown function found to have significantly altered expression profiles in a standard analysis of PHMB amended compared with PHMB unamended *E. coli* at same culture age

Gene	Blattner no.	Gene product description	Fold change
(a) Genes of known function			
Nucleic acid associated			
<i>stpA</i>	b2669	DNA-binding protein StpA	+9.1
<i>recA</i>	b2699	DNA strand exchange and recombination protein	+6.6
<i>dnaK</i>	b0014	Chaperone-heat-shock protein 70	+7.4
<i>xseA</i>	b2509	Exodeoxyribonuclease large subunit	+6.8
<i>mcrA</i>	b1159	5-Methylcytosine-specific restriction enzyme A	+21.9
<i>evgS</i>	b2370	Sensory histidine kinase regulating multidrug resistance	+6.9
Translation			
<i>rplY</i>	b2185	50S Ribosomal protein L25	+9.8
<i>hha</i>	b0460	Haemolysin expression modulator	+16.0
<i>rpsP</i>	b2609	30S Ribosomal subunit protein S16	+14.0
<i>miaA</i>	b4171	tRNA Δ -2-isopentenylpyrophosphate (IPP) transferase	+7.7
<i>ybcM</i>	b0546	DLP12 prophage, putative transcriptional regulator	+17.2
Amino acid metabolism			
<i>tnaL</i>	b3707	Tryptophanase leader peptide	-33.9
<i>tnaA</i>	b3708	Tryptophan deaminase	-29.3
<i>tdcR</i>	b3119	<i>tdcABC</i> operon (threonine dehydratase) transcriptional activator	+7.1
<i>cysB</i>	b1275	Transcriptional regulator of cysteine biosynthesis and regulator of sulphur assimilation	+8.2
<i>cbl</i>	b1987	Transcriptional regulator of cysteine biosynthesis	+12.2
<i>aspA</i>	b4139	Aspartate ammonia-lyase (aspartase)	-21.9
Energy metabolism			
<i>pflB</i>	b0903	Pyruvate formate lyase I, induced anaerobically	-18.5
<i>glpD</i>	b3426	Glycerol-3-phosphate dehydrogenase (aerobic)	+9.6
Transport and binding			
<i>lamB</i>	b4036	High-affinity receptor for maltose and maltoseoligosaccharides, phage lambda receptor	-10.1
<i>rbsD</i>	b3748	Membrane-associated component of high-affinity ribose transport system	-12.1
<i>gatB</i>	b2093	Phosphotransferase system, galactitol-specific IIB component	-19.0
Fimbriae and flagella			
<i>flgE</i>	b1076	Flagella hook protein FlgE	-9.8
<i>flgJ</i>	b1081	Flagella protein FlgJ	-11.3
<i>yadC</i>	b0135	Putative fimbrial-like protein	+31.9
<i>yehC</i>	b2110	Putative periplasmic fimbrial chaperone	+7.2
Surface and outer-membrane associated			
<i>osmB</i>	b1283	Osmotically inducible lipoprotein B precursor	+7.4
<i>vacJ</i>	b2346	VacJ lipoprotein precursor	+15.3
<i>rfaL</i>	b3622	O-antigen ligase	+11.6
<i>yefI</i>	b2032	Putative transferase	+26.0
<i>rfaC</i>	b2035	O-antigen polymerase	+5.6
<i>rfaX</i>	b2037	Putative O-antigen transporter	+16.9
Others			
<i>uspA</i>	b3495	Universal stress protein A	-37.2
<i>intB</i>	b4271	Prophage P4 integrase	+5.5
<i>cpxP</i>	b3913	Periplasmic repressor of Cpx regulon	+17.5
<i>b3914</i>	b3914	Putative periplasmic protein	+26.1

Table 3. cont.

Gene	Blattner no.	Gene product description	Fold change
(b) Genes of unknown function			
Rhs associated			
<i>rhsD</i>	b0497	RhsD protein precursor	+40.0
<i>b0499</i>	b0499	Conserved protein	+8.9
<i>ybbD</i>	b0500	Conserved hypothetical protein	+13.9
<i>b0501</i>	b0501	Unknown CDS	+6.6
<i>rhsE</i>	b1456	RhsE protein	+16.1
<i>ycdD</i>	b1457	Unknown CDS	+14.0
<i>ybfD</i>	b0706	H-repeat associated protein	+9.3
<i>rhsB</i>	b3482	RhsB core protein with unique extension	+14.3
<i>yhhH</i>	b3483	Unknown CDS	+19.8
<i>yhiJ</i>	b3488	Conserved hypothetical protein	+69.4
<i>yhiK</i>	b3489	Hypothetical protein	+11.6
<i>yhiL</i>	b3490	Hypothetical protein	+27.4
<i>yibJ</i>	b3595	Putative Rhs protein	+21.5
Other unknowns			
<i>hdeB</i>	b3509	Protein HDEB precursor	-14.8
<i>yeaC</i>	b1777	Conserved hypothetical protein	-6.4
<i>yaiN</i>	b0357	Conserved hypothetical protein	+41.0
<i>ycgV</i>	b1202	Putative membrane protein	+5.2
<i>ydjF</i>	b1770	Putative transcriptional regulator	+8.1
<i>ygiG</i>	b3046	Putative outer-membrane usher protein	+5.5
<i>b0299</i>	b0299	Putative IS transposase	+5.9
<i>ymgD</i>	b1171	Unknown CDS	+15.2
<i>b1172</i>	b1172	Conserved hypothetical protein	+22.5
<i>yhiW</i>	b3515	Putative transcriptional regulator	+6.6
<i>yhiX</i>	b3516	Putative transcriptional regulator	+19.0
<i>yahA</i>	b0315	Putative transcriptional repressor	+12.7
<i>ycgW</i>	b1160	Conserved hypothetical protein	+39.6
<i>b1228</i>	b1228	Unknown CDS	+44.2
<i>ydhA</i>	b1639	Conserved hypothetical protein	+10.0
<i>yehF</i>	b1203	Putative GTP binding protein	+8.4
<i>yefG</i>	b2034	Unknown CDS	+23.1
<i>ybaJ</i>	b0461	Conserved hypothetical protein	+7.6
<i>htrL</i>	b3618	Lipopolysaccharide biosynthesis	+8.6
<i>yiiG</i>	b3896	Conserved protein	+10.0
<i>ydjO</i>	b1730	Putative enzyme	+6.7
<i>yffW</i>	b2642	CP4-57 prophage	+13.9
<i>b1721</i>	b1721	Putative regulator	+8.4
<i>yhaB</i>	b3120	Conserved protein	+48.8
<i>b2854</i>	b2854	Conserved protein, lysozyme like	+19.7
<i>yjbM</i>	b4048	Conserved hypothetical protein	+8.4
<i>yebG</i>	b1848	DNA damage inducible gene in SOS regulon, dependent on cAMP, H-NS	+13.8
<i>yjcF</i>	b4066	Conserved protein	+10.2
<i>b1527</i>	b1527	Conserved protein	+6.0
<i>yedM</i>	b1935	Unknown CDS	+5.5
<i>yrrB</i>	b3446	Unknown CDS	+6.8
<i>b2863</i>	b2863	Unknown CDS	+37.5
<i>yeeN</i>	b1983	Conserved protein	+9.9
<i>b1963</i>	b1963	Unknown CDS	+13.2

damages bacterial membranes (Silva *et al.*, 1979). Thus the altered expression of *osmB* observed after exposure to PHMB is most easily attributed to cell envelope perturbation

although, in the absence of data showing changes in either RpoS or RscCDB, the enforced entry into the stationary phase may also be a factor.

Table 4. Phenotypic characterization of strains based upon MIC assay

The MIC of MG1655 was 3.5 mg l⁻¹. The scale for the mean MIC in three replicate experiments is as follows: -----, MIC ≤ 1.25 mg l⁻¹; ----, 1.25 mg l⁻¹ < MIC ≤ 1.75 mg l⁻¹; ---, 1.75 mg l⁻¹ < MIC ≤ 2.25 mg l⁻¹; --, 2.25 mg l⁻¹ < MIC ≤ 2.75 mg l⁻¹; -, 2.75 mg l⁻¹ < MIC < 3.25 mg l⁻¹; =, 3.25 mg l⁻¹ ≤ MIC ≤ 3.75 mg l⁻¹; +, 3.75 mg l⁻¹ < MIC < 4.50 mg l⁻¹; ++, 4.50 mg l⁻¹ ≤ MIC < 5.25 mg l⁻¹; + + +, 5.25 mg l⁻¹ ≤ MIC < 6.75 mg l⁻¹; + + + +, 6.75 mg l⁻¹ ≤ MIC. Knockout strains that were not available for analysis are indicated by NA.

Gene	MIC for strain in which indicated gene was:		
	Knocked out	Knockout complemented	Overexpressed
DNA/RNA-associated			
<i>hns</i>	---	--	+
<i>stpA</i>	NA	NA	---
<i>recA</i>	-	+	+ + + +
<i>yebG</i>	=	+	+ + +
Metabolism			
<i>tnaA</i>	NA	NA	=
Periplasm-associated			
<i>cpxP</i>	-	-	--
<i>hdeA</i>	--	----	+ +
<i>hdeB</i>	NA	NA	+ +
Outer membrane			
<i>osmB</i>	+	+ + + +	+ + + + +
Flagella and fimbriae			
<i>flgJ</i>	+	----	=
<i>flgE</i>	NA	NA	=
Rhs elements			
<i>rhsB</i>	NA	NA	+ + +
<i>rhsD</i>	NA	NA	+ +
<i>rhsE</i>	-	----	--
Unknown function			
<i>yaiN</i>	NA	NA	=
<i>ycgW</i>	NA	NA	--
<i>yhaB</i>	NA	NA	=

Rhs elements

Exposure to PHMB caused rapid and strong induction of *rhsD* (40.0), *rhsE* (16.1) and *rhsB* (14.3) together with a number of associated genes downstream from each of these (Table 3). Of the remaining two Rhs elements, expression of *rhsA* was unaffected and *rhsC* was significantly upregulated in two of the three replicate experiments (data not shown). However, PHMB also induced additional genes *yibJ* (b3595) and *ybfD* (b0706) located downstream from *rhsA* (b3593) and *rhsC* (b0700), respectively (Table 3), perhaps suggesting that all five elements were induced in response to the presence of PHMB. Because these *rhsABCDE* genes have similar sequences, cross-hybridization artefacts might account for some of the observed signals. However, because PHMB also induced the associated downstream genes that show little or no sequence similarity to each other or the *rhs* genes themselves, it is likely that the signals for the latter are true positives.

The function of the Rhs elements (first identified as being recombinational hot spots) has remained elusive since their

discovery over 20 years ago (Lin *et al.*, 1984). They are not essential but are conserved among *E. coli* strains. Hitherto, attempts to express them under laboratory conditions have failed, so the discovery of induction by exposure to PHMB is both remarkable and a potentially important step in the elucidation of gene function. The *rhs* core ORF (~3.71 kb) contains a peptide motif xxGxxRYxYDxxGRL(I or T)xxxx that is repeated 28 times. Hill *et al.* (1994) suggested that these are cell-surface ligand-binding proteins, based on similarity with the *Bacillus* WapA (wall-associated protein) gene sequence (Foster, 1993), and that the downstream ORFs associated with the *rhs* core ORFs may play roles in the transport and processing of the core ORFs (which lack obvious signal sequences).

Overexpression of the core *rhsB* or *rhsD* in the wild-type led to increased PHMB resistance (Table 4), consistent with their strong induction in the wild-type on exposure to PHMB and possibly indicative of a protective role. Overexpression of *rhsA* or *rhsC* caused no change in PHMB

sensitivity (data not shown), consistent with our observation that PHMB failed to induce these genes. Overexpression of *rhsE* was anomalous in producing little change, despite its strong induction by PHMB. However, absence of a strong phenotype in the overexpressing strain is consistent with the notion that *rhsE* is the only Rhs element that is 'non-functional' in *E. coli* K-12 (Sadosky *et al.*, 1991; Wang *et al.*, 1998). The absence of a clear pattern of PHMB resistance among strains overexpressing *rhs* genes and downstream ORFs confounds unequivocal interpretation of their role in PHMB resistance. Nevertheless, induction of these large putative cell-surface proteins could provide a physical barrier preventing PHMB from interacting directly with LPS in the outer membrane. Alternatively, these anionic proteins [theoretical pI 6.21, with excess of Glu and Asp residues (totalling 185) over Arg and Lys (totalling 139)] may possibly be released into the growth medium, perhaps sequestering the cationic PHMB, and indeed it has been suggested that the homologous WapA may actually be clipped from the membrane and secreted (Foster, 1993). Based on their possible cell-surface location (Hill *et al.*, 1994), these Rhs proteins could also play a role in cellular aggregation, thus reducing PHMB exposure by reducing the area of exposed cell surface (see below).

Flagella, fimbriae and pili

Exposure to PHMB caused induction of hypothetical pilus-assembly genes *yadC* and *yehC* (Table 3a), a putative adhesion gene *ycgV* and, downstream, a putative GTP-binding pilus chaperone *ychF* (Table 3b). At the same time, the flagella-associated genes *flgE* and *flgJ* were down-regulated. The latter are members of a flagella-associated operon that includes *flgBCDEFGHIJKL*. It is likely that all of the operon has had a decrease in expression after exposure to PHMB, but that only *flgE* and *flgJ* have been identified as being significantly altered. Reasons for this could include variation in transcript stability, primer efficiency in the reverse transcription step and hybridization. Pili are known to be used as adherence factors (Low *et al.*, 1987) and to link cells to form aggregates (when they become fimbriae). Thus the switch in production from flagella to pili is consistent with cell aggregation as a response to PHMB exposure. Cellular self-association mediated by pili might protect the innermost cells from PHMB until further changes in gene expression allow cells to recover and grow in the presence of PHMB. The observed rapid decrease in culture attenuation after challenge with higher concentrations of PHMB (Fig. 1) is consistent with cell-cell aggregation.

A *flgJ* knockout strain was slightly more resistant to PHMB in comparison with the wild-type strain. Overexpression of *flgJ* in this knockout (but not in the wild-type) strain led to PHMB sensitivity. FlgJ is bifunctional, containing a C-terminal muramidase to degrade peptidoglycan at the site of flagellum formation (Nambu *et al.*, 1999) and a putative flagellar motor rod-capping role (Hirano *et al.*, 2001). FlgJ, through its muramidase activity, is responsible for the hydrolysis of the peptidoglycan in the periplasm, creating

space for the rod assembly to penetrate. Presumably, overexpression of *flgJ* would increase the rate of breakdown of peptidoglycan in the periplasm, thus allowing PHMB easier access to the cytoplasmic membrane. The resistance of the *flgJ* knockout strain could therefore arise from diminished breakdown of peptidoglycan.

Periplasm

Although PHMB needs to traverse the periplasmic space en route to the cytoplasmic membrane, relatively few periplasmic-protein-encoding genes were changed in response to the presence of PHMB. Exposure to PHMB induced the periplasmic repressor of the Cpx regulon (CpxP) approximately 20-fold and, in keeping with its repressor function, no other members appeared to be induced. However the *cpx* system is complex and *cpxP* itself serves more than one function. The system is activated by misfolded periplasmic proteins and controls not only a stress response but also the assembly of pili. CpxP is a feedback repressor of the *cpx* pathway, dependent for its activity on the presence of the two-component sensor-kinase, CpxA. The ability to auto-activate and then subsequently repress the *cpx* pathway is believed (Raivio *et al.*, 1999) to allow for a temporary amplification of the Cpx response that may be important in rescuing cells from transitory stresses, as is the present case with sublethal exposure to PHMB. CpxP is also a periplasmic chaperone (DiGiuseppe & Silhavy, 2003; Duguay & Silhavy, 2004) known to bind to misfolded pilus subunits (Hung *et al.*, 2001) and it could be induced directly in response to damage to pili caused by PHMB.

The *hdeB* gene in the *hdeAB* operon was down-regulated around 15-fold immediately after exposure to PHMB (*hdeA* was down-regulated approximately eightfold initially, but only significantly in two of three experiments). These genes are linked to acid resistance and may act as chaperones by preventing the aggregation of denatured proteins and thus may also act in a similar fashion in response to the chaotropic action of PHMB.

The alterations in expression of genes encoding periplasmic proteins would be expected to be much greater if extensive damage to the contents of the periplasm was occurring. This relative stability in gene expression therefore suggests that the route to the cytoplasmic membrane by PHMB is direct and that it has very little interaction with the contents of the periplasm.

Cytoplasmic membrane

Many genes involved in transport/binding functions of the cytoplasmic membrane were altered in response to PHMB, in particular those involved in sugar transport. Exposure to PHMB caused *rbsD* to become down-regulated immediately 12-fold. RbsD is involved in the high-affinity transport of ribose. The *gat* operon, involved in the galactitol-specific phosphotransferase system, was also down-regulated on PHMB exposure. In experiments over a longer time frame in

which comparison of unexposed with exposed-recovered cells was made (data not shown), *lldP* (encoding a lactate permease) was down-regulated approximately sixfold. Shut-down of transport systems when challenged with a toxic substance seems a logical strategy but phenotypic analysis of the few available knockout strains gave no coherent set of results although the tolerance to PHMB was altered in some strains (data not shown).

Central metabolism

Tryptophan deaminase (*tnaA*) and an associated leader peptide (*tnaL*) were down-regulated approximately 29- and 34-fold, respectively (Table 2). Besides an apparent role in alkaline stress (Blankenhorn *et al.*, 1999; Bordi *et al.*, 2003; Stancik *et al.*, 2002; Yohannes *et al.*, 2004), TnaA converts tryptophan to indole, which is believed to act as a signal molecule (Wang *et al.*, 2001) in the onset of stationary phase (Lacour & Landini, 2004) and as a stimulator of biofilm production (Di Martino *et al.*, 2002, 2003). Thus, although PHMB exposure produced an enforced stationary phase, cells did not appear to use the *tnaAL* pathway to signal the need for biofilm formation.

The aspartase gene (*aspA*) was down-regulated 22-fold (Table 2) by PHMB exposure, and the corresponding knockout strain was much more resistant than the wild-type (Table 4). Complementing the knockout with the *aspA* expression plasmid restored some sensitivity, and over-expression in the wild-type produced hypersensitivity to PHMB. The *aspA* gene encodes an aspartate ammonia lyase (aspartase) that converts L-aspartate into fumarate and ammonia. The enzyme is repressed when aspartate is needed for other biosynthetic purposes (Kim *et al.*, 2004), but under anaerobic conditions the enzyme is induced in order to generate fumarate as an electron acceptor (Bronder *et al.*, 1982). However, the enzyme is also produced under aerobic conditions, suggesting it has multiple roles (Golby *et al.*, 1998). Repression of *aspA* gene expression has also been noted as an initial response in other stressful conditions (e.g. Brocklehurst & Morby, 2000; Polen *et al.*, 2003) and indeed AspA appears to interact with a conserved histidine-phosphotransferase domain of a two-component signalling system (Salinas & Contreras, 2003), suggesting involvement in transcriptional regulation. Nevertheless, although expression of the *aspA* gene significantly degraded resistance to PHMB, an adequate explanation remains to be found.

Expression of two genes that affect formate metabolism was altered by exposure to PHMB. The *frmR* (formerly *yaiN*) gene product, which is a repressor of *frmAB* that encodes enzymes for oxidation of formaldehyde to formate (Herring & Blattner, 2004), was markedly induced (41-fold) by PHMB. In addition, pyruvate-formate lyase (*pflB*) was repressed (Table 3a). Thus PHMB exposure suppressed formation of formate, which is regarded as a signature molecule in the fermenting *E. coli* cell (Sawers, 2005). Formate probably regulates the *hyf* operon encoding a respiration-linked proton-translocating formate hydrogen

lyase that generates dihydrogen from formate under anaerobic conditions (Andrews *et al.*, 1997). These results, together with the strong suppression of aspartase, suggest that part of the response to PHMB is to deny the cells any opportunity to switch to anaerobic metabolism.

It is possible that the repression of *tnaA* and *aspA* serves as an indirect response to preserve amino acids needed to replace proteins damaged by exposure to PHMB. This is consistent with the observed induction of the transcriptional master regulator of sulphur assimilation into cysteine, *cysB*, and its associated accessory element *cbl* (induced approximately 8- and 12-fold, respectively).

DNA-binding proteins

PHMB altered the expression of several genes encoding DNA-binding proteins, other than specific transcriptional regulators. For example, transcripts for *recA*, *xseA* (an exodeoxyribonuclease) and *stpA* [a H-NS homologue and potential H-NS chaperone (Dorman & Deighan, 2003)] were induced approximately seven-, seven- and ninefold, respectively, immediately after exposure to PHMB. Over-expression of *stpA* (encoding a chaperone and homologue of H-NS, the histone-like nucleoid structuring protein) increased the sensitivity to PHMB; a knockout strain was unavailable for assay. Given the strong sequence similarity between StpA and H-NS but their distinct expression patterns (Dorman *et al.*, 1999), we also assessed the PHMB tolerance of strains expressing *hns* variably. Interestingly, resistance to PHMB was lost in an *hns* knockout strain, partially regained in the complemented knockout, and enhanced (compared to the wild-type) in the overexpressing strain. Thus PHMB tolerance in *E. coli* was directly correlated with the level of H-NS expression. Because H-NS globally represses many unrelated genes (Lammi *et al.*, 1984), the apparent sensitivity in the *hns* knockout strain could be caused by a deregulation of H-NS-regulated genes, but it could also be caused by a change in DNA structure. H-NS regulates by binding to at least two patches of curved DNA to create loops in which RNA polymerase is trapped (Dorman, 2004; Dorman & Deighan, 2003). Moreover, the smallest functional units are dimers which may oligomerize and effectively cross-link adjacent DNA segments of DNA through an oligomeric matrix of H-NS (Dorman, 2004). This process could have a direct protective role against PHMB, because the latter is also known to interact strongly and cooperatively with DNA to create cross-links (Allen *et al.*, 2004) and thus potentially to interfere with DNA-metabolism (replication, repair, transcription, etc.). Competitive binding by H-NS would mitigate against such adverse effects of PHMB.

The regulatory networks in *E. coli* are highly integrated and complex, and rarely, if ever, rely on a single regulator in response to stress. Indeed, Table 3 shows that several other known or putative transcriptional regulators (e.g. *hha*, *ybcM*, *tdcR*, *cysB*, *cbl*) are induced by PHMB and their binding to DNA may also contribute to a DNA-shielding

effect. That *stpA* and *hns* give opposing PHMB tolerance phenotypes when expressed in wild-type *E. coli* provides additional evidence for the difference in biological role of the *stpA* and *hns* gene products.

The *recA* knockout was sensitive to PHMB and complementation of *recA* in this knockout strain led to modest PHMB resistance. Overexpression of *recA* in the wild-type strain led to a stronger resistance to PHMB. If PHMB is indeed gaining access to the cytoplasm and interacting with genomic DNA (Allen *et al.*, 2004), the induction of RecA could either help to repair the damage effected by PHMB, or be part of a damage signal that leads to other events affecting the phenotype, or contribute to an increased capacity to generate diversity (cf. *ycgW* below).

Expression of *yebG* (Table 3b) is a part of the SOS response to damaged DNA and part of the entry into stationary phase (Lomba *et al.*, 1997; Oh & Kim, 1999). A *yebG* knockout strain showed no difference to the wild-type in sensitivity to PHMB but the complemented knockout strain became slightly more resistant and overexpression in the wild-type strain produced strong resistance. Induction of *yebG* by PHMB may therefore be a response either to DNA damage or the entry to an enforced stationary (bacteriostatic) phase. Although the transcriptional control of *yebG* is partly characterized (it is dependent on H-NS and cAMP, but does not require RpoS), no biological function has been found, to date, for YebG (Oh & Kim, 1999).

Potential mutator gene

The *ycgW* gene, which was strongly induced by PHMB (Table 3), was previously isolated in a screen for mutator function (Yang *et al.*, 2004). Mutator cells can have increased fitness, and are selected during exposure to fluctuating environments (see Wright, 2004, for review). Conversely, the overexpression of a mutator (i.e. from a high-copy plasmid) would probably be detrimental to the cell if it conferred too great a mutational load, and this may explain the lesser tolerance seen to PHMB in *ycgW*-expressing cells (Table 4).

The global response of *E. coli* to PHMB

In terms of cellular location, *E. coli* responded to bacteriostatic levels of PHMB by altering the expression of many genes functioning at all levels of cell ultrastructure, i.e. the outer membrane, periplasm, inner membrane and cytoplasmic domains. On the other hand, some of the transcriptional changes were clustered around relatively few specific aspects of cell physiology. First, genes associated with stresses including acid resistance, alkali resistance, osmotic shock and cell-envelope perturbation were altered in expression alongside the controlling factors of the Evg and Cpx response systems. These systems are all involved with sensing and responding to environmental insults that affect the outer membrane, periplasm and cytoplasmic membrane. Since PHMB interacts initially with this cell

envelope, the induction of members of these systems is not surprising. Second, several transcriptional changes (loss of flagella, pilus formation, *cpx*-mediated repair to pili) point towards cell-cell aggregation as a strategy to minimize exposure of cell surface to PHMB. This hypothesis is in keeping with observations that *E. coli* MG1655 cells, when viewed by light microscopy, both aggregate and elongate on exposure to PHMB at the same concentrations as used in the transcript profiling above (data not shown). The third and most novel and intriguing feature of transcript profiles was the induction of the σ^H (heat shock) response, SOS (DNA damage) response and other DNA metabolism-associated genes, which implied that significant damage was occurring to DNA in the cytoplasm. Although this would be almost predictable at bactericidal levels of PHMB (when severe disruption of the inner membrane is thought to occur), these experiments were performed at bacteriostatic levels of PHMB from which the cells, shortly after, recover. These findings led us to expand the theory for the PHMB mechanism of action, as discussed below.

Despite these rationalizations in terms of stress response, aggregation, and DNA-damage limitation, much of the *E. coli* response could not be interpreted as clearly. For example, lipopolysaccharide is thought to be an important exclusion barrier against PHMB, but relatively few genes associated with LPS metabolism were transcriptionally altered. Those that were affected are mostly involved in the synthesis of the O-antigen (a polysaccharide attached to the lipid core of a LPS). However, the O-antigen is not thought to be functional in K-12 strains of *E. coli*. Furthermore, the physiological reasons behind the strong induction of the Rhs elements still remain to be elucidated. Since their discovery, the function of the Rhs elements has remained elusive, particularly because no conditions have previously been found that lead to their induction.

A new theory for the PHMB mechanism of action

Recently we demonstrated a strong cooperative binding between PHMB and DNA, leading to co-precipitation *in vitro* (Allen *et al.*, 2004). This discovery, taken together with the present results, indicates that the critical factor underlying the different effects at bacteriostatic and bactericidal levels of PHMB may not be the ability to disrupt the inner membrane (as previously thought), but the direct interaction between PHMB and genomic DNA. At lower (bacteriostatic) concentrations, the damage caused by the interaction between PHMB and DNA could be tolerable and even repairable. Since binding is highly cooperative, small increases in PHMB concentrations can cause a massive increase in perturbation of DNA function and/or precipitation, thus leading to cell death. This could account for the dose dependence observed by Broxton *et al.* (1983) and Davies *et al.* (1968) and the aggregation of phosphorus near the cell wall in PHMB-treated *Acanthamoeba* (Khunkitti *et al.*, 1998), and could help explain the rapid switch, over a small concentration range, between bacteriostatic and bactericidal levels seen in this study (Fig. 1). This theory

also underpins the central feature that allows widespread use of PHMB as a disinfectant, namely that it has very low mammalian toxicity and that eukaryotes tested tend to have higher MICs than prokaryotes. In eukaryotic cells, the much more extensive compartmentation of genomic DNA and the presence of internal structures are further barriers blocking the direct interaction between PHMB and DNA.

ACKNOWLEDGEMENTS

We would like to thank Dr Simon Andrews for supplying the *E. coli* ORF overexpression plasmids, Cardiff University for financial support and Avecia for supply of specialty chemicals

REFERENCES

- Allen, M. J., Morby, A. P. & White, G. F. (2004). Cooperativity in the binding of the cationic biocide polyhexamethylene biguanide to nucleic acids. *Biochem Biophys Res Commun* **318**, 397–404.
- Andrews, S. C., Berks, B. C., McClay, J., Ambler, A., Quail, M. A., Golby, P. & Guest, J. R. (1997). A 12-cistron *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate hydrogen-lyase system. *Microbiology* **143**, 3633–3647.
- Blankenhorn, D., Phillips, J. & Slonczewski, J. L. (1999). Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* **181**, 2209–2216.
- Blattner, F. R., Plunkett, G. R., Bloch, C. A. & 14 other authors (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Bordi, C., Theraulaz, L., Mejean, V. & Jourlin-Castelli, C. (2003). Anticipating an alkaline stress through the Tor phosphorelay system in *Escherichia coli*. *Mol Microbiol* **48**, 211–223.
- Boulanger, A., Francez-Charlot, A., Conter, A., Castanie-Cornet, M. P., Cam, K. & Gutierrez, C. (2005). Multistress regulation in *Escherichia coli*: expression of *osmB* involves two independent promoters responding either to sigmaS or to the RcsCDB His-Asp phosphorelay. *J Bacteriol* **187**, 3282–3286.
- Brocklehurst, K. R. & Morby, A. P. (2000). Metal-ion tolerance in *Escherichia coli*: analysis of transcriptional profiles by gene-array technology. *Microbiology* **146**, 2277–2282.
- Bronder, M., Mell, H., Stupperich, E. & Kroger, A. (1982). Biosynthetic pathways of *Vibrio succinogenes* growing with fumarate as terminal electron acceptor and sole carbon source. *Arch Microbiol* **131**, 216–223.
- Broxton, P., Woodcock, P. M. & Gilbert, P. (1983). A study of the antibacterial activity of some polyhexamethylene biguanides towards *Escherichia coli* ATCC 8739. *J Appl Bacteriol* **54**, 345–353.
- Broxton, P., Woodcock, P. M. & Gilbert, P. (1984). Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli*. *J Appl Bacteriol* **57**, 115–124.
- Cazzaniga, A., Serralta, V., Davis, S., Orr, R., Eaglstein, W. & Mertz, P. M. (2002). The effect of an antimicrobial gauze dressing impregnated with 0.2-percent polyhexamethylene biguanide as a barrier to prevent *Pseudomonas aeruginosa* wound invasion. *Wounds-Compend Clin Res Pract* **14**, 169–176.
- Cohen, S. S. (1998). *A Guide To Polyamines*, pp. 185–230. Oxford: Oxford University Press.
- Conter, A., Sturny, R., Gutierrez, C. & Cam, K. (2002). The RcsCB His-Asp phosphorelay system is essential to overcome chlorpromazine-induced stress in *Escherichia coli*. *J Bacteriol* **184**, 2850–2853.
- Cox, N. A., Bailey, J. S. & Berrang, M. E. (1998). Bactericidal treatment of hatching eggs. I. Chemical immersion treatments and salmonella. *J Appl Poult Res* **7**, 347–350.
- Cox, N. A., Berrang, M. E., Buhr, R. J. & Bailey, J. S. (1999). Bactericidal treatment of hatching eggs. II. Use of chemical disinfectants with vacuum to reduce salmonella. *J Appl Poult Res* **8**, 321–326.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Davies, A., Bentley, M. & Field, B. S. (1968). Comparison of the action of vantocil, cetrime and chlorhexidine on *Escherichia coli* and its spheroplasts and the protoplasts of gram positive bacteria. *J Appl Bacteriol* **31**, 448–461.
- DiGiuseppe, P. A. & Silhavy, T. J. (2003). Signal detection and target gene induction by the CpxRA two-component system. *J Bacteriol* **185**, 2432–2440.
- Di Martino, P., Merieau, A., Phillips, R., Orange, N. & Hulen, C. (2002). Isolation of an *Escherichia coli* strain mutant unable to form biofilm on polystyrene and to adhere to human pneumocyte cells: involvement of tryptophanase. *Can J Microbiol* **48**, 132–137.
- Di Martino, P., Fursy, R., Bret, L., Sundararaju, B. & Phillips, R. S. (2003). Indole can act as an extracellular signal to regulate biofilm formation of *Escherichia coli* and other indole-producing bacteria. *Can J Microbiol* **49**, 443–449.
- Donoso, R., Mura, J. J. & Lopez, M. (2002). *Acanthamoeba* keratitis treated with propamidine and polyhexamethyl biguanide (PHMB). *Rev Med Chil* **130**, 396–401.
- Dorman, C. J. (2004). H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* **2**, 391–400.
- Dorman, C. J. & Deighan, P. (2003). Regulation of gene expression by histone-like proteins in bacteria. *Curr Opin Genet Dev* **13**, 179–184.
- Dorman, C. J., Hinton, J. C. & Free, A. (1999). Domain organization and oligomerization among H-NS-like nucleoid-associated proteins in bacteria. *Trends Microbiol* **7**, 124–128.
- Duguay, A. R. & Silhavy, T. J. (2004). Quality control in the bacterial periplasm. *Biochim Biophys Acta* **1694**, 121–134.
- Foster, S. J. (1993). Molecular analysis of three major wall-associated proteins of *Bacillus subtilis* 168: evidence for processing of the product of a gene encoding a 258 kDa precursor two-domain ligand-binding protein. *Mol Microbiol* **8**, 299–310.
- Gerdes, S. Y., Scholle, M. D., Campbell, J. W. & 18 other authors (2003). Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. *J Bacteriol* **185**, 5673–5684.
- Gilbert, P., Pemberton, D. & Wilkinson, D. E. (1990a). Barrier properties of the Gram-negative cell envelope towards high molecular weight polyhexamethylene biguanides. *J Appl Bacteriol* **69**, 585–592.
- Gilbert, P., Pemberton, D. & Wilkinson, D. E. (1990b). Synergism within polyhexamethylene biguanide biocide formulations. *J Appl Bacteriol* **69**, 593–598.
- Golby, P., Kelly, D. J., Guest, J. R. & Andrews, S. C. (1998). Transcriptional regulation and organization of the *dcuA* and *dcuB* genes, encoding homologous anaerobic C4-dicarboxylate transporters in *Escherichia coli*. *J Bacteriol* **180**, 6586–6596.
- Gottesman, S. (1995). Regulation of capsule synthesis: modification of the two-component paradigm by an accessory unstable regulator. In *Two-Component Signal Transduction*, pp. 253–262. Edited by J. A. Hoch & T. J. Silhavy. Washington, DC: American Society for Microbiology.
- Gray, T. B., Gross, K. A., Cursons, R. T. M. & Shewan, J. F. (1994). *Acanthamoeba*-keratitis – a sobering case and a promising new treatment. *Aust N Z J Ophthalmol* **22**, 73–76.

- Herring, C. D. & Blattner, F. R. (2004). Global transcriptional effects of a suppressor tRNA and the inactivation of the regulator *frmR*. *J Bacteriol* **186**, 6714–6720.
- Hill, C. W., Sandt, C. H. & Vlazny, D. A. (1994). Rhs elements of *Escherichia coli* – a family of genetic composites each encoding a large mosaic protein. *Mol Microbiol* **12**, 865–871.
- Hirano, T., Minamino, T. & Macnab, R. M. (2001). The role in flagellar rod assembly of the N-terminal domain of *Salmonella* FlgJ, a flagellum-specific muramidase. *J Mol Biol* **312**, 359–369.
- Hiti, K., Walochnik, J., Haller-Schober, E. M., Faschinger, C. & Aspöck, H. (2002). Viability of *Acanthamoeba* after exposure to a multipurpose disinfecting contact lens solution and two hydrogen peroxide systems. *Br J Ophthalmol* **86**, 144–146.
- Hung, D. L., Raivio, T. L., Jones, C. H., Silhavy, T. J. & Hultgren, S. J. (2001). Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili. *EMBO J* **20**, 1508–1518.
- Ikeda, T., Tazuke, S., Bamford, C. H. & Ledwith, A. (1984). Interaction of polymeric biguanide biocide with phospholipid membranes. *Biochim Biophys Acta* **54**, 796–799.
- Jung, J. U., Gutierrez, C., Martin, F., Ardourel, M. & Villarejo, M. (1990). Transcription of *osmB*, a gene encoding an *Escherichia coli* lipoprotein, is regulated by dual signals. Osmotic stress and stationary phase. *J Biol Chem* **265**, 10574–10581.
- Khunkitti, W., Hann, A. C., Lloyd, D., Furr, J. R. & Russell, A. D. (1998). Biguanide-induced changes in *Acanthamoeba castellanii*: an electron microscopic study. *J Appl Microbiol* **84**, 53–62.
- Khunkitti, W., Hann, A. C., Lloyd, D., Furr, J. R. & Russell, A. D. (1999). X-ray microanalysis of chlorine and phosphorus content in biguanide-treated *Acanthamoeba castellanii*. *J Appl Microbiol* **86**, 453–459.
- Kim, Y. H., Park, J. S., Cho, J. Y., Cho, K. M., Park, Y. H. & Lee, J. (2004). Proteomic response analysis of a threonine-overproducing mutant of *Escherichia coli*. *Biochem J* **381**, 823–829.
- Kusnetsov, J. M., Tulkki, A. I., Ahonen, H. E. & Martikainen, P. J. (1997). Efficacy of three prevention strategies against legionella in cooling water systems. *J Appl Microbiol* **82**, 763–768.
- Lacour, S. & Landini, P. (2004). SigmaS-dependent gene expression at the onset of stationary phase in *Escherichia coli*: function of sigmaS-dependent genes and identification of their promoter sequences. *J Bacteriol* **186**, 7186–7195.
- Lammi, M., Paci, M., Pon, C. L., Losso, M. A., Miano, A., Pawlik, R. T., Gianfranceschi, G. L. & Gualerzi, C. O. (1984). Proteins from the prokaryotic nucleoid: biochemical and ¹H NMR studies on three bacterial histone-like proteins. *Adv Exp Med Biol* **179**, 467–477.
- Lin, R. J., Capage, M. & Hill, C. W. (1984). A repetitive DNA sequence, *rhs*, responsible for duplications within the *Escherichia coli* K-12 chromosome. *J Mol Biol* **177**, 1–18.
- Lomba, M. R., Vasconcelos, A. T., Pacheco, A. B. & de Almeida, D. F. (1997). Identification of *yebG* as a DNA damage-inducible *Escherichia coli* gene. *FEMS Microbiol Lett* **156**, 119–122.
- Low, D., Braaten, B. & Woude, V. D. (1987). Fimbriae. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Messick, C. R., Pendland, S. L., Moshirfar, M., Fiscella, R. G., Losnedahl, K. J., Schriever, C. A. & Schreckenberger, P. C. (1999). In-vitro activity of polyhexamethylene biguanide (PHMB) against fungal isolates associated with infective keratitis. *J Antimicrob Chemother* **44**, 297–298.
- Mori, H., Isono, K., Horiuchi, T. & Miki, T. (2000). Functional genomics of *Escherichia coli* in Japan. *Res Microbiol* **151**, 121–128.
- Nambu, T., Minamino, T., Macnab, R. M. & Kutsukake, K. (1999). Peptidoglycan-hydrolyzing activity of the FlgJ protein, essential for flagellar rod formation in *Salmonella typhimurium*. *J Bacteriol* **181**, 1555–1561.
- Narasimhan, S., Madhavan, H. N. & Therese, L. K. (2002). Development and application of an in vitro susceptibility test for *Acanthamoeba* species isolated from keratitis to polyhexamethylene biguanide and chlorhexidine. *Cornea* **21**, 203–205.
- Oh, T. J. & Kim, I. G. (1999). Identification of genetic factors altering the SOS induction of DNA damage-inducible *yebG* gene in *Escherichia coli*. *FEMS Microbiol Lett* **177**, 271–277.
- Payne, J. D. & Kudner, D. W. (1996). A durable antiodor finish for cotton textiles. *Text Chem Color* **28**, 28–30.
- Polen, T., Rittmann, D., Wendisch, V. F. & Sahm, H. (2003). DNA microarray analyses of the long-term adaptive response of *Escherichia coli* to acetate and propionate. *Appl Environ Microbiol* **69**, 1759–1774.
- Raivio, T. L., Popkin, D. L. & Silhavy, T. J. (1999). The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J Bacteriol* **181**, 5263–5272.
- Rosin, M., Welk, A., Bernhardt, O., Ruhnau, M., Pitten, F. A., Kocher, T. & Kramer, A. (2001). Effect of a polyhexamethylene biguanide mouthrinse on bacterial counts and plaque. *J Clin Periodontol* **28**, 1121–1126.
- Rosin, M., Welk, A., Kocher, T., Majic-Todt, A., Kramer, A. & Pitten, F. A. (2002). The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth. *J Clin Periodontol* **29**, 392–399.
- Sadosky, A. B., Gray, J. A. & Hill, C. W. (1991). The Rhsd-E subfamily of *Escherichia coli* K-12. *Nucleic Acids Res* **19**, 7177–7183.
- Salinas, P. & Contreras, A. (2003). Identification and analysis of *Escherichia coli* proteins that interact with the histidine kinase NtrB in a yeast two-hybrid system. *Mol Gen Genomics* **269**, 574–581.
- Sawers, R. G. (2005). Formate and its role in hydrogen production in *Escherichia coli*. *Biochem Soc Trans* **33**, 42–46.
- Serres, M. H., Goswami, S. & Riley, M. (2004). GenProtEC: an updated and improved analysis of functions of *Escherichia coli* K-12 proteins. *Nucleic Acids Res* **32**, D300–D302.
- Silva, M. T., Sousa, J. C. F., Polonia, J. J. & Macedo, P. M. (1979). Effects of local anesthetics on bacterial cells. *J Bacteriol* **137**, 461–468.
- Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhart, D. M., Yoncheva, Y. N. & Slonczewski, J. L. (2002). pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J Bacteriol* **184**, 4246–4258.
- Vijayanathan, V., Thomas, T. & Thomas, T. J. (2002). DNA nanoparticles and development of DNA delivery vehicles for gene therapy. *Biochemistry* **41**, 14085–14094.
- Wallace, H. M. (2003). Polyamines and their role in human disease – an introduction. *Biochem Soc Trans* **31**, 354–355.
- Wang, Y. D., Zhao, S. & Hill, C. W. (1998). Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. *J Bacteriol* **180**, 4102–4110.
- Wang, D., Ding, X. & Rather, P. N. (2001). Indole can act as an extracellular signal in *Escherichia coli*. *J Bacteriol* **183**, 4210–4216.
- Wright, B. E. (2004). Stress-directed adaptive mutations and evolution. *Mol Microbiol* **52**, 643–650.
- Yang, H., Wolff, E., Kim, M., Diep, A. & Miller, J. H. (2004). Identification of mutator genes and mutational pathways in *Escherichia coli* using a multicopy cloning approach. *Mol Microbiol* **53**, 283–295.
- Yohannes, E., Barnhart, D. M. & Slonczewski, J. L. (2004). pH-dependent catabolic protein expression during anaerobic growth of *Escherichia coli* K-12. *J Bacteriol* **186**, 192–199.