Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses

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Benzalkonium chloride (BC) is a commonly used disinfectant and preservative. This study describes changes in expression level at the transcriptomic and proteomic level for *Escherichia coli* K-12 gradually adapted to a tolerance level to BC of 7–8 times the initial MIC. Results from DNA arrays and two-dimensional gel electrophoresis for global gene and protein expression studies were confirmed by real-time quantitative PCR. Peptide mass fingerprinting by MALDI-TOF MS was used to identify differentially expressed proteins. Changes in expression level in adapted cells were shown for porins, drug transporters, glycolytic enzymes, ribosomal subunits and several genes and proteins involved in protection against oxidative stress and antibiotics. Adapted strains showed increased tolerance to several antibiotics. In conclusion, *E. coli* K-12 adapted to higher tolerance to BC acquired several general resistance mechanisms, including responses normally related to the multiple antibiotic resistance (Mar) regulon and protection against oxidative stress. The results revealed that BC treatment might result in superoxide stress in *E. coli*.

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INTRODUCTION

Quaternary ammonium compounds (QACs) such as benzalkonium chloride (BC) and cetrimide are frequently used for disinfection and preservation. QACs are relatively stable, non-corrosive compounds with low toxicity and efficacy over a wide pH range. The destructive mechanism of QACs against bacteria is still not known in detail, but is thought to involve a general perturbation of the lipid bilayer in bacterial membranes. This leads to leakage of cytoplasmic material, damaging and ultimately killing the bacterial cell. For a review see Gilbert & Moore (2005). The QACs have been actively deployed since the 1930s and one has not seen a

resistance development similar to that seen for antibiotics in the same period. Still, there are numerous reports of QAC resistance (Heir *et al.*, 1999; Langsrud *et al.*, 2003; Wright & Gilbert, 1987), most often associated with acquisition, or hyperexpression, of multi-drug efflux pumps (Li & Nikaido, 2004; Tikhonova & Zgurskaya, 2004), which has also been associated with changes in MIC of therapeutically important antibiotics serving as substrates to such pumps (Langsrud *et al.*, 2004; Poole, 2004, 2005). Because of their relatively impermeable outer membrane, Gram-negative bacteria are intrinsically more resistant to QACs than Gram-positives (McDonnell & Russell, 1999), but both Gram-negative and Gram-positive BC-resistant bacteria have been isolated from the food industry (Aase *et al.*, 2000; Heir *et al.*, 1995; Langsrud *et al.*, 2003; Soumet *et al.*, 2005).

Several studies have linked intrinsic/natural resistance of Gram-negative bacteria to tenside-based disinfectants, such as QACs, to the low permeability of the outer membrane, or broad-spectrum efflux systems (Denyer & Maillard, 2002; Nikaido, 2001). Acquisition of resistance in *Escherichia coli* has been related mainly to changes in the composition of lipopolysaccharide (LPS) and fatty acids in the membrane (Ishikawa *et al.*, 2002; Sakagami *et al.*, 1989). In contrast to

Abbreviations: 2-DE, two-dimensional gel electrophoresis; BC, benzalkonium chloride; Omp, outer-membrane protein; PPP, pentose phosphate pathway; QAC, quaternary ammonium compound; RTQ-PCR, real-time quantitative polymerase chain reaction.

The array data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE6712.

A supplementary data file is available with the online version of this paper.

antibiotics, which have very specific targets within the bacterial cell, biocides affect multiple cellular components. We have earlier demonstrated that adaptation of *E. coli* to BC is followed by enhanced efflux activity and may result in cross-resistance to several antibiotics (Langsrud *et al.*, 2004). In this study we describe changes in expression level at the transcriptomic and proteomic level for *E. coli* K-12 cells gradually adapted to a higher tolerance level to BC. The results reveal that BC treatment may result in superoxide stress in Gram-negative bacteria.

METHODS

***E. coli* cell culture.** The *E. coli* K-12 strain selected for this work is a laboratory strain that was purchased from the American Type Culture Collection (ATCC 47076). The 4.6 Mbp genome of *E. coli* K-12 is the most thoroughly characterized amongst micro-organisms and contains 4290 predicted ORFs (Blattner *et al.*, 1997). Cultures were stored in 16% (v/v) glycerol at -80°C . Unless otherwise stated the strain was cultivated at 37°C with shaking (200 r.p.m.).

Adaptation to benzalkonium chloride. *E. coli* K-12 was inoculated (two drops) into 5 ml TSB with BC in a range of concentrations in $2\text{ }\mu\text{g ml}^{-1}$ steps. The cultures were incubated overnight (20–24 h) and inspected visually for growth. The tube with highest BC concentration with visible growth was reinoculated (two drops) into tubes with fresh TSB, with a range of BC concentrations in steps of $2\text{ }\mu\text{g ml}^{-1}$. The cultures were incubated overnight and inspected visually for growth. This procedure was continued for a total of 30–40 days until five reinoculations did not result in growth at higher concentrations of BC. The adaptation process was performed three times, leading to the three biological repeats of adapted strains (A1, A2 and A3). Adapted strains and samples from several steps of the adaptation process were grown overnight in TSB without BC and kept frozen in 16% glycerol until use. PFGE was run to confirm the identity of the final adapted strain, using a Chef Electrophoresis Cell and Pulswave 760 (Bio-Rad), and essentially following the protocol of Bohm & Karch (1992). All adapted strains were tested for stability of the tolerance level after freezing by four reinoculations and growth overnight in TSB without BC.

Harvesting of cells for RNA, DNA and protein analysis. Frozen cultures (control, A1, A2 and A3) were inoculated into 5 ml TSB and incubated overnight. One millilitre of culture was inoculated in 99 ml TSB in a 500 ml baffled shake flask and incubated at 37°C with shaking. Adapted cultures were in addition grown in TSB with $50\text{ }\mu\text{g BC ml}^{-1}$. Bacterial cells were harvested at mid-exponential phase ($\sim 10^8$ c.f.u. ml^{-1}). For protein samples the culture was centrifuged at 5000 g for 10 min and washed three times in TE buffer pH 7.5 (20 mM Tris, 5 mM EDTA and 5 mM MgCl_2), resuspended in 1 ml TE buffer and frozen at -80°C . For DNA arrays, bacterial cells were harvested directly from the shake flask into RNeasyprotect (Qiagen) to secure stable RNA before cell lysis. One volume of culture (1×10^8 c.f.u. ml^{-1}) was added to two volumes of RNeasyprotect, vortexed and incubated at room temperature for 5 min. The sample was then centrifuged at 5000 g for 10 min and the supernatant removed.

Cultures for transcriptional and proteomic analysis were harvested three times on different days, resulting in three control samples and three samples for each adapted strain. Transcriptional analysis was performed for all samples. Protein analysis was performed for controls, A1 and A3 with 4–8 gels per sample.

Protein extraction – total soluble fraction. The protocols were mainly based on methods described by Molloy *et al.* (1999). The

sample was centrifuged at 10°C and 7500 g for 10 min. The pellet was resuspended in 1 ml buffer 1 (40 mM Tris pH 9.5, 5 mM MgCl_2 and 5 mM EDTA). Then $10\text{ }\mu\text{l}$ DNase I and $10\text{ }\mu\text{l}$ RNase A were added and the tube was vortexed and kept at room temperature for 15 min. The sample was then split into two fractions of $500\text{ }\mu\text{l}$ each and sonicated using a Vibra-cell sonifier (Biorblock Scientific) with a Microsonde $l=138\text{ mm}$ and coupler $l=90\text{ mm}$. The sonication was run on ice as five cycles, alternating between 30 s at constant power level 5 and 2 min at power level 5 and 50% of the active cycle, separated by 1 min on ice between each single step. Five hundred microlitres of buffer 2 (40 mM Tris pH 9.5, 8 M urea and 4%, w/v, CHAPS) was added to each tube and the tubes vortexed. Just before use, $25\text{ }\mu\text{l}$ 40% (v/v) ampholyte 3–10 and $20\text{ }\mu\text{l}$ 0.2 M tributylphosphine were added to each tube. The samples were then centrifuged at 10°C , $13\,000\text{ g}$ for 15 min. The supernatant was collected as the cellular protein fraction. The protein concentrations were determined for each fraction by the method of Bradford (1976) using the Bio-Rad protein assay kit with bovine serum albumin as the standard.

Two-dimensional electrophoresis (2-DE). 2-DE was performed essentially according to the method previously described by Folio *et al.* (2004). For isoelectric focusing, 18 cm pH 3–10 non-linear (NL) immobilized pH gradient (IPG) strips (Bio-Rad) were rehydrated overnight with $400\text{ }\mu\text{l}$ IEF buffer containing $60\text{ }\mu\text{g}$ solubilized proteins (Folio *et al.*, 2004). To improve focusing of protein spots in the gels, 50% trifluoroethanol was added to the rehydration buffer according to Deshusses *et al.* (2003).

Proteins were separated using a Multiphor II electrophoresis unit (Amersham Biosciences). After running the first dimension overnight, the IPG strips were equilibrated as previously described (Folio *et al.*, 2004) and then placed on top of 12% acrylamide gels. The second dimension (SDS-PAGE) was carried out in a Multicell Protean II XL system (Bio-Rad) at 15 mA per gel and 40 V constant for 1 h and then at 150 V constant for about 15 h.

Gels were silver stained according to the procedure of Rabilloud *et al.* (1992). For the identification of protein spots, semipreparative conditions were applied with $800\text{ }\mu\text{g}$ of proteins and spots were detected using colloidal Coomassie blue stain according to the protocol of Neuhoff *et al.* (1988).

Gel analysis. Analysis of gel images was carried out using the softwares Z3 (Compugen) and Melanie 4.0 (GeneBio). 2-DE images of adapted and control *E. coli* were compared and qualitative and quantitative differences in spot patterns were analysed. Mean values using at least four gels from two independent protein extractions were taken into account for comparative analyses. The molecular masses and pI values were estimated via standard protein markers, and with theoretical IPG pH gradient and theoretical parameters of five identified spots.

Identification of proteins by MALDI-TOF-MS. Proteins were identified by MALDI-TOF-MS analysis (Proteomic Platform, INRA site de Theix, Clermont-Ferrand, France), using the peptide mass fingerprinting approach. One microlitre each of sample and saturated α -cyano-4-hydroxycinnamic acid matrix (10 mg ml^{-1}) in 50% acetonitrile and 0.1% TFA were mixed onto the target and allowed to dry. Positive-ion MALDI mass spectra were recorded in the reflectron mode of a MALDI-TOF-MS (Voyager DE-Pro; PerSeptive BioSystems), using Voyager software for data collection and analysis. The MS was calibrated with a standard peptide solution (Proteomix; LaserBio Labs). Trypsin autolysis peptides were used for internal calibration of samples. Monoisotopic peptide masses were assigned and used for NCBI database searches with the Mascot software (<http://www.matrixscience.com>). The following parameters were considered for the searches: a maximum fragment

ion mass tolerance of ± 25 p.p.m., a maximum of one missed enzymic cleavage, modification of cysteines by carbamidomethylation and possible oxidation of methionine.

Arrays. Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's recommendations. The concentration of RNA was determined by measuring the absorbance at 260 nm and 280 nm in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The ratio between the measured absorbance at the two wavelengths gives an estimate of RNA purity with respect to contamination (Qiagen Bench guide).

RNA degradation was checked with an Agilent 2100 Bioanalyser according to the userguide from Agilent Technologies. Before cDNA labelling, variations in RNA concentrations were adjusted to $1 \mu\text{g } \mu\text{l}^{-1}$ for all array hybridizations. Labelled cDNA was prepared according to the Panorama *E. coli* gene array protocol (Sigma Genosys), with some modifications. Superscript II RNase H⁻ reverse transcriptase (Invitrogen) and sheared salmon sperm DNA (Ambidon) replaced the equivalent components given in the Panorama protocol. The unincorporated nucleotides were removed with MicroSpinTM G-25 columns (Amersham Pharmacia Biotech), according to the protocol given. Purified labelled cDNAs were hybridized to the Panorama *E. coli* gene array, according to the protocol supplied. The membranes were wrapped in clear plastic film and exposed to a Storage Phosphor Screen (Amersham Biosciences) for 6–7 days. [$\alpha^{32}\text{P}$]dCTP from Amersham Bioscience was used for all hybridizations but one, where the isotope was purchased from Montebello Diagnostics. The arrays were stripped between hybridizations according to the Panorama *E. coli* gene arrays technical protocol (Sigma Genosys).

The software ImageJ (BioDiscovery) was used for quantification of data and the software Genespring (Silicon Genetics) for normalization.

Real-time quantitative PCR (RTQ-PCR). The quality of the Taqman data was secured through the use of two references, house-keeping genes *gapA* and *accD*. The Taqman probes and primers (Table 1) were found using the software SDS 2.2 and purchased from Applied Biosystems. The protocol used was essentially after Rudi *et al.* (2003). PCR samples and controls were prepared in triplicate. The reaction tubes were 96-Well Optical Reaction Plate and tube caps were Optical Adhesive Covers. Applied Biosystems supplied all consumables. Reactions were performed in the 7900HT Sequence Detection System from Applied Biosystems.

Antibiotic resistance. All strains were screened for antibiotic resistance using panels for monitoring of resistance in Gram-negative bacteria, VetMIC GN-mo (version 3) purchased from the National Veterinary Institute in Sweden. VetMIC is a microdilution method made and carried out in accordance with the recommendations and quality control methods recommended by NCCLS (2002), using known reference strains. The method is used in the Swedish (<http://www.sva.se>), SVARM, Norwegian (<http://www.zoonose.no>),

NORM-VET and Finnish (<http://www.eela.fi>), FINRES, monitoring programmes (Petersen *et al.*, 2003).

Overnight cultures in TSB (37 °C, 200 r.p.m.) were diluted to 10^4 – 10^5 c.f.u. ml^{-1} and 50 μl was added to each of the 96 wells of the panel containing an antibiotic gradient for 13 different antibiotics. The panels were incubated for 2 days at 37 °C and inspected for visible growth indicated by a pellet at the bottom of the wells after 1 and 2 days. The screening was repeated twice.

Statistics. The data were log-transformed and evaluated statistically according to the approach of Langsrud (2002, 2005) for analysing designed experiments with multiple responses. The analysis was based on an unbalanced analysis of variance model with the following model terms (degrees of freedom in parentheses): *Strain* (3), *BC conc.* (1), *Biological repeats* (2), *Strain* $\times$ *BC conc.* (2), *Biological repeats* $\times$ *Strain* (6). The presence of the *Biological repeats* $\times$ *Strain* interaction complicated the analysis. To ensure that our conclusions were general, we treated *Biological repeats* as a random factor. This means that the data were analysed according to a mixed-model approach where *Strain* was tested against *Biological repeats* $\times$ *Strain* as its error term. Since the current software for Langsrud's approach (available at <http://www.matforsk.no/ola/program.htm>) does not handle such models, a specialized routine was programmed in Matlab. To separate the adapted effect from the general *Strain* difference, the main effect of *Strain* was decomposed into two parts: Adapted (one degree of freedom) and the remaining part with two degrees of freedom.

With several multiple responses, ordinary significance testing leads to a lot of false significant results. Therefore, the *P*-values for each gene were adjusted by rotation testing according to a false discovery rate criterion (Moen *et al.*, 2005).

In the analysis of a total of 4033 genes, observed difference needed to be relatively large to be judged as significant. Therefore, in addition to this analysis of (nearly) all genes, we ran an extra analysis with a smaller group of 25 pre-selected genes (*acrABEF*, *cysDK*, *emrABDKY*, *fliY*, *marABR*, *mltAB*, *ompFR*, *soxS*, *tolC*, *yciQ*, *yhfZ*, *yhiUV*). These 25 genes had been chosen from the literature for their relevance to resistance and stress prior to the analyses. In this case it was easier to detect significant results, since these genes did not need to compete with all the other 4008 (randomly varying) genes.

RESULTS

Adaptation of *E. coli* K-12 to growth in BC

The initial MIC of BC for *E. coli* ATCC 47076 was $13 \mu\text{g } \text{ml}^{-1}$; this level was increased to 80–90 $\mu\text{g } \text{ml}^{-1}$ in three independently adapted strains. The level of BC

Table 1. Primers and probes used for RTQ-PCR

Gene	<i>T_m</i> (°C)	Forward primer	<i>T_m</i> (°C)	Taqman MGB probe	<i>T_m</i> (°C)	Reverse primer	<i>T_m</i> (°C)
<i>acrB</i>	59	GCGCTTTCTCGAAATCAA	69	TGCGATGGTTTTTCG	59	CGATTGCGGGCAGGT TAA	59
<i>marA</i>	59	GGATCGAGGACAACCTGGAA	68	CGCCACTGTCACTGG	59	CCCGAACGCTCTGACACTTT	57
<i>mdaB</i>	58	GTATGCCAGCGATGGTCGTA	68	CCGCAAAAGATCCGTC	59	CCGCCGAACCGTATTTTT	60
<i>nfnB</i>	59	AAAAACCGCGATGGACGAT	69	TCTGGCTGAAGCTGGT	60	TCGGCATCTTCTGGTCAAC	60
<i>soxS</i>	58	CATCAGACGCTTGCGATT	70	CATTGCGCAACGCC	58	AACGGCGGCCAGTAACAG	61
<i>zwf</i>	59	TGCTGCAAAATCTTTGCATGA	69	TGCGATGTCTCCGCC	59	CTGTCTGCGCTCAGGTCAGA	60

tolerance was still above $80 \mu\text{g ml}^{-1}$ after freezing and after four passages in TSB containing no BC. PFGE confirmed that no contamination had occurred during the adaptation process by showing the same PFGE pattern for the adapted and the control strains.

Transcriptional and proteomic analysis

Initial experiments showed that it was difficult to extract sufficient protein from adapted cells pregrown in BC, and only results from adapted cells grown in TSB without BC were analysed further. More than 50 protein spots showed differences in expression between control and adapted strains, but for many of these spots there was high variation in expression between adapted strains, or it was not possible to identify the protein by MALDI-TOF-MS. Only identified proteins showing similar change of expression for all adapted strains and biological repeats analysed are discussed in the following.

Based on these criteria, the analyses of the protein data revealed 13 identified proteins (Table 2). For some proteins, such as RpsF and NfnB (Fig. 1; Table 2), several spots were identified and analysed.

The analysis of the array data revealed eight genes that showed significantly changed expression ($P < 0.05$) based on statistical analysis of a total of 4033 genes. In the statistical analysis based on 25 pre-selected genes, another five genes showed significant changes ($P < 0.05$). Results from the two analyses were then compared and the gene *acrB* was found to show significantly changed expression ($P < 0.05$) in both analyses. In addition *marA* showed a significance of $P < 0.12$ in the 25 genes analysis and was included in the RTQ-PCR analysis (Table 3). The PCR confirmed the array data and confirmed that *marA* was upregulated in all adapted strains, although at only a small level in one of them. The effect of BC in the medium (*BC conc.*) varied between adapted strains and no genes showed significance ($P < 0.05$) for this. The statistical model term *Strain* $\times$ *BC conc.* showed significance ($P < 0.05$) for 105 genes (results not shown). These 105 genes are listed in supplementary material available with the online version of this paper (and also at <http://www.matforsk.no/lab/ebo/1>). Significance for this interaction indicates that there is some difference in genetic expression between $50 \mu\text{g ml}^{-1}$ and no BC in the medium, but this effect varied between adapted strains (A1, A2, A3) and no significant main effect for *BC conc.* was shown. These genes are therefore not further discussed in the following.

Table 2. Genes and proteins differentially expressed in adapted vs control strain

Variations corresponding to arrays and protein gels given in *italic* and **bold** respectively. All strains for protein analysis and DNA array analysis were grown without BC in the medium. In addition the statistical analysis of the array data includes data from the adapted strains grown with $50 \mu\text{g BC ml}^{-1}$ in the medium.

Function	Gene or protein	Ratio A/C	Comment
Global regulators	<i>soxS</i>	13.4	Oxidative stress protection
	<i>marA</i>	4.7	Multiple antibiotic resistance
Stress related	<i>nfnB</i> *	7.1	Oxygen-insensitive NAD(P)H nitroreductase
	NfnB *	2–5	
	Ssb	0.16–0.54	Single-stranded DNA-binding protein
	<i>ybjC</i> *	9.2	Conserved hypothetical protein, part of <i>sox</i> regulon
	MnSOD *		Manganese superoxide dismutase
	<i>fldA</i> *	3.4	Flavodoxin
	MdaB	4.3	NADPH quinone reductase
Metabolic enzymes	FumC *	2.0	Fumarate hydratase
	<i>zwf</i> *	3.6	PPP enzyme glucose-6-phosphate dehydrogenase
	<i>gcd</i>	4.2	PPP enzyme membrane-bound glucose dehydrogenase
	<i>gapA</i>	2.1	Glyceraldehyde-3-phosphate dehydrogenase A, glycolysis
RpoS regulon	WrbA	0.31	Trp repressor-binding protein
	Dps	0.12	DNA-binding protein
Transporter genes	<i>yeeF</i>	0.44	Amino acid APC transporter
	<i>yhiV</i>	2.2	Multidrug transporter
	<i>acrA</i> *, <i>acrB</i> *	2.5, 3.7	Multidrug exporter
Ribosomal subunits	RpsF	See Fig. 1	30S ribosomal subunit S6
Outer-membrane proteins	<i>tolC</i>	2.2	Associated with efflux pumps
	OmpA, OmpF, OmpT	0.17–0.29	Porins
	OmpC	3.9	Porin
	FecA	0.27	Outer-membrane ferric citrate receptor

**sox/mar* regulated.

Fig. 1. Typical 2-DE-gel images of *E. coli* ATCC 47076 control strain (A) and *E. coli* ATCC 47076 adapted to BC (B). The first-dimension separation of proteins according to *pI* is shown along the non-linear pH gradient (*pI* 3–10) on the horizontal axis. The second-dimension separation of proteins according to molecular mass is seen along the vertical axis (kDa). The enlarged detail (C) is a composite picture in colours showing the expression of ribosomal subunit RpsF in control (green) and BC-adapted (pink) bacterial cells. The colours are complementary, meaning that equal expression levels in both strains shows as black spots. For each spot a dominant green colour indicates a downregulated and a dominant pink colour an upregulated level of expression in the adapted strain.

Table 3. RTQ-PCR results showing differential expression in adapted vs control strain

Results are given as *n* in 2ⁿ, representing fold change in relation to the housekeeping *gapA* and *accD* genes.

Housekeeping gene	Adapted line	<i>soxS</i>	<i>marA</i>	<i>nfnB</i>	<i>acrB</i>	<i>zwf</i>	<i>mdaB</i>	<i>ompF</i>
<i>gapA</i>	A1	4.50	5.90	3.07	2.40	2.40	2.55	−2.47
	A2	2.79	−0.77	0.01	−0.63	0.71	−1.25	−6.28
	A3	6.44	1.15	1.95	2.08	3.03	1.18	0.33
<i>accD</i>	A1	3.86	5.05	2.43	1.75	1.76	1.91	−3.11
	A2	6.19	1.82	3.41	2.76	4.11	2.15	−2.88
	A3	6.24	0.36	1.75	1.88	2.83	0.98	0.13

Global regulators *soxS* and *marA* and genes they regulate

The array data showed that expression of the global regulator *soxS* increased significantly in adapted cells (Table 2). A large upregulation of the global regulator *marA* was also seen in two of the three adapted lines (A1 and A2) but only a small increase in the third adapted line (A3) placed *marA* outside the 5 % significance level ($P=0.118$). A significant effect of adapted line variation was also calculated for *marA* ($P=0.018$). RTQ-PCR confirmed that *marA* was upregulated in all adapted lines, but that the upregulation in one adapted line was small. In this strain (A3) the expression level of *marA* increased early in the adaptation process, but then came close to the expression level of the control strain towards the end of the adaptation process (Fig. 2). Our results showed a significant increase in expression of *acrAB* and *tolC*. Testing for adapted line variation showed significance for *acrB* ($P=0.051$), but RTQ-PCR confirmed the upregulation of *acrB* in all adapted lines (Table 3). RTQ-PCR also showed (Fig. 2) that the upregulation of *acrB* took place in the early part of the adaptation, without any further increase in the level of expression throughout the adaptation process.

SoxS is the activator of the *soxSR* regulon, which includes at least 15 genes, including several whose products play a direct role in responding to oxidative stress. Our results show elevated expression for *soxS* and several *sox* regulon-related genes. RTQ-PCR was used to study how the expression level of some of these genes developed (Table 3, Fig. 2), through the gradual increase in BC tolerance level during the adaptation process. Both macroarrays and RTQ-PCR showed a striking upregulation for *soxS*. The curve

throughout the adaptation process (Fig. 2) clearly shows high upregulated levels of *soxS* already in the early adaptation steps, but also that the level continued to increase throughout the adaptation process.

Although at a much lower level of overexpression than *soxS*, the *sox* regulon genes *zwf* and *nfnB* were both significantly upregulated in the adapted strains. The gene *zwf* encoding glucose-6-phosphate dehydrogenase, which is the first enzyme leading into the pentose phosphate pathway (PPP), is known to be transcriptionally activated by SoxS when exposed to oxidative stress (Greenberg *et al.*, 1990) and by antibiotics through *marA* (Cohen *et al.*, 1993). Another glucose dehydrogenase gene, *gcd*, was also shown to be upregulated in adapted strains at a similar ratio to *zwf* (Table 2), but the relation of this gene to the *soxRS* regulon has never been described.

NfnB is a minor *E. coli* oxygen-insensitive nitroreductase that uses both NADPH and NADH as cofactors. Transcription of *nfnB* is known to be highly increased by induced or constitutive chromosomal expression of *marA*, but is unaltered by constitutive expression of chromosomal *soxS* (Barbosa & Levy, 2002). In the protein gels, NfnB was identified in three spots with the same molecular mass, but different pI values (Fig. 1).

Other transporter genes

Besides the *sox*- and *mar*-regulated *acrAB* multidrug exporter, the gene *yhiV* also shows a significant increase in its level of expression. This gene is a component of the YhiUV multidrug transporter *tolC*, which is also upregulated in the macroarrays, and is required for proper YhiUV

Fig. 2. RTQ-PCR data showing the fold change in expression level of the genes *soxS* (◆), *marA* (△), *nfnB* (□), *acrB* (▲), *zwf* (◇), *mdaB* (●) and *ompF* (■) through the stepwise adaptation of *E. coli* ATCC 47076 to BC from an initial MIC of 13 µg ml⁻¹ to a final MIC of 92 µg ml⁻¹ in the adapted strain A3. An expression level of 1 in the graph indicates an equal level of expression in control and adapted strain. An expression level >1 indicates increased expression while an expression level <1.00 indicates decreased expression level of a gene through adaptation. The data in the figure are normalized to the expression of the housekeeping gene *accD*.

transporter function (Zgurskaya & Nikaido, 2000). The gene *yeeF* encoding an amino acid APC transporter showed a reduced level of expression (Table 2).

Outer-membrane proteins (Omps)

The 2-DE shows a change in expression for several Omps, including TolC already mentioned (Table 2). OmpA, OmpF and OmpT were found to be downregulated while OmpC was upregulated in adapted strains. OmpF, OmpC and OmpT are general bacterial porins and it is well known that when osmotic pressure increases, OmpF decreases and OmpC increases and vice versa. Ishikawa *et al.* (2002) also found a higher relative content of OmpC than OmpF in accordance with higher BC tolerance in *E. coli*. Our Taqman analysis for *ompF* showed an initial response opposed to that of *soxS*, with a steep decrease in expression for initial adaptation steps. Then, the level of expression increased again and normalized half way through the adaptation process (Fig. 2) and stayed close to normal level for the last adaptation steps ($\sim 50\text{--}100\ \mu\text{g ml}^{-1}$). The Taqman results for the end-point in the adaptation of the different adapted lines (Table 3) indicate that this normalization of *ompF* expression level occurred in only one of the three adapted lines. The two other adapted strains remained down-regulated to a significant level at the end of the adaptation process (Table 3).

Modifications of ribosomal subunits

A cluster of spots (Fig. 1) in the lower range of both pI and molecular mass was identified by MALDI-TOF as RpsF protein (30S ribosomal subunit S6). Two of these spots disappeared in patterns of control cells while RpsF spots with slightly higher molecular mass and lower pI appeared in patterns of adapted cells. These modifications indicate that RpsF seems to be post-translationally modified in the adapted strain. RpsF has two glutamic acid residues (Glu) in its C-terminus and it is known that additional Glu residues can be added post-translationally to the C-terminus by the enzyme RimK (Kang *et al.*, 1989; Wilcox *et al.*, 2001). Mutations in ribosomal subunits have been observed to confirm resistance to antibiotics (Allen & Noller, 1989; Bilgin *et al.*, 1990; Chittum & Champney, 1994; Funatsu *et al.*, 1972), and thus it can be assumed that the new RpsF spots observed in adapted cells correspond to post-translational modifications of the protein involved in cell tolerance to BC.

Other stress-related genes

The MdaB protein (NADPH quinone reductase) was significantly upregulated in the 2-DE gels (Table 2). Little has been published on this protein, but Wang & Maier (2004) concluded that it plays an important role in oxidative stress resistance in *Helicobacter pylori*. Moreover, Barbosa & Levy (2000) showed that the gene was upregulated along with constitutive expression of MarA. The *mdaB* gene seems to increase less during the first adaptation step than the *sox*

regulon genes (Fig. 2), but it still showed a significant increase in the level of expression when compared to the control and it continued to increase throughout the adaptation process.

Ssb, the single-stranded DNA-binding protein, binds to single-stranded DNA at the lagging strand of the replication fork in *E. coli* cells (Eisenman & Craig, 2004; Witte *et al.*, 2003). The 2-DE patterns show Ssb to be underexpressed in adapted strains. Others have recently shown Ssb to be upregulated in pH-tolerant oral streptococci (Len *et al.*, 2004) and Ssb has also been shown to be induced upon UV treatment, but downregulated by mitomycin C-induced damage (Courcelle *et al.*, 2001; Khil & Camerini-Otero, 2002).

The major global regulator gene *dps* showed a significant change in expression in the protein gels (Fig. 1). Dps (DNA-binding protein) is primarily produced in stationary phase and has been shown to be regulated by OxyR, σ^{38} and σ^{70} (Altuvia *et al.*, 1994; Azam *et al.*, 1999; Lomovskaya *et al.*, 1994). Nishino & Yamaguchi (2004) showed that another global regulator, H-NS, contributes to multidrug resistance, including to BC, by regulating the expression of multidrug exporter genes. They showed that a double deletion mutant *hns acrAB* had increased resistance to BC while no change was observed for an *hns* mutant and concluded that H-NS represses the expression of *acrEF* and *yhiUV* (*mdtEF*). However, our protein data for H-NS varied significantly between adapted lines, making it difficult to draw any final conclusions on the importance of this regulator.

Antibiotic tolerance of adapted strains

Table 4 shows antibiotic tolerance concentrations for the control and adapted strains. All adapted strains showed an increase in antibiotic tolerance for chloramphenicol, florfenicol, ciprofloxacin, nalidixic acid, ampicillin and cefotaxime. Increased susceptibility was shown for gentamicin, streptomycin and kanamycin.

DISCUSSION

Acquisition of surfactant resistance in *E. coli* has been related mainly to changes in the composition of LPS and fatty acids in the membrane (Ishikawa *et al.*, 2002; Sakagami *et al.*, 1989). We have earlier demonstrated that adaptation of *E. coli* to BC is followed by enhanced efflux activity and may result in cross-resistance to several antibiotics (Langsrud *et al.*, 2004). The results of the current work showed that the BC-adapted *E. coli* strains have acquired defence mechanisms normally related to oxidative protection, and increased tolerance to antibiotics was confirmed.

soxS was by far the most upregulated gene in our arrays. It is known that *soxS* and *marA* contribute to intrinsic resistance to several antibiotics and chemicals in *E. coli* (for a review, see Randall & Woodward, 2002), and that overproduction of SoxS leads to a higher superoxide resistance than

Table 4. Panel for monitoring of antibiotic resistance in Gram-negative bacteria

The control strain and four adapted lines were tested. The numbers given are the MIC of each antibiotic ($\mu\text{g ml}^{-1}$): Am, ampicillin; Ci, ciprofloxacin; Nal, nalidixic acid; Gm, gentamicin; Ce, ceftiofur; Sm, streptomycin; Tc, tetracycline; Ff, florfenicol; Km, kanamycin; Cm, chloramphenicol; Ctx, cefotaxime. Results that differed between repeated experiments are given as intervals (e.g. 2–4). All other results did not differ when repeated.

Strain	Am	Ci	Nal	Gm	Ce	Sm	Tc	Ff	Km	Cm	Ctx
Control	4	0.06	8	2–4	0.5	16–32	2	8	16	8	0.06
A1	8	0.25	64	1	2	4–8	4	64	2–4	128	0.5
A2	4–8	0.25	32	2	1	8–16	4	16–32	8	8–16	0.12
A3	8	0.25	32	2	2	16	4–8	32	8	16–32	0.25

overproduction of MarA (Martin *et al.*, 2000). Our results showed a striking upregulation of several genes known to belong to the *soxRS* regulon, as would be expected for cells exposed to an oxidative stress component. Basically, *E. coli* cells possess two genetic defence systems for response to oxidative stress: one specific defence against peroxides, mediated by the transcriptional activator OxyR (Zheng *et al.*, 2001), and one against superoxide (Pomposiello *et al.*, 2001) that is controlled by the two-stage *soxRS* system (for a review, see Cabiscol *et al.*, 2000). Induction of defence mechanisms against oxidative stress has been linked to other stress responses such as acid stress responses. Maurer *et al.* (2005) showed that low pH accelerates acid consumption and proton export in *E. coli*, while co-inducing oxidative stress and heat shock regulons. Others have shown that overexpression of *soxS* in *E. coli* produces resistance to the antimicrobial agent triclosan (McMurry *et al.*, 1998). When it comes to QACs such as BC, a recent study on BC effects on Chang conjunctival cells showed that QACs induced superoxide synthesis at low concentrations (Debbasch *et al.*, 2001). To our knowledge there has not been published any clear link between acquired resistance to QACs, such as BC, and oxidative stress response in bacteria.

Cationic surfactants, including QACs, are known to disrupt cell membranes and interrupt protein functions, releasing K^+ and other cell constituents and inducing cell autolysis. Majtan *et al.* (1995) reported that the QAC (1-methyldodecyl)trimethylammonium bromide inhibits respiratory activity in *Pseudomonas aeruginosa*. Under aerobic growth, disruption of the cell membrane by BC activity could disrupt the electron-transport chain and thereby increase the level of highly reactive oxygen radicals in the cell. This indirect oxidative stress would cause a depletion of NADPH followed by activation of the *soxRS* regulon as observed in our results. 2-DE showed a significant increase in the expression level of manganese superoxide dismutase (MnSOD) (Table 2, Fig. 1). MnSOD is the primary antioxidant enzyme that protects cells from oxidative stress by catalysing dismutation of superoxide ($\text{O}_2^{\cdot-}$) to

hydrogen peroxide and oxygen. It is uniformly distributed throughout the cytoplasm in prokaryotic cells, binds to DNA and protects it from oxidative damage (Steinman & Weinstein, 1993; Steinman *et al.*, 1994). The MnSOD gene *sodA* is part of the *soxRS* regulon and is transcriptionally regulated by at least six control systems (Compan & Touati, 1993; Nunoshiba *et al.*, 1992; Wu & Weiss, 1992). The increase in MnSOD levels in the adapted cells helps to minimize depletion of NADPH, as does also induction of the PPP through Zwf, by increasing production of NADPH. Besides *zwf*, we also observed an increase in the expression of *gcd*, encoding another dehydrogenase. Gcd is known to be involved in the formation of membrane potentials, feeding electrons obtained from the oxidation of glucose to ubiquinone and ubiquinol oxidase (van Schie *et al.*, 1985; Yamada *et al.*, 1993b). Gcd is negatively regulated by cAMP and positively by oxygen (Yamada *et al.*, 1993a).

FldA (flavodoxin I), one of the two known flavodoxins in *E. coli* and also known to belong to the *soxRS* oxidative stress regulon, was significantly upregulated in the adapted strains (Table 2). The same was the case for *fumC* (fumarase C) (Paterson *et al.*, 2002). Upregulation of *fumC*, as observed in our data (Table 2), is also a part of the cellular defence against oxidative stress through the replacement of redox-sensitive isozymes by redox-resistant isozymes (fumarase C). FumC activity has also been shown to be upregulated as a response to high oxygen levels (Liochev & Fridovich, 1992; Tseng *et al.*, 2001).

Our results showed significant upregulation of the *nfnB* gene (synonym *nfsB*), encoding an oxygen-insensitive nitroreductase, while we saw no evidence for increased expression of *nfsA*. On the other hand, we found the *ybjC* gene (conserved hypothetical protein) to be upregulated in adapted strains. This gene is located directly upstream of *nfsA* in the *sox* regulon, cotranscribed with *nfsA*, which suggests that these two genes form an operon (Paterson *et al.*, 2002). NfsA is the major nitroreductase and uses NADPH as the electron donor. In a macroarray study, Barbosa & Levy (2000) showed *nfnB*, as well as *nfsA*, to be MarA responsive and they found a significant change for *nfnB* and *ybjC*, but not for *nfsA* under constitutive expression of MarA. Their data suggested that MarA is preferred over SoxS in upregulating *nfnB* and further work by the same authors supported this conclusion (Barbosa & Levy, 2002). Our results corroborate those of Barbosa & Levy (2000) for many of the observed changes in our adapted strains. The expression level of *nfnB* is lower for the adapted line (Table 3, A3) that showed the lowest level of *marA* expression. Due to the increase in expression we observed for *marA* and several genes known to be affected by constitutive expression of MarA, it is likely that this protein plays a regulatory role in the increased resistance to BC in the adapted cells. However, the variation between adapted lines for this gene, as opposed to the highly statistically significant upregulation of *soxS*, points to *soxS* as the most important regulator in the general adapted resistance to BC.

Both *marA* and *soxS* encode positive regulators of *acrAB* and upregulate *acrAB*, which encodes a stress-induced multi-drug efflux pump formed by the AcrAB–TolC complex across the membranes (Ma *et al.*, 1995). This AcrAB–TolC efflux pump has been linked to intrinsic resistance to BC (Moken *et al.*, 1997). Our results showed a significant increase in expression of *acrAB* and *tolC*.

There was no support in our results for any variation of gene or protein expression in the OxyR system, except a reduced expression of Dps in adapted cells as observed in 2-DE gels. *dps* is also part of the RpoS growth phase regulon and most work published on this protein refers to upregulation of its expression as a defence mechanism against oxidative damage by peroxides during the exponential growth phase (Almiron *et al.*, 1992; Choi *et al.*, 2000; Martinez & Kolter, 1997; Martinez-Martinez *et al.*, 2000). Our data also showed a downregulated level of another member of the RpoS regulon, the WrbA protein. It therefore stands to reason that the RpoS regulon could be repressed in the adapted strains, although further work would have to be done to confirm this. Results of bactericidal tests (not shown) showed a much higher survival ratio for *E. coli* control cells in the exponential phase than in the stationary phase. In the stationary phase, Dps is known to be active in DNA protection by presence in high molecule numbers and against multiple stress factors such as UV, copper, thermal, acid and base shock (Almiron *et al.*, 1992; Nair & Finkel, 2004; Wolf *et al.*, 1999). Kirkpatrick *et al.* (2001) showed repressed levels of Dps and other RpoS regulon proteins for *E. coli* exposed to formate stress, as opposed to acetate stress, which led to increased expression of the same proteins. They hypothesized that the level of acetyl-CoA could be involved in regulation of this response. The activation of the *soxRS* regulon leads to induction of the PPP through glucose-6-phosphate dehydrogenase (Zwf) with corresponding reversion of the glycolysis (glyconeogenesis) to meet the urgent demand for reducing power (NADPH). The need to metabolize pyruvate through other pathways then arises, resulting in a reduction of acetyl-CoA levels, which could explain the reduced level of the Dps protein in our adapted strains.

Changes in the cell membrane with alteration of the LPS composition have been described for QACs and this might lead to a tighter membrane structure, making it harder to extract proteins. On the other hand, recent work by Braoudaki & Hilton (2005) concluded that a sublethal concentration of BC did not lead to any significant changes in outer membrane or LPS, but that preadapted strains proved less hydrophobic than resistant strains. We observed that the pellets of cells grown in the presence of BC were dark brownish in colour and stuck strongly to the inside of the test tubes. The lower OD₆₀₀ we observed for adapted cells at mid-exponential phase (10^8 c.f.u. ml⁻¹) could also indicate a change in cell size.

Observed changes in Omps and in particular the upregulation of the small porin OmpC and the downregulation of the larger porin OmpF is well described in the literature as a

mechanism to reduce access through the cell membrane (Ishikawa *et al.*, 2002). The Taqman results for *ompF*, which showed a normalization of the *ompF* level after an initial decrease in expression level for one of the adapted lines, could indicate that the *ompF* response is not critical for the BC-resistant strain. One explanation could be that BC cannot pass through the OmpF porin, but that the switch from the large porin OmpF to the smaller porin OmpC is a general stress response, not actually protecting the cell from BC. Another explanation could be that as other adapted resistance mechanisms become stronger, the OmpF down-regulation in early adapted cells is no longer needed to cope with the BC threat. This presupposes that BC can enter the cell through the OmpF porin, which is yet to be verified. Loss of OmpF has been shown to be one of the resistance mechanisms elaborated by *E. coli* against β -lactam antibiotics (Martinez-Martinez *et al.*, 2000) and fluoroquinolones (Tavio *et al.*, 1999). It is known that β -lactams such as ampicillin, which are molecules close to BC in size, can bind to and pass through the OmpF porin (Nestorovich *et al.*, 2002) and interestingly the antibiotic panel testing (Table 2) showed an increase in tolerance to ampicillin.

The protein data indicate post-translational modifications of the S6 ribosomal subunit encoded by the *rpsF* gene. Several of the antibiotics showing increased tolerance for adapted strains in our screening tests (gentamicin, tetracycline, chloramphenicol, streptomycin) have killing mechanisms linked to ribosomal activity. One might speculate whether ribosomal modification is a likely cross-resistance mechanism between BC and certain antibiotics. Further work is needed to clarify this issue.

The variation in curve profiles for the adaptation process indicates that different genetic approaches could lead to the same resistance level and/or that some adaptation steps could be more difficult to acquire. This is further supported by the significant differences between adapted lines in the expression of genes such as *marA* and *ompF*.

The *gapA* gene is a common reference for Taqman results, but our results showed a variation in the level of expression of the GapA protein. Therefore, we decided to use another commonly used reference and housekeeping gene, *accD*, as a second reference for these experiments. The results showed that one of the adapted lines differed from the others when *gapA* was used as a reference. This confirmed that the expression of *gapA* was more strongly upregulated in some adapted lines than in others. For two out of three adapted lines, however, the *gapA* reference gave the same pattern of change in level of expression as *accD*. The change in *gapA* level in adapted strains could be explained by increased flux through the PPP, producing NADPH and returning glyceraldehyde 3-phosphate to glycolytic metabolism and then running either glyconeogenesis, or the second part of glycolysis to pyruvate.

The average signal in the DNA arrays was relatively low. Therefore there are probably smaller (<2-fold) but

significant changes in the expression of several genes that are not seen in these results. The data presented represent the largest changes in expression level between adapted strains and control strain and are clearly significant for repeated experiments. There is overlap between protein data and DNA array data, and the PCR data confirm the other data.

In summary, *E. coli* K-12 adapted to higher tolerance to BC acquired several general resistance mechanisms including responses normally related to the multiple antibiotic resistance (Mar) regulon and protection against oxidative stress. Adapted strains showed increased tolerance to several antibiotics and the results revealed that BC treatment might result in superoxide stress in *E. coli*.

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