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Indole-3-acetic acid improves *Escherichia coli's* defences to stress

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Abstract Indole-3-acetic acid (IAA) is a ubiquitous molecule playing regulatory roles in many living organisms. To elucidate the physiological changes induced by IAA treatment, we used *Escherichia coli* K-12 as a model system. By microarray analysis we found that 16 genes showed an altered expression level in IAA-treated cells. One-third of these genes encode cell envelope components, or proteins involved in bacterial adaptation to unfavourable environmental conditions. We thus investigated the effect of IAA treatment on some of the structural components of the envelope that may be involved in cellular response to stresses. This showed that IAA-treated cells had increased the production of trehalose, lipopolysaccharide (LPS), exopolysaccharide (EPS) and biofilm. We demonstrated further that IAA triggers an increased tolerance to several stress conditions (heat and cold shock, UV-irradiation, osmotic and acid shock and oxidative stress) and different toxic compounds (antibiotics, detergents and dyes) and this correlates with higher levels of the heat shock protein DnaK. We suggest that IAA triggers an increased level of alert and protection against external adverse conditions by coordinately enhancing different cellular defence systems.

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Introduction

Indole-3-acetic acid (IAA) is a ubiquitous molecule able to induce, in prokaryotic and eukaryotic organisms, changes in gene and protein expressions leading to different physiological alterations. In particular, IAA, the main phytohormone with an auxin activity, regulates many plant developmental and cellular processes (Kende and Zeevaart 1997). Despite this, the molecular mechanism of its action remains unknown.

Soil bacteria (such as *Pseudomonas, Azospririllum, Agrobacterium* and *Rhizobium*) synthesize IAA as part of a system to communicate with their host plant, and many of them use IAA in pathogenic interactions such as tumours and hairy roots. These bacteria mainly synthesize IAA from tryptophan via the indoleacetamide (IAM) (Lemcke et al. 2000), or indole-3-pyruvate (Zimmer et al. 1998) biosynthetic pathways.

IAA is also involved in the morphogenetic development of *Saccharomyces cerevisiae*. At high concentrations, IAA blocks the growth of yeast cells, whereas at lower concentrations it induces filamentation and adhesion leading to plant infection (Prusty et al. 2004). In mammals Folkes et al. 2002 have demonstrated that IAA, in combination with a peroxidase activity (HRP), can be an alternative prodrug compound for targeted cancer therapy. Indeed, the IAA/HRP combination induces the loss of membrane integrity, DNA fragmentation and chromatin condensation (De Melo et al. 2004).

In *Escherichia coli* K-12, IAA and other indole derivatives are able to circumvent the cAMP requirement for the induction of the *araBAD* operon, involved in L-arabinose metabolism, in *cya*$^-$ strains (*cya* encodes adenylate cyclase, required for cAMP synthesis). The IAA-dependent induction does not require the catabolite gene activator protein CAP (the *crp* gene

product) (Ebright and Beckwith 1985). In addition, the over-expression of arabinose operon is not observed in isogenic wild-type (cya^+) strains (Kline et al. 1980). Moreover, IAA and other small metabolites can induce the *ilvB* gene product, the acetohydroxy acid syntase, that catalyses the first step common to isoleucine and valine synthesis (De Felice et al. 1986). This is observed in both cya^+ and cya^- derivative of *E. coli* K-12, but exactly how these indole derivatives mediate their effects is yet to be understood.

To analyse changes in the transcription profiles of *E. coli* cells treated with IAA we employed high-density oligonucleotide arrays (GeneChip^R *E. coli* Genome Array, Affymetrix), derived from the sequenced K-12 MG1655 strain (Blattner et al. 1997). This array contains probes for all 4,218 annotated open-reading frames (ORFs) and many of the intergenic (Ig) regions. The analysis revealed that alterations in transcription induced by IAA treatment were mostly connected to genes regulating the general defence mechanisms that are activated under stress conditions. However, these alterations were different from those observed in the typical stress and SOS responses.

Since both the integrity of the cell envelope and the synthesis of protective compounds may help the cell to overcome stressful environmental conditions, we evaluated the production of some structural cellular components such as LPS, EPS and biofilm and the synthesis of chemical and molecular chaperones (trehalose and DnaK, respectively). We found that the increased production of trehalose and LPS, the higher release of slim polysaccharides and the enhanced synthesis of the DnaK molecular chaperone correlated with the higher resistance to stress conditions (UV, heat, cold, low pH, high salt, H_2O_2, antibiotics, detergent and dye) observed for IAA-treated cells. Taken together, our data suggest that IAA may work as a stress manager by activating different protective pathways to synergistically enhance stress tolerance.

Materials and methods

Bacterial growth conditions

Cells were grown, aerobically, at 37°C in M9 minimal medium containing: 20 mg uracil l^{-1}, 1 mg thiamine l^{-1}, 0.4% (w/v) L-arabinose as carbon source and supplemented with 40 mg casein acid hydrolysate l^{-1}. Solid media contained 15 g agar l^{-1} (Difco) in TY [0.5% (w/v) yeast extract, 0.8% (w/v) NaCl and 1% (w/v) tryptone] or minimal medium. Exponentially growing *E. coli* K-12 (MG1655) cultures ($OD_{600} = 0.6$) were split into six aliquots; to five aliquots, an IAA solution was added to a final concentration of 0.125, 0.25, 0.5, 1.0 or 2.0 mM and the last one was left untreated (control). After 2 h ($OD_{600} = 1.2$ for both cultures) different cell batches, taken from IAA-treated and untreated cells, were aliquoted, freezed in liquid nitrogen for 5 min and stored at −80°C for use in experiments. We found that neither the growth rate nor the viability of the *E. coli* cells was affected by 0.5 mM IAA treatment, as already shown by Kline et al. (1980) for other *E. coli* K-12 wild-type strain. We did not yet investigate stationary phase growing cells that will require a specific analysis in a chemostat. For biofilm analysis, resistance to SDS and UV-irradiation cultures were also treated with indole as for IAA treatment. The IAA and indole stock solutions were prepared using 50% (v/v) ethanol as solvent. To avoid solvent interference control cells were treated with similar amount of ethanol solution. At least three independent experiments were performed for all the results presented in this work. When more than three repeated experiments were done, the number of repetitions is indicated in the table note or figure legend.

GeneChip^R *E. coli* genome array

Total RNA was isolated from cells using the protocol accompanying the MasterPure complete DNA/RNA purification kit from Epicentre Tecnologies (Madison, WI, USA) as suggested by the microarray manufacturer (Affymetrix Inc., Santa Clara, CA, USA). Isolated RNA was dissolved in diethylpirocarbonate (DEPC)-treated water, quantified based on absorption at 260 nm and stored at −20°C until further use. Enrichment of mRNA was done as described in the Affymetrix Expression Handbook (Affymetrix Inc.). In brief, a set of oligonucleotide primers, specific for either 16S or 23S rRNA, were mixed with total RNA isolated from bacterial cultures. After annealing at 70°C for 5 min, 500 U MMLV reverse transcriptase (Epicentre Technologies) was added to synthesize cDNA strands complementary to the two rRNA species. The cDNA strand synthesis allowed selective degradation of 16S and 23S rRNA by RNaseH. Treatment of RNA/cDNA mixture with DNase I (Amersham Pharmacia Biotech) removed the cDNA molecules and oligonucleotide primers, which results in an RNA preparation enriched for mRNA. For direct labelling of RNA, 20 µg enriched bacterial RNA was fragmented at 95°C for 30 min in a total volume of 88 µl of 1×NEB buffer for T4 polynucleotide kinase (New England Biolabs). After cooling to 4°C, 100 µM γ-S-ATP (Roche Molecular Biochemicals) and 100 U T4 polynucleotide kinase (Roche Molecular Biochemicals) were added to the fragmented RNA, the reaction was incubated for 10 min at 65°C and the RNA was subsequently ethanol precipitated to remove excess γ-S-ATP. After centrifugation the RNA pellet was dissolved in 90 µl of DEPC-treated water, and 6 µl of 500 mM MOPS, pH 7.5, and 4 µl of 50 mM PEO-iodoacetyl-biotin (Pierce Chemical) solutions were added to introduce the biotin label. The reaction was incubated at 37°C for 1 h and the labelled RNA was purified using the RNA/DNA Mini-Kit from QIAGEN as recommended by the manufacturer. Eluted RNA was

quantified by absorption at 260 nm and hybridized to the oligonucleotide array.

The hybridization solution contained 100 mM MES, 1 M NaCl, 20 mM EDTA and 0.01% Tween 20, pH 6.6 (referred to as 1×MES). In addition, the solution contained 0.1 mg/ml herring sperm DNA, 3 nM control Oligo B2 (Affymetrix). Samples were placed in the array cartridge, and the hybridization was carried out at 45°C for 16 h with mixing on a rotary mixer at 60 rpm. Following hybridization, the sample solution was removed and the array was washed and stained as recommended in the technical manual (Affymetrix Inc.). In brief, to enhance the signals 10 μg/ml streptavidin and 2 mg/ml BSA in 1×MES were used as first staining solution. After streptavidin solution was removed, an antibody mix was added as the second stain, containing 0.1 mg/ml goat IgG, 5 μg/ml biotin-bound anti-streptavidin antibody and 2 mg/ml in 1×MES. Nucleic acid was fluorescently labelled by incubation with 10 μg/ml streptavidin–phycoerythrin (Molecular Probes) and 2 mg/ml BSA in 1×MES. The arrays were read at 570 nm with a resolution of 3 μm using a confocal laser scanner (Affymetrix). For signals intensity normalization the MAS 5.0 software was used by performing a background correction across the entire arrays and by assigning an expression call (i.e call P: gene is expressed; call A: gene is not expressed; call M: gene is marginally expressed) to each probe set. After data scaling, performed to minimize discrepancies due to variables as sample preparation, hybridization conditions, staining, or array lot, a filtering procedure was applied. The filtering step was done using as threshold the number of call A detected for each probe in all the arrays under analysis, gathering out all probe sets called A in above 90% of the analysed arrays. Filtered data were statistically validated using the SAM programme, developed by Tusher et al. (2001), to measure the strength of the relationship between gene expression and the response variable. We performed SAM analysis selecting, after a full set of permutations (>700), an FDR (false discovery rate) of 0%, a SAM threshold tuning parameter of $\Delta = 0.4$ and a fold change variation $=1.5$. To obtain a robust set of differentially expressed data we validated SAM data by the statistical programme CyberT, developed by Baldi and Long (2001). This tool uses a Bayesian approach to calculate a background variance for each of the genes under analysis and uses such value to balance experimental fluctuations within a limited number of replicates. The statistical analysis was performed selecting the following parameters: confidence of 50, window of 80 and Bonferroni correction of 0.25. Genes found differentially expressed by SAM analysis were mapped on a plot in which differential expressions values are plotted with respect to CyberT *P* values (data not shown), calculated from the same data set used in SAM analysis. We considered a gene differentially expressed only if it passed the SAM test and if present in the top score results generated by CyberT.

RT-PCR studies

Total RNA was isolated using a RNasy Mini Kit (QIAGEN) following the manufacturer's protocol. Residual DNA present in the RNA preparations was removed by RNase-free DNase I treatment (Epicentre Tecnologies). cDNA were synthesized with the StrataScript™ reverse transcription reagents (Stratagene) and random hexamers as primers. One "no RT" control (without reverse transcriptase) for each RNA sample and one "no RNA" control (replacing RNA with dH_2O) for each primer and probe set were also performed. Specific primer pairs were designed using the Primer3 software. Primer for *rrsA* of the 16S rRNA gene was also designed, and this gene was included in all the Q-RT-PCR analyses for the purpose of data normalization. RT-PCR was performed with each specific primer pair by using DyNamo HS SYBR Green qPCR kit (FINNZYMES). The reactions were performed with the DNA Engine OPTICON 2 system (MJ Research). RT-PCR amplification for each cDNA sample was performed in triplicate wells. During the reactions the fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. Results were recorded as relative gene expression changes after normalizing for *rrsA* gene expression and computed using the comparative C_T method ($2^{-\Delta\Delta C_T}$) method described in detail by Livak and Schmittgen (2001).

Microbiological and biochemical analyses

Stress tests

UV-irradiation of the cell suspensions (10 ml) was performed with a germicidal lamp (254 nm) at 100 J/m^2 in a Petri dish (5 cm in diameter). For osmotic shock cells were incubated with 0.5 M NaCl for up to 4 h at 37°C. For acid pH assay culture samples were harvested and the cells were then washed with M9 medium at pH 3 (adjusted with HCl) and re-suspended in the M9 medium at pH 3.0. The cell suspensions were shaken at 37°C for 2 h. Control samples received the same treatment except that M9 medium at pH 7.0 was used throughout the procedure. For oxidative stress cells were exposed to hydrogen peroxide, at final concentration of 2 mM, for up to 2 h at 37°C. For heat shock cells were exposed to 55°C for 5 min by immersion of the cultures in a shaking water bath. For cold treatment diluted cultures were plated on TY agar plates. The plates were then sealed in plastic bags to prevent drying and stored at 4°C. At different times, the number of colonies that survived were measured by transferring the plates to 37°C. Fraction of viable cells after each treatment was determined by plating appropriate dilutions of the cultures on TY agar plates. For survival calculation the number of colonies formed by control cultures were set to 100%

and that formed by stressed cultures were normalized accordingly.

For antibiotic resistance and for sensitivity to detergent and dye diluted cultures were plated on TY agar plates supplemented with gentian violet (GV) (20 μg ml^{-1}), benzalkoniumchloride (BCL) [0.002% (w/v)], sodium dodecyl sulphate (SDS) [2% (w/v)], novobiocin (250 g l^{-1}), erythromycin (50 g l^{-1}), rifampicin (5 g l^{-1}), penicillin (25 g l^{-1}) and vancomycin (250 g l^{-1}). The growth, after overnight incubation at 37°C, was compared with that on nutrient agar plates (Sukupoli et al. 1984). The percent of viable cells was calculated by adjusting the values for the growth on nutrient agar plates to 100% and those for the growth on supplemented plates were normalized accordingly.

SDS-PAGE and immunoblot analysis

Cells were harvested, washed twice with 25 mM Tris–HCl pH 7.5 (containing 1 mM EDTA and 2 mM DTT) and dissolved in the same buffer plus protease inhibitors (antipain, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, pefabloc SC, EDTA-Na_2 and aprotinin, all at 10 μg ml^{-1} final concentration). Cells were then destroyed by sonication (seven times for 10 s at 20 s intervals at medium power, using MSE Soniprep sonicator) and centrifuged for 30 min at 17,000 g at 4°C. Total proteins were quantified by Bradford's assay using BSA as standard. The resulting crude cell-free extracts were immediately subjected to SDS-PAGE (Laemmli 1970) using 12.5% polyacrilamide gels. After electrophoresis, proteins were blotted onto PVDF membranes according to the standard procedures. Blots were probed with anti-DnaK monoclonal (Stressgen) as primary antibody and alkaline phophatase-conjugated anti-mouse IgG (Sigma) as secondary antibodies and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Developed immunoblots were scanned using an EPSON PERFECTION 1670 scanner and quantified using Quantity One software (BIO-RAD). For immunoblot quantification purified DnaK protein was subjected to SDS-PAGE and immunoblotting.

Determination of carbonyl content—the protein carbonyl content of crude extracts was measured by using the reagent 2,4-dinitrophenyl hydrazine (DNPH) and the method described by Dalle-Donne et al. (2003).

Trehalose, LPS, EPS and biofilm analysis—trehalose was extracted and assayed as described by Lillie and Pringle (1980). Exopolysaccharides (EPS) production in liquid culture was detected by adding calcofluor-withe to 0.02% (w/v) as previously reported (Marroquì et al. 2001). EPS isolation was achieved as described by Leigh et al. (1985). For biofilm analysis cultures were incubated at room temperature in microtiter plates made of polystirene (100 μl/well). After 20 h of incubation, unbounded cells were removed by inversion of the microtiter plate, followed by vigorous tapping on adsorbent paper. Subsequently, adhered cells were fixed, stained and quantified as described by Stepanovic et al. (2000). Quantitative lipopolysaccharide (LPS) determination was performed by EDTA treatment and alcohol precipitation as described by Leive (1965). The resulting LPS samples were subjected to SDS-PAGE (Laemmli 1970) and the gels were then stained by periodic acid/silver for carbohydrates. Isolated LPS were analysed for sugar composition as follows. Total lipopolysaccharide-bound 2-keto-3-deoxyoctonate (KDO) was determined after acid hydrolysis by the thiobarbituric acid (TBA) method (Ashwell 1966). Heptose was determined by cysteine–H_2SO_4 reaction (Osborn 1963). For glucosamine, isolated LPS was hydrolysed at 95°C for 5 h in 4 N HCl, neutralized with NaOH, and assayed for hexosamine by the method of Davidson (1966). Glucose and galactose were determined by the phenol–H_2SO_4 method (Dubois et al. 1951).

Results

Effect of IAA treatment on gene expression

We screened DNA microarrays with total RNA (see Materials and methods) to compare the transcription patterns of 0.5 mM IAA-treated and control cells. Among genes with altered expression levels 16 (11 up-regulated and 5 down-regulated) showed a P value of ≤ 0.05 and a fold difference in the expression ratio between the two conditions of at least 1.5 ($\log_2$ fold change $= |0.6|$), the threshold used to assess the confidence of the expression ratios. Table 1 shows that the genes differentially expressed encode proteins that are predicted to perform cellular functions such as cell envelope biogenesis (35%), metabolism (24%) and translation (18%).

Many genes displaying altered expression levels encode proteins involved in bacterial responses to multiple stress conditions and in cell envelope composition. In particular, we found that IAA treatment induced the expression of the *cfa* gene coding for a cyclopropane fatty acid synthase involved in the conversion of unsaturated fatty acids (UFAs) to saturated fatty acids (CFAs). This conversion in the phospholipid composition of the inner membrane produces acid resistance (Chang and Cronan 1999).

We observed an increased expression level for the genes *yggB*, coding for a small mechanosensitive channel (MscS) induced at high osmolarity and in stationary phase (Edwards et al. 2004), *yacH*, coding for a putative membrane protein, *asmA*, whose product is indirectly involved in the assembly of outer membrane proteins (Deng and Misra 1996), and *smpA*, coding for a protein belonging to a family of novel outer membrane lipoproteins, which probably have a structural role in maintaining cell envelope integrity (Rezuchova et al. 2003).

Table 1 MG1655 genes whose relative expression level increases or decreases after treatment with 0.5 mM IAA

Gene	Known or predicted function	Functional classification[a]	Fold change[b]	Bayes *P* value
celD	Negative DNA-binding trascriptional regulator for cellobiose uptake	Metabolism	1.04	0.002
rpsN	30S ribosomal subunit protein S14	Translation	0.96	0.004
yacH	Putative membrane protein	Cell envelope biogenesis	0.92	0.003
cynR	*cyn* operon positive regulator. Cyanate metabolism	Metabolism	0.87	0.006
lysA	Diaminopimelate decarboxylase. Lysine biosinthesis, last step	Metabolism	0.78	0.012
fusA	GTP-binding protein chain elongation factor EF-G	Translation	0.68	0.001
cfa	Cyclopropane fatty acyl phospholipid synthase	Cell envelope biogenesis	0.68	0.016
yggB	Putative transport protein. Belong to the MscS family	Cell envelope biogenesis	0.66	0.014
rpsQ	30S ribosomal subunit protein S17	Translation	0.65	0.001
asmA	Suppressor of OmpF assembly mutants	Cell envelope biogenesis	0.64	0.032
intB	Prophage P4 integrase	Extrachromosomal	0.60	0.022
smpA	Small membrane protein. Inner membrane	Cell envelope biogenesis	−0.60	0.018
ygiA	Hypothetical protein	–	−0.65	0.007
msyB	Acid protein suppresses mutants lacking function of protein export	Transport	−0.78	< 0.001
nudD	GDP-mannose mannosyl hydrolase	Cell envelope biogenesis	−1.14	< 0.001
sfmC	Putative chaperone. Belong to the periplasmic pilus chaperone family	Information transfer	−2.70	< 0.001

[a]Based on the GenProtEC databases (http://www.genprotec.mbl.edu/)
[b]Log_2 (expression ratio) of relative transcript levels for IAA-treated cells to transcript levels for untreated cells. The 'fold change' is positive for genes that are more highly expressed in IAA-treated cells and negative for genes that are more highly expressed in control cells

Among the five genes, whose expression was reduced by IAA treatment we found *nudD* that codes for a newly identified *E. coli* enzyme, the GDP-mannose mannosyl hydrolase involved in the synthesis of β-glucan constituents of cell wall (Yoda et al. 2000).

Validation of transcriptome data by RT-PCR

Six genes (either up-, or down-regulated from distinct functional categories) were selected for RT-PCR studies. The results of this analysis confirmed the microarray hybridization data, although the absolute values of fold changes were different, especially in the case of the *cfa* gene (Table 2). This difference is probably due to the more sensitive RNA quantitative measures of RT-PCR analysis.

Table 2 RT-PCR analysis

Gene	Known or predicted function	Relative level[a]
CelD	Negative DNA-binding trascriptional regulator for cellobiose uptake	1.5 ± 0.2
FusA	GTP-binding protein chain elongation factor EF-G	1.4 ± 0.1
Cfa	Cyclopropane fatty acyl phospholipid synthase	3.0 ± 0.3
MsyB	Acid protein suppresses mutants lacking function of protein export	0.55 ± 0.06
NudD	GDP-mannose mannosyl hydrolase	0.28 ± 0.01
SfmC	Putative chaperone. Belong to the periplasmic pilus chaperone family	0.67 ± 0.11

[a]Relative gene expression levels from comparative C_T method; $2^{-\Delta\Delta CT} > 1$, gene more highly expressed in IAA-treated cells; $2^{-\Delta\Delta CT} < 1$, gene more highly expressed in control cells

Stress resistance

Since control over expression of cell wall remodelling components is critical for cell integrity, we tested whether the increased transcription of genes (e.g. *cfa* and *yggB*) implicated in the modulation of cell wall structure might provide greater protection against different stress conditions that alter cell wall stability.

Survival at low pH and high osmolarity

To verify if IAA treatment could increase the resistance to acid shock the survival of cells at low pH (pH 3.0) was examined. We found that the sensitivity of control cells and cells treated with IAA for 2 h was significantly different. Treated cells showed a higher level of tolerance (up to 80% cell survival) to the acid challenge compared to untreated cells (more than 50% reduction in viable cell number). The pH of cell-broth mixtures was measured over the course of the challenge and remained constant at pH 3.0 (data not shown), ruling out alkalinization of the medium as a possible explanation for resistance.

To determine the survival of cells in hyperosmotic conditions they were transferred into fresh prewarmed medium, or into a medium containing 0.5 M NaCl. After osmotic stress about 50% of control cells died, whereas 70% of treated cells remained viable (Table 3).

Resistance to temperatures exceeding normal cell growth range

Cold and heat shock are physical stresses that drastically modify all chemico-physical parameters of a living cell, including solute diffusion rate, enzymes kinetics, membrane fluidity, DNA, RNA, and protein topology,

Table 3 Resistance of *E. coli* cells to various stress conditions and toxic compounds

	Survival (%)	
	Control	IAA-treated
Acid shock (pH 3.0)	46 ± 5	81 ± 2
Osmotic shock (0.5 M NaCl)	52 ± 1	72 ± 1
UV irradiation (100 J/m^2)	51 ± 4	92 ± 7
Heat-shock (55°C)	1.7 ± 0.3	21.5 ± 5
Oxidative stress (2 mM H_2O_2)	56 ± 2	97 ± 6
Novobiocin	0.4 ± 0.1	0.8 ± 0
Erythromycin	79 ± 4.2	100 ± 0
Rifampicin	1.4 ± 0.1	5.2 ± 0.8
Penicillin	0.3 ± 0.1	5.2 ± 0.8
Vancomycin	9.1 ± 1.0	5.6 ± 1.1
SDS	15 ± 2	37 ± 1
BCL	85 ± 2	100 ± 0
GV	24 ± 2	44 ± 3

The values reported in the table are the averages ± standard deviation of at least five measurements

flexibility and their reciprocal interactions (Polissi et al. 2003). To asses the importance of IAA on cell viability at temperatures that prevent normal growth, diluted cultures were plated on TY agar plates and maintained at 4°C. At different times plates were transferred to 37°C and the number of colony-forming units (cfu) was measured. As shown in Fig. 1 cells lost viability at quite different rates. After 5 days at 4°C only 58% of control cells survived, whereas nearly 100% of IAA-treated cells were viable. This effect was much more marked after 19 days, when surviving colonies counted for treated cells were 83% compared to 31% for control cells. Finally, heat challenge at 55°C showed that although 21% of IAA-treated cells survived 5 min of exposure, only 1.7% of control cells survived (Table 3).

Oxidative damage

Oxidative stress mediated by highly reactive hydroxyl radicals generated by the presence of hydrogen peroxide is one of the most frequently occurring damages. To test the ability of IAA to protect cells against reactive oxygen species, cultures of IAA-treated and control cells were challenged with 2 mM hydrogen peroxide. The results presented in Table 3 show that IAA-treated cells were significantly more resistant to hydrogen peroxide than control cells. This protective effect was associated with a decrease of oxidative damage that was evaluated by the measurement of protein carbonyl content, the commonly used biomarker of severe oxidative protein damage (Table 4).

Moreover, the analysis of oxidative damage by western blot immunoassay using an anti-DNP antibody showed that several proteins stained less noticeably in treated compared to control cells (not shown). No significant difference in the electrophoretic patterns between the two protein extracts was observed (data not shown).

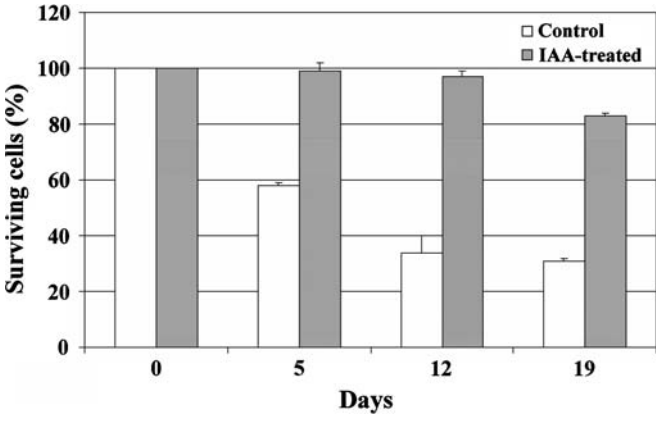

Fig. 1 Cell viability at 4°C. Surviving colonies were determined at the indicated times as described in the Materials and methods section

UV sensitivity

UV survival analysis revealed that IAA-treated cells were significantly more resistant (over 90% of surviving cells) to UV-irradiation compared to control cells (nearly 50% of surviving cells) (Table 3).

Intracellular DnaK molecular chaperone level

To test whether IAA treatment could affect the level of proteins that protect cells against high temperatures and a variety of stress conditions by preventing the aggregation of unfolded or improperly folded polypeptides, western blots of crude extracts were probed with anti-DnaK monoclonal antibody (Fig. 2). The standard curve of purified DnaK protein was used to determine amounts per microgram of cell protein present in sample extracts. We found that this chaperone was considerably more abundant (sixfold) in treated compared to control cells. This result was compatible with data obtained from transcription analysis. Indeed, the $\log_2$ expression ratio of *dnaK* gene was 0.76, whereas that of similarly regulated heat shock genes, as *dnaJ* and *grpE*, was 0.5. These genes were not included among genes differentially expressed either because of an extremely high *P* value (*dnaK*) or because of a fold change right below the fixed threshold of 0.6 (*dnaJ* and *grpE*).

Analysis of *E. coli* polymeric structures (trehalose, LPS, EPS and biofilm)

To investigate the general protection systems activated under stress conditions, we analysed the intracellular content of trehalose, a sugar that acts as a stress protector in a wide variety of organisms and under different environmental insults. We found that IAA treatment induced trehalose accumulation in *E. coli* cells: the

Table 4 Carbonyl content of crude extracts after exposure to oxidative stress conditions

Culture conditions	Carbonyl content (nmol/mg)	
	Control	IAA-treated
M9	1.9 ± 0.1	2.0 ± 0.1
M9 + 2 mM H_2O_2	2.4 ± 0	2.1 ± 0

The values reported in the table are the averages ± standard deviation of at least five measurements

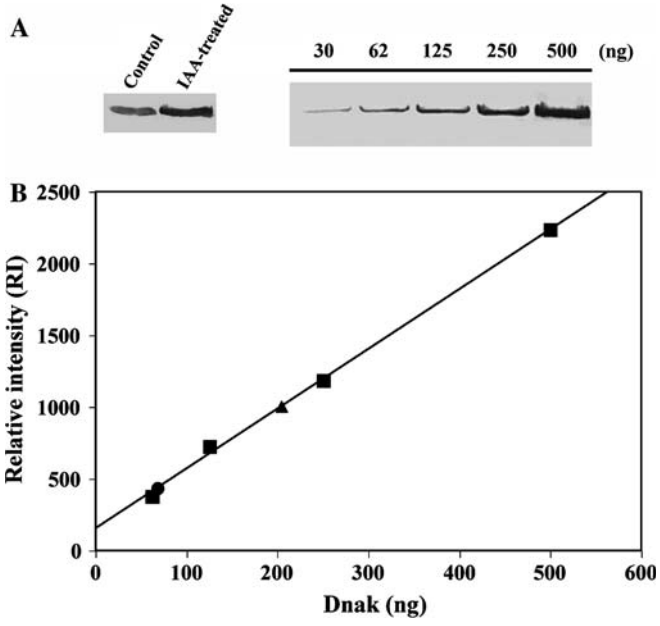

Fig. 2 DnaK western blot analysis. Crude extracts (50 μg) from control and IAA-treated cells and standard of purified protein were subjected to SDS-PAGE followed by immunoblotting (**a**) and quantification (**b**). *Circle* relative intensity (RI) of control cells; *triangle* RI of IAA-treated cells

amount of trehalose extracted from IAA-treated cells was higher (up to 44%) than that obtained from control cells (Table 5).

In addition, since alterations of the outer membrane fluidity and permeability may play an important role in the stress response, we measured the level of LPS as it contributes to the outer membrane permeability barrier of gram-negative enteric bacteria. LPS from control and 0.5 mM IAA-treated cells was purified and then subjected to electrophoretic and chemical analysis, as described in the Materials and methods section. We found that the total weight of LPS obtained from IAA-treated cells was 60% higher than that obtained from control cells (Table 5). However, purified LPS exhibited the same low-molecular-weight bands on SDS-PAGE (data not shown) and the same chemical composition with respect to galactose, glucose, glucosamine, heptose and KDO (data not shown). Finally, it is known that members of Enterobacteriaceae constitutively produce exopolysaccharides, but when the cells are traumatized by stress that destabilize the outer membrane the production of exopolysaccharides markedly increases in the form of capsular material such as biofilm. Biocides, detergents, antibiotics and stress are often ineffective against cells protected within biofilm. We found that the culture supernatant of 0.5 mM IAA-treated cells exhibited a stronger blue–green fluorescence with calcofluor-white than that observed for control cells (data not shown). Quantitative analysis of EPS produced in liquid cultures confirmed that 0.5 mM IAA-treated cells produce about 33% more EPS than control cells (Table 5).

Table 5 Yield of lipopolysaccharides (LPS), exopolysaccharides (EPS), trehalose and biofilm from *E. coli* cells

	LPS (mg/g cells)	EPS (mg/g cells)	Trehalose (μmol/g cells)	Biofilm (OD_{570})[a]
Control	21 ± 3	690 ± 50	0.08 ± 0.005	0.40 ± 0.03
IAA-treated	34 ± 4	920 ± 70	0.120 ± 0.01	0.53 ± 0.06

The values reported in the table are the averages ± standard deviation of at least five measurements
[a]Crystal violet staining quantification

Using a simple assay for the attachment to a polystirene abiotic surface, we examined the ability of exogenous IAA to affect biofilm formation of *E. coli* cells. We found that biofilm formation, as well as the bacterial growth, was not affected when IAA was added at concentrations of 0.125 and 0.25 mM (not shown). On the contrary, at 0.5 mM concentration, the biofilm formation of IAA-treated cells increased (up to 30%) as compared to control cells (Table 5). At 1.0 mM concentration IAA-treated cells showed the same induction level as that at 0.5 mM (not shown). These variations in biofilm formation were not related to differences in bacterial growth as measured by optical density at 600 nm. An increase in IAA concentration to 2.0 mM resulted in a decrease of bacterial growth (not shown).

Drug resistance

To analyse the effects of IAA treatment on the function of the outer membrane as a permeability barrier for potentially toxic detergents and hydrophobic compounds (e.g. dye and antibiotics), the sensitivity to a number of these substances was tested (Table 3). We found that IAA-treated cells were more tolerant to hydrophobic dye and detergents. In addition, our results indicate that IAA might increase the rate of mutations conferring antibiotic-resistance (with the exception of vancomycin).

Effects of indole treatment

To test whether an IAA precursor, indole, might lead to effects similar to those observed for IAA, we evaluated the fraction of viable cells in the presence of one toxic compound (SDS) and one stress condition

(UV irradiation) among those selected for IAA. These analyses revealed that indole treatment, at all tested concentrations, neither significantly affect the survival rate nor affect the production of polymeric structures such as biofilm (not shown).

Discussion

In the present study we have shown that IAA, at concentrations that did not appear harmful for *E. coli* MG1655, induced a transcriptional response that involved changes in the expression of a selected groups of genes coding for functions related to adaptation to stress. We exploited the microarray information to more deeply investigate using biochemical and microbiological analyses, the physiological consequences of IAA treatment.

The microarray analysis revealed that one-third of genes induced after IAA exposure coded for proteins involved in cell envelope biogenesis and in cellular responses activated under stress conditions. Among them were the genes *cfa, yggB* and *nudD* and RT-PCR results confirmed the down-regulation of *nudD* and the up-regulation of *cfa* expression and demonstrated that the changes were the highest among the genes induced by IAA.

nudD codes for a newly identified *E. coli* enzyme, GDP-mannose mannosyl hydrolase that potentially participates in the regulation of cell wall biosynthesis by influencing the cell concentration of GDP-mannose. Genetic and biochemical studies showed that this ß-glucan with a fibrous nature has a critical role in the maintenance of cell-wall integrity (Yoda et al. 2000). The product of *yggB* belongs to a family of small mechano-sensitive channels (MscS) and solute release through these channels reduces pressure on the cell wall to prevent lysis upon hypo-osmotic shock. Recent work has shown that the expression of MscS and MscL (the large mechano-sensitive channel) is coordinated with cellular response to growth under conditions of high osmolarity (Edwards et al. 2004). The *cfa* gene codes for an enzyme involved in cyclopropane fatty acids (CFAs) formation, a postsynthetic modification of bacterial lipid bilayers that has the same effect as an increase in bilayer thickness. This lipid modification probably provides the greatest protection against acid shock and other stress conditions such as high salt concentration, ethanol and hyperbaric oxygen (Chang and Cronan 1999). Accordingly, the analysis of the effects of acid shock on cell viability showed that IAA treatment increased the resistance to pH 3.0. In addition, we also verified that IAA increased the cell viability under many other stress conditions, although to varying extents for the different stresses. These results prompted us to analyse whether increased resistance to rapid environmental changes was due to cellular defence systems induced as a response of *E. coli* MG1655 to IAA.

It is known that one of the major damages induced by stress is the aggregation of unfolded proteins and that the activity of the abundant heat-shock protein 70 (Hsp70), together with its chaperones is essential to identify and refold these denatured or improperly folded proteins, preventing their aggregation (Bukau and Hesterkamp 1998; Morimoto 1993). Moreover, a part of the defence system active in bacteria under stress involves the intracellular accumulation of protective compounds that shield macromolecules from damage (Caldas et al. 1999; Diamant et al. 2001; Purvis et al. 2005). *E. coli* utilizes a variety of compounds for this purpose, including proline, trehalose and betaine. However, in the absence of other supplements, trehalose, with its ability to suppress aggregation, serves as primary protective osmolyte (Kaushik and Bhat 2003). Even at high concentration this osmolyte does not interfere with enzyme activity, since it is degraded far more rapidly than are Hsps soon after the stress disappears (Singer and Lindquist 1998).

We verified that the DnaK chaperone and the trehalose thermo-protector osmolyte accumulated upon exposure of the cells to IAA. The results presented in Table 3 show that the IAA-treated cells were markedly more resistant (up to 13-fold) to heat challenge at 55°C and significantly less UV-sensitive than the control cells, in agreement with published studies showing the involvement of DnaK in nucleotide excision repair (Zou et al. 1998). In addition, the analysis of tolerance of *E. coli* cells to cold shock revealed that IAA-treated cells were more resistant to low temperatures displaying more than 80% survival, even after 19 days at 4°C. This may be connected with the higher intracellular trehalose content found in these cells. Indeed, the ability of trehalose to enhance viability of *E. coli* cells during freezing has been demonstrated (Kandror et al. 2002; Lodato et al. 1999). Similar to other bacteria and yeast, this protection presumably involves the stabilization of membranes by preventing the denaturation and aggregation of membrane proteins whose folding, as well as refolding, is important after cold shock. By simultaneously inducing both molecular and chemical chaperones, IAA also enhanced cell resistance to other toxic damages such as the exposure to high external salinity and to oxidative stress. The intracellular accumulation of trehalose in salt-stressed *E. coli* cells constitutes a strategy to control the internal water activity, to maintain the appropriate cell volume and turgor pressure and to protect intracellular macromolecules. On the other hand, trehalose concentrations increase as a part of a cellular response to free-radical-generating system (H_2O_2), which irreversibly damages proteins and causes cell death (Tamarit et al. 1998; Benaroudj et al. 2001). In addition, Echeve et al. (2002) shown that synthesis of DnaK also protects several proteins from oxidative damage in *E. coli*. The data reported in Table 3 show that IAA-treated cells were able to withstand sudden changes in the osmolarity of the environment, showing 20% greater survival in minimal medium with elevated osmotic strength. In addition, proteins of these cells were less susceptible to covalent damage by free radicals, as shown

by a decrease of the protein carbonyl concentration in their crude extracts (see Results section).

Our results suggest that these protective mechanisms might be connected to the increased expression of DnaK and the synthesis of trehalose induced by IAA and might involve an interaction between these two different kinds of chaperones. Indeed, trehalose and Hsps are likely to be both required for improving the ability of cells to control protein stability, aggregation, disaggregation and refolding during and after the stress. Trehalose might prevent inactivation of the end product of the folding reaction catalyzed by chaperones and contribute to the stabilization of the molecular chaperones themselves. We also suggest the existence of a correlation between the induction of LPS, EPS and biofilm formations and the higher resistance to potentially toxic agents, observed for IAA-treated cells. The outer membrane of *E. coli* shows a low permeability to hydrophobic molecules, as demonstrated by direct measurement of penetration kinetics (Niakaido 1976). This low permeability is caused by the low permeability of the LPS monolayer that comprises the outer half of the bilayer. If solute penetration is induced by the temporary dislocation of lipids to produce 'cavities', then both the increased lateral interactions between LPS molecules and their large size will make the production of such cavities more difficult in IAA-treated cells. In addition, surface-attached bacterial communities, or biofilms, can become 10–1,000 times more resistant to the effects of antimicrobial agents such as biocides, detergents and antibiotics compared to their planktonic counterparts (Mah et al. 2001). The increased production of LPS and EPS induced by IAA might be relevant in the soil bacteria (such as *Pseudomonas, Azospirillum, Agrobacterium* and *Rhizobium*) that normally synthesize IAA and use LPS and EPS as a communication signal in both symbiotic and pathogenic interactions with plants. We, thus, speculate that IAA might be a signal able to co-ordinate bacterial behaviour to enhance protection against damage by adverse conditions. Greater understanding of the molecular mechanisms by which this molecule promotes resistance to toxic insults may lead to the development of some more environment-specific opportunities that enable growth to occur undisturbed by adverse changes in environmental conditions.

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