Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of *Escherichia coli*

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We recently reported that carbon monoxide (CO) has bactericidal activity. To understand its mode of action we analysed the gene expression changes occurring when *Escherichia coli*, grown aerobically and anaerobically, is treated with the CO-releasing molecule CORM-2 (tricarbonyldichlororuthenium(II) dimer). Microarray analysis shows that the *E. coli* CORM-2 response is multifaceted, with a high number of differentially regulated genes spread through several functional categories, namely genes involved in inorganic ion transport and metabolism, regulators, and genes implicated in post-translational modification, such as chaperones. CORM-2 has a higher impact in *E. coli* cells grown anaerobically, as judged by the repression of genes belonging to eight functional classes which are not seen in the response of aerobically CORM-2-treated cells. The biological relevance of the variations caused by CORM-2 was substantiated by studying the CORM-2 sensitivity of selected *E. coli* mutants. The results show that the deletion of redox-sensing regulators SoxS and OxyR increased the sensitivity to CORM-2 and suggest that while SoxS plays an important role in protection against CORM-2 under both growth conditions, OxyR seems to participate only in the aerobic CORM-2 response. Under anaerobic conditions, we found that the heat-shock proteins IbpA and IbpB contribute to CORM-2 defence since the deletion of these genes increases the sensitivity of the strain. The induction of several *met* genes and the hypersensitivity to CORM-2 of the $\Delta metR$, $\Delta metI$ and $\Delta metN$ mutant strains suggest that CO has effects on the methionine metabolism of *E. coli*. CORM-2 also affects the transcription of several *E. coli* biofilm-related genes and increases biofilm formation in *E. coli*. In particular, the absence of *tqsA* or *bhsA* increases the resistance of *E. coli* to CORM-2, and deletion of *tsqA* leads to a strain that has lost its capacity to form biofilm upon treatment with CORM-2. In spite of the relatively stable nature of the CO molecule, our results show that CO is able to trigger a significant alteration in the transcriptome of *E. coli* which necessarily has effects in several key metabolic pathways.

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INTRODUCTION

Carbon monoxide (CO) formed in nature results from the incomplete oxidation of organic matter and as the by-product of the activity of the haem oxygenase enzyme, which oxidizes haem to biliverdin (Li *et al.*, 2007). In the latter process, which can occur in bacteria, plants and animals, the amount of CO endogenously produced by the

Abbreviations: CORM, CO-releasing molecule; CORM-2, tricarbonyldichlororuthenium(II) dimer; FDR, false discovery rate.

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various cell types is low. In humans, the CO formed has several physiological functions in the neuronal and cardiovascular systems (Wu & Wang, 2005). However, high concentrations of CO have a toxic effect in mammals due to the binding of CO to haemoglobin; the consequent impairment of oxygen storage and transport by haemoglobin causes hypoxia (Wu & Wang, 2005). CO binds preferentially to transition-metal-containing proteins, including haem-based proteins/sensors, giving rise to structural modifications and alteration of their biological function which may also account for the toxic effects of CO. However, it has been shown that living cells can tolerate CO at the concentration of 0.01 % (100 p.p.m.) for several hours (Otterbein & Choi, 2000), and rodents could be exposed continuously for up to 2 years to 500 p.p.m. CO without deleterious effects (Otterbein & Choi, 2000; Stupfel & Bouley, 1970; Verma *et al.*, 1993).

Following the discovery that carbon monoxide is physiologically important, molecules acting as CO-carriers, namely 'CO-releasing molecules' (CORMs), were developed. In order to mimic the role of CO derived from haem oxygenase (Ryter *et al.*, 2006), these molecules have to be able to liberate CO in a biological environment. This liberation is triggered by some physical, chemical or enzymic stimulus. For many CORMs that carry molecular CO as a ligand to a central transition metal, the lack of a pharmacological effect of a similar compound devoid or depleted of CO has been taken as a positive proof for the biological action of CO (Dulak *et al.*, 2002). Following this principle, the beneficial therapeutic action of several CORMs in a wide range of vasoactive and anti-inflammatory situations and models has been attributed to CO (Motterlini *et al.*, 2005).

Recently, we reported that four transition-metal-based CORMs at micromolar concentrations have antibiotic activity, reducing the viability of bacteria such as *Escherichia coli* and *Staphylococcus aureus*, under both aerobic and anoxic conditions (Nobre *et al.*, 2007). In that work we also showed that the bactericidal action of those CORMs was only effective when CO was taken up by the cells. In fact, we demonstrated that one of the CORMs led to extensive accumulation of the transition metal molybdenum inside bacterial cells. In all cases, the addition of haemoglobin to the medium, before addition of the CORM, prevented any bactericidal action. Separate experiments (Motterlini *et al.*, 2002; J. D. Seixas, personal communication) showed that these CORMs transfer CO to haemoglobin at a very fast rate. Taken together these results strongly suggest that the bactericidal action of a given CORM depends on its ability to enter bacterial cells and enable CO to reach specific targets.

We have now extended these studies to the analysis of the global transcriptome of *E. coli* treated with CORM-2, which reflects the organism's immediate response to the environment. Additionally, we have examined the phenotype of 12 mutant strains to test the role of each deleted gene in the *E. coli* CORM-2 response. Regulatory genes and

genes involved in methionine metabolism and biofilm formation were found to play an important role when the micro-organism is exposed to the bactericidal action of CORM-2.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* MG1655 was grown, at 37 °C and 150 r.p.m., in minimal medium salts (MS) (Nobre *et al.*, 2007) in the presence or absence of oxygen. Aerobic experiments were performed in flasks filled with 1/5 of their volume and anaerobic experiments in rubber-sealed flasks that, once filled with medium and closed, were extensively flushed with nitrogen gas. Cultures were grown to an OD₆₀₀ of 0.3, and at this point cells were left untreated or treated with CORM-2 (Sigma).

RNA isolation. The hot-phenol method was used to extract total RNA from two independent cultures of cells grown either aerobically or anaerobically and treated with 250 µM CORM-2 for 15 min. The concentration of the drug added to the cultures is equivalent to 0.5 times the minimal inhibitory concentration of 500 µM which was previously determined (Nobre *et al.*, 2007). After the isolation procedure, RNA was incubated with DNase (Ambion), and once the absence of any residual DNA was confirmed, concentration and purity were determined in a Nanodrop ND-1000 UV-visible spectrophotometer. RNA integrity was checked in an Agilent 2100 Bioanalyser coupled to an RNA Nano-Assay (Agilent Technologies).

Microarray analysis. Total RNA (10 µg) was processed for use in the Affymetrix GeneChip *E. coli* Genome 2.0 Arrays, according to the manufacturer's instructions. Arrays were scanned in an Affymetrix GeneChip scanner 2500 and analysed first with Affymetrix MAS 5.0 software to obtain Absent/Present calls and to ensure that all quality parameters were within the recommended range. Hybridization, scanning and detection procedures were done at the Genomics Unit of the Instituto Gulbenkian de Ciência (Portugal). The advantage of using the Affymetrix GeneChip is that it contains 20 probe pairs to detect each ORF, providing multiple independent measurements for each transcript. The subsequent analysis was performed with the dChip software program (Li & Wong, 2001). The invariant set method (Li & Hung Wong, 2001) was used to normalize arrays at the probe cell level for comparison purposes, and the model-base (Perfect Match-only model) was used to probe selection and computing expression values. The expression profiles of 4306 genes encoding proteins of *E. coli* K-12 MG155 were analysed (intergenic regions were not included) and genes were considered to have significant differential expression when they showed a transcriptional fold change >2 (with a 90 % confidence interval) and a *P*-value <0.05 for a paired Student's *t*-test. To minimize occurrence of false positives the comparison criterion was carefully chosen to ensure that the empirical false discovery rate (FDR) was low (random permuting of the samples 200 times led to a median of FDR of 0 % and 1.1 % for the arrays acquired for cells grown anaerobically and aerobically, respectively).

Gene ontology analysis. To investigate the biological relevance of the differential gene expression profile, the microarray data were analysed with the FatiGO algorithm (Al-Shahrour *et al.*, 2004, 2006). Briefly, FatiGO considers two lists of genes (in this case the genes up- or downregulated by CORM-2 anaerobically or aerobically were compared with the unchanged genes) and converts them into two lists of gene ontology (GO) terms. Subsequently, FatiGO determines separately the percentage of the relative frequency of genes in each group that is annotated with a particular GO term (biological process or molecular function), and finally, a Fisher's exact test is used to

determine if the GO term is significantly over- or under-represented in one of the groups with respect to the other one. Hence, it retrieves information about the predominant biological process or molecular function for the genes that are differently expressed in a certain experimental condition. In this study, the GO terms were considered differently represented when they exhibited a FDR-adjusted *P*-value <0.2. FatiGO is implemented in a web tool at: <http://www.fatigo.org> and within the Babelomics environment (<http://www.babelomics.org>).

Quantitative real-time RT-PCR. DNA microarray data were validated by quantitative real-time RT-PCR. To this end, 2.5 µg *E. coli* total RNA, derived from two independent samples grown aerobically or anaerobically and treated with 250 µM CORM-2 or with 500 µM Ru(II)Cl₂(DMSO)₄, was used to synthesize cDNA, using the first-strand synthesis protocol of the Universal RiboClone cDNA Synthesis System (Promega). Each cDNA synthesis reaction was performed using 40 µg ml⁻¹ of random primers, 40 U RNasin RNase inhibitor, 4 mM sodium pyrophosphate and 30 U AMV reverse transcriptase. After amplification, cDNA was purified by phenol extraction and ethanol precipitation. Real-time PCRs were performed in a LightCycler Instrument using the LightCycler FastStart DNA Master SYBER Green I kit according to the manufacturer's instructions (Roche Applied Science). For each target gene, specific oligonucleotides were designed to amplify nucleotide fragments of 200–580 bp (see Supplementary Table S4, available with the online version of this paper, for oligonucleotide sequences). The amplification reactions were carried out with equal amounts of cDNA (120 ng), as initial template, and each reaction contained 0.5 µM primers, 2 mM MgCl₂, and hot-start PCR mix (Roche Applied Science). The expression ratio of the target gene was determined relative to a reference gene, the *E. coli* glyceraldehyde-3-phosphate dehydrogenase gene (*gapA*), which did not show variation in transcription abundance under any of the conditions tested by microarray analysis or real-time RT-PCR experiments.

Viability assays and complementation studies. In order to compare the sensitivity to CORM-2 of the *E. coli* parental strain versus the various mutant strains, cell viability was determined by evaluation of the number of colony-forming units (c.f.u. ml⁻¹), after 7 h growth in the presence or in the absence of CORM-2. The percentage survival was calculated as the number of colonies obtained from CORM-2-treated cultures divided by the number of colonies formed upon plating untreated cultures. The experiments were performed in duplicate, with a minimum of two independent cultures of the mutant strain and of its corresponding parental strain (see Supplementary Table S5 for details of bacterial strains); the results are presented as means ± SE. For complementation purposes, *ibpAB* and *metR* genes were amplified from *E. coli* genomic DNA using the appropriate oligonucleotides (Supplementary Table S4), and cloned into pUC19 to generate plasmids *pibpAB* and *pmetR* (see Supplementary Table S6 for details of plasmids). These plasmids or the empty vector were transformed into the correspondent mutant strain and the phenotype was analysed by plating on agar successive dilutions of the cultures treated with CORM-2 or left untreated. For *ΔsoxS*, *ΔoxyR*, *ΔtsqA* and *ΔbhsA*, the complementation experiments were performed using plasmids pWB31, pAQ17, pCA24NydG⁺ and pCA24NycfR⁺, respectively (Supplementary Table S6).

Biofilm assays. Biofilm formation was quantified by the crystal violet method (Ren *et al.*, 2005; Zhang *et al.*, 2007). Briefly, overnight LB-grown cultures of wild-type *E. coli*, *ΔtsqA* and *ΔbhsA* mutant strains were used to inoculate fresh medium to an OD₆₀₀ of 0.05 and then incubated for 6 h, at 37 °C. At this stage, 250 µM CORM-2 was added and the plates were incubated for another 18 h, at 37 °C. The total biofilm formed was stained with a solution of 0.1 % crystal violet for 30 min and quantified by measuring the A₅₄₀. Relative biofilm formation was calculated as the total biofilm formed by the cultures

treated with CORM-2 divided by the total biofilm obtained in untreated cultures; each data point represents the mean of six replicated wells from four independent cultures.

RESULTS AND DISCUSSION

Analysis of the *E. coli* transcriptome of cells treated with CORM-2

Microarray technology was utilized to analyse the genome-wide transcriptional pattern of *E. coli* cells, grown aerobically and anaerobically, in the presence of CORM-2. The complete list of genes that exhibited a twofold, or higher, increase or decrease (with *P* < 0.05) in transcription is presented in Supplementary Tables S1 (anaerobic growth) and S2 (aerobic growth), available with the online version of this paper. In order to independently confirm the microarray results, quantitative real-time PCR was carried out on ten selected genes that were found to be upregulated or downregulated by the action of CORM-2. The values acquired in the real-time PCR experiments were in agreement with the fold variation obtained in the microarrays (Table 1). To facilitate subsequent analysis, the differentially expressed genes were divided into functional categories, based on the NCBI (<http://www.ncbi.nlm.nih.gov>) and EcoCyc (<http://ecocyc.org>) gene annotations and on the protein databases that were used to search for homologues of hypothetical gene products. Since a better correlation between gene expression and function is found for groups of genes instead of individual genes, the FatiGO algorithm (Al-Shahrour *et al.*, 2004, 2006, 2007) was also used to interpret the data.

Anaerobically grown cells. The microarray data acquired for cells grown under anaerobic conditions and treated with CORM-2 showed that 396 genes had their transcription altered (~9 % of the total genome). Of these, the transcription of 228 genes was repressed (~5 % of the transcriptome); these genes were dispersed through nearly all functional categories, particularly amino acid transport and metabolism (Fig. 1A). The FatiGO analysis showed that the genes downregulated are over-represented in cellular metabolic processes (which correspond to 84 % of all repressed genes), namely in catabolic processes, nucleotide metabolism, and energy production by oxidation of organic compounds (Fig. 2A), indicating that CORM-2 induces metabolic adaptation.

The largest class of genes that were upregulated belongs to the category hypothetical proteins (~39 %). Apart from these, the genes that showed increased transcription are divided essentially into three classes: inorganic ion transport, post-translational modification and transcription (Fig. 1A). Further analysis revealed that the genes upregulated by CORM-2 were significantly over-represented within the classes transcriptional regulation and protein folding (Fig. 2B). Among the latter, genes encoding two heat-shock proteins that are directly connected with

Table 1. Quantitative real-time RT-PCR analysis performed in cells exposed to CORM-2 and to the CO-free Ru(II)Cl₂(DMSO)₄

Gene	Anaerobic			Aerobic		
	Microarray	Real-time PCR*		Microarray	Real-time PCR*	
		CORM-2	Ru(II)Cl ₂ (DMSO) ₄		CORM-2	Ru(II)Cl ₂ (DMSO) ₄
<i>ibpB</i>	40	427 ± 33	-2 ± 0	79	2798 ± 27	12 ± 8
<i>soxS</i>	10	14 ± 0	-3 ± 0	15	268 ± 63	-3 ± 0
<i>marA</i>	14	14 ± 0	2 ± 1	10	56 ± 3	-2 ± 0
<i>frmA</i>	10	57 ± 1	-1 ± 0	16	133 ± 0	-2 ± 0
<i>lrhA</i>	16	14 ± 5	-2 ± 0	5	23 ± 8	-2 ± 0
<i>yncJ</i>	42	85 ± 8	12 ± 2	62	162 ± 49	7 ± 2
<i>bhsA</i>	26	33 ± 2	3 ± 2	—	—	—
<i>metR</i>	—	—	—	21	432 ± 85	4 ± 0
<i>gadX</i>	-10	-21 ± 0	2 ± 1	-4	-4 ± 0	-2 ± 0
<i>sucA</i>	-9	-6 ± 0	1 ± 0	-5	-4 ± 0	-1 ± 0

*Fold change ± SE.

protein stability, *ibpA* and *ibpB*, were strongly induced (19- and 40-fold, respectively). The data also showed that, under anaerobic conditions, the addition of CORM-2 had effects on iron metabolism, since several genes related to this function were altered, e.g. those encoding ferritin (*ftn*), and bacterioferritin (*bfr*) (Supplementary Table S1).

Aerobically grown cells. *E. coli* cells grown aerobically and exposed to CORM-2 displayed altered transcription of 175 genes, ~4 % of the global gene expression profile, with 46 genes repressed and 129 genes induced. Apart from genes encoding hypothetical proteins, three functional categories exhibited a similar number of transcriptionally repressed genes, namely the classes energy production, and amino acid and carbohydrate transport and metabolism (Fig. 1B). The FatiGO algorithm revealed over-representation of repressed genes in coenzyme catabolic processes, tricarboxylic acid cycle and aerobic respiration classes (Fig. 2C). This trend would ultimately lead to the inhibition of aerobic respiratory metabolism.

The genes whose expression was induced by CORM-2 were spread through several functional classes, the inorganic ion transport category containing the second-highest number of genes transcriptionally modified (Fig. 1B). Interestingly, the FatiGO analysis indicates that under aerobic conditions the addition of CORM-2 to *E. coli* cells led to a significant upregulation of genes involved in sulfur metabolism, such as *tauABC*, *ssuAD*, *cysWA* and *sbp*, and in methionine metabolism, like the gene clusters *metNI* and *metBLF* (Fig. 2D).

Transcriptional alterations caused by CORM-2 are dependent on CO release

Administration of any CORM produces some metabolite(s) along with the CO that is liberated. Therefore, we

determined whether the biological effect of a given CORM actually results from the liberated CO and not from any of its metabolites or the CORM's molecular skeleton itself. The commercially available tricarbonyldichlororuthenium(II) dimer, [Ru(CO)₃Cl₂]₂, also known as CORM-2, is by far the most tested CORM and we have reported its bactericidal activity (Nobre *et al.*, 2007). The dimer complex is insoluble in aqueous media and its solubilization, usually carried out in DMSO, entails a number of chemical transformations. According to Motterlini *et al.* (2002), 23 min after dissolution three species are already formed, namely RuCl₂(CO)₃(DMSO) and two isomers of RuCl₂(CO)₂(DMSO)₂. Of necessity, a certain amount of free CO is liberated into the DMSO solution and eventually lost to the atmosphere at an undetermined rate. More species appear upon standing or warming of the solution and further CO loss to give RuCl₂(CO)(DMSO)₃, and eventually RuCl₂(DMSO)₄ is to be expected since they are all synthetically interrelated in the presence of CO gas (Alessio *et al.*, 1995). Nothing is presently known about the biological activity of any of these pure species individually. However, the octahedral structure, the oxidation state as well as the ligands other than CO are retained along this series, strongly supporting our choice of Ru(II)Cl₂(DMSO)₄ as a control for the bactericidal activity of the CO-free Ru(II)Cl₂(DMSO)_x fragment. It must also be mentioned that, regardless of the species present in the DMSO solution of [Ru(CO)₃Cl₂]₂, when an aliquot of this solution is added to the cell culture medium no CO is released to the gas phase, as previously reported (Nobre *et al.*, 2007). We challenged *E. coli* with 500 µM CO-free Ru(II)Cl₂(DMSO)₄ and analysed the expression of several genes. Table 1 shows that no significant alteration in transcription was observed when the CO-free compound was used, which allows us to conclude that the main biological activity is due to the release of CO from CORM-2.

Fig. 1. Effect of CORM-2 on the *E. coli* transcriptome analysed according to the distribution of the genes with altered expression into functional categories. The differentially regulated genes in cells grown under anaerobic (A) and aerobic (B) conditions were divided into 21 functional categories according to the NCB and EcoCyc databases. The black and white bars represent the number of genes whose transcription was up- and downregulated by CORM-2, respectively. The functional categories that include genes up- and downregulated by CORM-2 in both growth conditions are highlighted in bold, and the eight functional classes containing genes that were repressed by CORM-2 under anaerobic conditions and are absent in aerobically grown cells are shown in italics.

Genes transcriptionally regulated in response to CORM-2, independently of oxygen

The *E. coli* microarray data showed that upon exposure to CORM-2 the cells grown anaerobically exhibited more repressed genes than the cells grown aerobically. In particular, eight functional classes containing genes that were repressed by CORM-2 under anaerobic conditions were not detected in aerobically grown cells (Fig. 1A, B). These results are in agreement with our previous observation that the decrease in cell viability caused by CORMs is higher for cells grown under anaerobic conditions (Nobre *et al.*, 2007). However, a large set of genes were found in common between the aerobic and anaerobic conditions, and the fold induction or repression generated by CORM-2 did not vary significantly between them (Table 2). There is also no example of a gene that was repressed under anoxic conditions and induced under aerobic conditions, and vice versa. The major fold difference was observed for the genes

encoding a heat-shock protein (*ibpB*), two hypothetical proteins (*yncJ* and *ymgG*), a flagellar repressor (*lrhA*) and an envelope-stress-induced periplasmic protein (*spy*). In all these cases, the genes showed a higher fold CORM-2-dependent induction in aerobically grown cells than in cells cultured anaerobically (Table 2).

Phenotypic analysis of *E. coli* regulators induced by CORM-2

To gain further insight into the function of the genes whose transcription was perturbed by treatment with CORM-2 we analysed the phenotype of 12 *E. coli* mutant strains to test if the deleted gene has a role in the *E. coli* CORM-2 response. The genes were chosen on the basis that they showed a high fold induction or repression and/or exhibited variation in transcript abundance in cells grown under both aerobic and anaerobic conditions (see Table 2).

Fig. 2. Biological processes modified by CORM-2. The genes down- and upregulated by CORM-2 anaerobically (A and B) or aerobically (C and D) were compared with those unaltered by CORM-2 (reference genes), using the FatiGO algorithm (see Methods). The y-axis shows the biological processes that were considered differentially represented between the two groups compared in each case. The specificity of the biological process increases from the top to the bottom of the y-axis, as indicated by the numbers in parentheses. The x-axis represents the differences in percentages of the relative frequency of genes annotated for each biological process. The right and left part of the x-axis corresponds to the biological processes over- and under-represented, in the downregulated genes (A and C) and the upregulated genes (B and D), respectively. TCA, tricarboxylic acid cycle.

Hence, genes encoding regulators (*soxS*, *oxyR*, *zntR*, *metR*, *gadX*), a putative stress-combating protein (*cpxP*), heat-shock proteins (*ibpA* and *ibpB*), proteins involved in biofilm formation [*ydgG* (*tqsA*), *ycfR* (*bhsA*)], an antiporter protein (*chaA*), and genes involved in methionine metabolism (*metN*, *metI*) were studied.

Study of the *E. coli* single mutant strains deleted in the genes *zntR*, *gadX* and *chaA* revealed that these strains did not display a CORM-2 growth-induced delay when compared to the parent strain (data not shown).

The *soxS* gene, whose transcription is controlled directly by SoxR, showed a high fold induction upon treatment with CORM-2, under both aerobic and anaerobic conditions (Table 2). Together with SoxR, SoxS participates in the regulation of several genes involved in the response to oxidative stress (Wu & Weiss, 1992). However, with the exception of the *marRAB* operon, none of the other known members of the SoxRS regulon was induced. A similar behaviour, which so far remains unexplained, was previously observed in other microarray experiments with *E. coli* grown in the presence of nitric oxide and hydrogen peroxide (Justino *et al.*, 2005; Zheng *et al.*, 2001). The phenotypic study of the *E. coli* strain deficient in the *soxS*

gene showed that the mutation leads to an increased sensitivity to CORM-2 of the cells grown under aerobic (>70 %) and anaerobic conditions (>35 %) (Fig. 3A, B).

The *oxyR* gene, whose encoded protein participates in the regulation of genes involved in the response to oxidative stress (Zheng *et al.*, 2001), was upregulated by CORM-2 under aerobic and anaerobic conditions (≥ 4 -fold) (Table 2). Under aerobic conditions and in the presence of CORM-2, the sensitivity of the *oxyR* mutant strain was found to be ~70 % higher than that of the wild-type strain (Fig. 3C), while no differences were observed for growth under anaerobic conditions.

The microarray data showed that in aerobically grown *E. coli* the transcription of *metR* was upregulated by CORM-2. Analysis of the $\Delta metR$ strain revealed that this mutant displays a sensitivity ~80 % higher than that of the parental strain for cells grown aerobically (Fig. 3F), while no effect was detected in anoxic conditions (data not shown).

In all cases, the wild-type behaviour was restored upon expression of the genes from a plasmid containing SoxR, OxyR or MetR (Fig. 3A₁–C₁ and F₁), showing that these transcription factors have an important role in the *E. coli* regulatory mechanisms triggered by exposure to CORM-2.

Table 2. Genes of *E. coli* differentially regulated by CORM-2 under both anaerobic and aerobic conditions

Gene name*	Locus	Gene product	Fold change	
			Anaerobic	Aerobic
Inorganic ion transport and metabolism				
<i>alx</i>	b3088	Predicted inner-membrane protein	12.3	3.3
<i>yheL</i>	b3343	Predicted intracellular sulfur oxidation protein	6.8	7.4
<i>exuT</i>	b3093	Hexuronate transporter	6.5	2.5
<i>chaA</i>	b1216	Calcium/proton antiporter	6.0	8.4
<i>yqjH</i>	b3070	Predicted siderophore-interacting protein	4.4	2.7
<i>metN</i>	b0199	D-Methionine transport ATP-binding protein	4.3	9.9
<i>metI</i>	b0198	D-Methionine transport system permease	4.0	6.5
<i>pspE</i>	b1308	Phage shock protein E precursor	2.4	7.0
<i>yegH</i>	b2063	Putative transport protein	−2.4	−2.5
<i>apaG</i>	b0050	Uncharacterized protein associated with Co ²⁺ /Mg ²⁺ efflux	−3.6	−2.4
Transcription				
<i>lrhA</i>	b2289	DNA-binding transcriptional repressor of flagella	15.5	4.6
<i>cpxP</i>	b4484	Periplasmic protein combating stress	14.0	12.2
<i>soxS</i>	b4062	DNA-binding transcriptional dual regulator	10.0	14.5
<i>frmR</i>	b0357	Regulator protein that represses <i>frmRAB</i> operon	8.5	6.7
<i>marA</i>	b1531	Multiple antibiotic-resistance protein	7.3	9.5
<i>lexA</i>	b4043	LexA repressor	6.8	3.8
<i>zntR</i>	b3292	Zinc-responsive transcriptional regulator	5.1	6.1
<i>oxyR</i>	b3961	Hydrogen peroxide-inducible genes activator	4.5	4.1
<i>gadX</i>	b3516	DNA-binding transcriptional dual regulator	−10.4	−4.3
Post-translational modification, protein turnover, chaperones				
<i>ibpB</i>	b3686	16 kDa heat-shock protein B	39.6	79.3
<i>ibpA</i>	b3687	16 kDa heat-shock protein A	19.2	18.7
<i>hspQ</i>	b0966	DNA-binding protein, hemimethylated	7.7	4.8
<i>yeeD</i>	b2012	Predicted redox protein	7.4	3.3
<i>yggG</i>	b2936	Predicted peptidase	6.0	2.7
<i>hslO</i>	b3401	Hsp33-like chaperonin	4.6	5.4
<i>dnaJ</i>	b0015	Chaperone protein	3.7	4.1
<i>clpB</i>	b2592	Protein disaggregation chaperone	3.1	4.8
Signal transduction mechanisms				
<i>ydeH</i>	b1535	Hypothetical protein with diguanylate-cyclase (DGC) or GGDEF domain	17.5	23.5
<i>pspB</i>	b1305	Phage shock protein B	4.4	10.5
<i>pspG</i>	b4050	Phage shock protein G	3.9	11.4
<i>narP</i>	b2193	Nitrate/nitrite response regulator protein	2.7	2.8
<i>ydiV</i>	b1707	Cyclic-di-GMP phosphodiesterase class I	−3.7	−5.9
Energy production and conversion				
<i>frmA</i>	b0356	Alcohol dehydrogenase class III	15.8	8.1
<i>nemA</i>	b1650	N-Ethylmaleimide reductase	3.1	3.6
<i>sucA</i>	b0726	2-Oxoglutarate decarboxylase	−9.1	−5.1
Translation, ribosomal structure and biogenesis				
<i>rrmJ</i>	b3179	rRNA large subunit methyltransferase J	4.6	3.2
<i>hslR</i>	b3400	Ribosome-associated heat-shock protein Hsp15	4.2	5.1
<i>miaA</i>	b4171	tRNA Δ ² -isopentenylpyrophosphate transferase	2.5	2.5
Amino acid transport and metabolism				
<i>ybaT</i>	b0486	Predicted transporter protein	−3.4	−3.7
<i>gss</i>	b2988	Fused glutathionylspermidine amidase	−3.5	−3.1
Carbohydrate transport and metabolism				
<i>tktB</i>	b2465	Transketolase	−2.5	−2.9
<i>otsA</i>	b1896	α,α-Trehalose-phosphate synthase	−4.9	−2.8
Replication, recombination and repair				
<i>fimE</i>	b4313	Tyrosine recombinase	5.6	3.9
<i>recN</i>	b2616	Recombination and repair protein	5.4	8.4

Table 2. cont.

Gene name*	Locus	Gene product	Fold change	
			Anaerobic	Aerobic
Defence mechanisms				
rdoA	b3859	Thr/Ser kinase implicated in Cpx stress response	2.8	3.5
aidB	b4187	Isovaleryl-CoA dehydrogenase	−2.6	−2.7
Coenzyme transport and metabolism				
btuD	b1709	Vitamin B12-transporter ATPase	−3.1	−2.3
Cell wall/membrane/envelope biogenesis				
amiC	b2817	N-Acetylmuramoyl-L-alanine amidase	2.4	2.4
Secondary metabolites biosynthesis, transport and catabolism				
ybbA	b0495	Hypothetical ABC transporter	7.7	7.1
Intracellular trafficking, secretion, and vesicular transport				
spy	b1743	Envelope-stress-induced periplasmic protein	16.0	30.0
Cell cycle control, cell division, chromosome partitioning				
sulA	b0958	Cell division inhibitor	9.5	5.4
Hypothetical proteins				
yncJ	b1436	Hypothetical protein	42.2	62.0
yjfN	b4188	Hypothetical protein	19.8	22.9
ycfJ	b1110	Hypothetical protein	18.0	13.5
yebE	b1846	Hypothetical protein	16.3	22.9
ycfS	b1113	Hypothetical protein	12.0	16.9
yhdV	b3267	Hypothetical protein	9.0	9.0
yneM	b4599	Hypothetical protein	8.4	6.2
yqjA	b3095	Hypothetical protein	8.0	5.7
ypfG	b2466	Hypothetical protein	6.7	8.6
fxsA	b4140	FxsA protein	6.7	13.8
yaiY	b0379	Hypothetical protein	6.5	5.8
tqsA	b1601	Predicted permease	6.3	9.1
yeeE	b2013	Predicted inner-membrane protein	6.3	2.7
ybeD	b0631	Hypothetical protein	6.1	6.0
yqaE	b2666	Hypothetical protein	5.9	4.5
ytfK	b4217	Hypothetical protein	5.3	5.3
ycjX	b1321	Hypothetical protein	5.0	7.7
ybfA	b0699	Hypothetical protein	4.5	4.5
bax	b3570	BAX protein	4.3	3.3
ymgG	b1172	Hypothetical protein	4.2	16.0
sraF	b4448	Unknown RNA	3.5	2.5
yrfG	b3399	Predicted hydrolase	3.4	4.3
ynfD	b1586	Hypothetical protein	3.4	4.5
yciS	b1279	Hypothetical protein	3.3	3.2
ycjF	b1322	Hypothetical protein	3.2	10.0
yhdN	b3293	Hypothetical protein	3.1	4.3
yciC	b1255	Hypothetical protein	2.6	2.3
slp	b3506	Outer-membrane protein Slp precursor	−2.2	−4.1
yahK	b0325	Predicted oxidoreductase, Zn-dependent and NAD(P)-binding	−4.0	−3.6
yciX	b4523	Hypothetical protein	−4.6	−8.0

*Strains mutated in the genes shown in bold were analysed phenotypically.

Identification of other genes involved in the CORM-2 sensitivity of anaerobically grown *E. coli* cells

A significant variation of the expression of *ibpA* and *ibpB* genes was measured in aerobically and anaerobically grown cells of *E. coli* treated with CORM-2 (Table 2). These genes

encode two small heat-shock proteins, *IbpA* and *IbpB*, that bind to protein aggregates and inclusion bodies formed during heterologous protein expression (Lethanh *et al.*, 2005). The two proteins are known to cooperate with *ClpB* and *DnaK*, forming a functional triad of chaperones (Lethanh *et al.*, 2005; Thomas & Baneyx, 1998). Interestingly, the transcription of *clpB* and *dnaK* as well as that of

Fig. 3. Analysis of the sensitivity of various *E. coli* mutant strains to CORM-2. (A–E) The parental strain (grey bars), and mutants (white bars) were grown aerobically (panels A and C) or anaerobically (panels B, D and E) and exposed to 150 μ M or 100 μ M CORM-2, respectively. The *E. coli* $\Delta cpxP$ mutant and its parental strain were treated with 125 μ M CORM-2 (panel E). In panel F, the $\Delta metR$ (striped bars), $\Delta metI$ (light grey bars) and $\Delta metN$ (white bars) mutant strains were grown aerobically and treated with 150 μ M CORM-2. The error bars represent the standard error of the mean values obtained from at least two independent biological samples performed in duplicate (* $P < 0.01$, ** $P < 0.05$). Panels A₁–F₁: complementation analysis of $\Delta soxS$ (A₁, B₁), $\Delta oxyR$ (C₁), $\Delta ibpAB$ (D₁) and $\Delta metR$ (F₁) using the plasmids indicated in each panel.

genes coding for other chaperones, such as *dnaJ*, *grpE* and *hspG*, was also upregulated anaerobically by CORM-2 (Supplementary Table S1). Under anaerobic conditions, inactivation of the *ibpAB* genes led to a 45 % increase in CORM-2 sensitivity relative to the parental strain (Fig. 3D), while no differences were observed under aerobic conditions (data not shown). As expected, complementation of *ibpAB* mutation induced the rescue of the wild-type phenotype (Fig. 3D₁).

The transcription of *E. coli* *cpxP*, which encodes a periplasmic protein putatively involved in combating extracytoplasmic protein-mediated toxicity (Danese & Silhavy, 1998), was upregulated 12–14-fold in cells exposed to CORM-2 and cultured in both aerobic and anaerobic conditions. CpxP belongs to the *E. coli* Cpx system, which senses perturbations in the bacterial cell envelope and responds through the upregulation of many gene products involved in protein folding and degradation (Fleischer *et al.*, 2007). Accordingly, we verified that together with the induction of *cpxP* occurred a high number of transcriptionally modified genes involved in cell wall biogenesis and in protein folding, including genes encoding heat-shock proteins (*ibpAB*, *hslJ*, *hspX*), chaperones (*dnaJ*, *hspG*, *clpB*) and proteases (*ftsH*) (Supplementary Table S1), mainly under anaerobic conditions. These genes are under the control of *rpoH* (σ^{32}) (Zhao *et al.*, 2005), a gene that was also found to be upregulated by CORM-2 under anaerobic conditions. The study of the growth behaviour of the Δ *cpxP* mutant revealed that this strain is approximately 35 % more sensitive to CORM-2 than the parental strain, but only under anaerobic conditions (Fig. 3E), not under aerobic conditions (data not shown).

CORM-2 interferes with methionine biosynthesis

Addition of CORM-2 to aerobically grown cells of *E. coli* caused a marked increase in the transcription of several genes implicated in methionine biosynthesis and uptake, with *metR* being the gene that showed the highest induction (21-fold) (Supplementary Table S2). Apart from the genes listed in Supplementary Table S2, induction of the expression of *metA* (12.4-fold; $P=0.0527$) and *metE* (5.5-fold; $P=0.0565$) was also observed. To further clarify this issue, growth experiments were conducted aerobically in the presence of CORM-2 for the *E. coli* Δ *metR*, Δ *metI* and Δ *metN* mutant strains. All these mutants were hypersensitive to CORM-2 (80 %, 66 % and 40 % higher sensitivity, respectively; Fig. 3F). Furthermore, the complementation assay showed that *E. coli* Δ *metR* regains resistance to CO upon expression of the regulator MetR (Fig. 3F₁). These findings suggest that, under aerobic conditions, CORM-2 affects the metabolism of methionine by *E. coli*.

CORM-2 influences biofilm formation

Previous microarray studies revealed that apart from the *E. coli* genes that are directly implicated in the process of biofilm formation, such as *tqsA*, *mqsR*, *bhsA*, *yceP* (*bssS*)

and *yliH* (*bssR*), genes involved in the response to stress conditions are also induced during biofilm formation, namely *ibpAB*, *soxS*, *cpxP* and *spy* (Beloin *et al.*, 2004; Ren *et al.*, 2004). Many of these genes were also found to be transcriptionally modified when *E. coli* is exposed to CORM-2 (Tables 2 and S3). In particular, comparison of the microarray data showed that approximately 40 % of the genes that are transcriptionally modified during the formation of *E. coli* biofilm (Ren *et al.*, 2004) are in common with those altered by CORM-2 under anaerobic conditions, and 12 % of the common genes are observed when growth is performed aerobically (Supplementary Table S3).

To evaluate the effect of CORM-2 in the process of biofilm formation, quantification of the total biofilm formed during aerobic growth in LB and in the presence of the compound was performed. The results showed that CORM-2 increased by 1.6-fold the total biofilm of the *E. coli* wild-type strain (Fig. 4A). This indicates that *E. coli* produces more biofilm as a defensive response against CORM-2, as previously observed for other stress conditions (Zhang *et al.*, 2007).

The *bshA* gene, which is related to biofilm formation (Zhang *et al.*, 2007), was significantly induced under anaerobic conditions (26-fold). Although the Δ *bhsA* mutant showed an elevated resistance to CORM-2 anaerobically (Fig. 4B), and the complementation experiment performed with a multiple-copy clone containing the *bhsA* regulatory region rescued the wild-type behaviour (Fig. 4B₂), the values of total biofilm measured for the mutant strain exposed to CORM-2 matched those of the parental strain (Fig. 4A).

In *E. coli*, the *tqsA* gene is proposed to encode a putative transport protein involved in biofilm formation (Herzberg *et al.*, 2006). This gene was upregulated in aerobically and anaerobically grown CORM-2-treated cells, with a 9- and 6-fold increase, respectively. Analysis of the *E. coli* Δ *tqsA* mutant revealed that deletion of *tqsA* yielded a strain with higher resistance to CORM-2 than the parental strain, under both aerobic and anaerobic growth conditions (Fig. 4B, C), and the phenotype of the *tqsA* mutant strain could be fully complemented by a clone containing the *tqsA* gene (Fig. 4B₁–C₁). This result is in agreement with those obtained by Herzberg *et al.* (2006), which showed that inactivation of *tqsA* increases the resistance to several antibiotics. Additionally, deletion of *tqsA* abolished the increase in biofilm formation observed in the wild-type strain upon exposure to CORM-2 (Fig. 4A).

Conclusion

In this study, we provide the first microarray analysis of a micro-organism treated with the new bactericide CORM-2. The choice of *E. coli* was based on the fact that, besides being a model bacterium, this Gram-negative micro-organism is able to live in aerobic environments and also adapts to anaerobic niches as part of its normal

Fig. 4. Effect of CORM-2 on biofilm formation and cell viability of *E. coli* $\Delta tqsA$ and $\Delta bhsA$ mutant strains. (A) Biofilm was assayed in *E. coli* wild-type and in $\Delta tqsA$ and $\Delta bhsA$ mutant strains, in the absence or presence of 250 μ M CORM-2. (B, C) Cell viability of *E. coli* parental strain (grey bars), $\Delta tqsA$ (white bars) and $\Delta bhsA$ (black bars) mutants grown anaerobically (b) or aerobically (c) in the presence of 100 μ M or 180 μ M CORM-2, respectively. The number of c.f.u. was determined for at least two biological samples and in duplicate. Error bars represent the standard error of mean values (* $P < 0.01$, ** $P < 0.05$). Complementation analysis of $\Delta tqsA$ and $\Delta bhsA$ (B₁ and B₂, anaerobic conditions) and $\Delta tqsA$ (C₁, aerobic conditions) mutant strains, using the plasmids indicated in each panel, is also shown.

colonization–transmission cycle within a host. Hence, it allowed us to compare the transcriptional response of a bacterium when treated with CORM-2 in both anaerobic and aerobic environments. The broad effect of CORM-2 on gene expression levels is evident from the distribution of induced and repressed genes over the spectrum of all functional categories. In particular, the higher number of genes affected in anaerobically CORM-2-exposed cells illustrates fundamental differences between the way in which microbes control external stress under the two oxygen conditions, and highlights the importance of

specific investigations to understand the different adaptation strategies.

Although it has been assumed that the targets of CO are haem-containing enzymes or proteins, they have not yet been identified and little is known about the trigger event or mechanism that mediates the transfer of CO from the CORM to the haem target. The present data show that the action of CO extends beyond the action on haem proteins since a wide range of transcriptional modifications is observed in cells grown under fermentative conditions and exposed, aerobically or anaerobically, to CORM-2.

The changes in the expression level of key transcription regulators together with the phenotypic analysis of the mutant strains reveal that CORM-2 triggers a complex network of responses. As observed in all other microarray studies performed in *E. coli* cells submitted to toxic chemicals such as hydrogen peroxide and NO, exposure to CORM-2 increases the transcription of the *soxS* regulator but it does not induce any of the genes of the regulon, a result that requires further studies for clarification. A particular observation, common to all transcriptional studies, is the large representation of ‘unknown’ genes, which leaves a wide field to be still explored, until bacterial physiology is fully understood and indeed ready for the application of a systems biology approach. Nevertheless, the data provided by this study will certainly be valuable for guiding future research on the pharmacological application of CORMs.

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