Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators*[S]

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Running title: Bacterial targets of CO-releasing molecules

The authors declare no conflict of interest.

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[S]The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1-S6 and Tables S1-S4.

Abbreviations: CO-RM, CO-releasing molecule; CORM-3, $Ru(CO)_3Cl(glycinate)$; ICP-AES, inductively coupled plasma-atomic emission spectrometry

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Carbon monoxide, a classical respiratory inhibitor, also exerts vasodilatory, anti-inflammatory and anti-apoptotic effects. CO-releasing molecules (CO-RMs) have therapeutic value, increasing phagocytosis and reducing sepsis-induced lethality. Here we identify for the first time the bacterial targets of CORM-3, $Ru(CO)_3Cl(glycinate)$, a ruthenium-based carbonyl that liberates CO rapidly under physiological conditions. Contrary to the expectation that CO would be preferentially inhibitory at low oxygen tensions or anaerobically, *Escherichia coli* cultures were also sensitive to CORM-3 at concentrations equimolar with oxygen. CORM-3, assayed as Ru, was taken up by bacteria and rapidly delivered CO intracellularly to terminal oxidases. Microarray analysis of CORM-3-treated cells revealed extensively modified gene expression, notably down-regulation of genes encoding key aerobic respiratory complexes. Genes involved in metal metabolism, homeostasis or transport, were also differentially expressed and free intracellular zinc levels were elevated. Probabilistic modelling of transcriptomic data identified the global transcription regulators ArcA, CRP, Fis, FNR, Fur, BaeR, CpxR and IHF as targets and potential CO sensors. Our discovery that CORM-3 is an effective inhibitor and global regulator of gene expression, especially under aerobic conditions, has important implications for administration of CO-releasing agents in sepsis and inflammation.

It has been recognised for over 80 years that carbon monoxide (CO) combines with ferrous hemoglobin, competing with oxygen and inhibiting respiration (1). Latterly, however, CO has been shown to have unexpected and profound physiological effects in higher organisms. Inducible heme oxygenase-1 (HO-1) and

constitutive heme oxygenase-2 (HO-2) degrade heme to generate CO that has vasodilatory, antiflammatory and anti-apoptotic effects (2). The CO is thought to target transition elements in biological systems, particularly hemes (in oxidases, globins and CO sensors, for example) (3), nickel (in CO dehydrogenase) and complex Ni-, Fe- and S-containing clusters (2). The impact of CO in physiology and medicine has prompted development of metal carbonyl compounds (carbon monoxide-releasing molecules, CO-RMs) to deliver CO in biological environments. Such molecules are pharmacologically active, eliciting vasodilation in isolated aorta and mediating hypotension *in vivo* (4). Newer water-soluble CO-RMs have revealed roles for CO in suppression of inflammation, protection against hypoxia-reoxygenation and oxidative stress and in mitigation of myocardial infarction and other disorders (5).

Considering the potent biological activities of CO and CO-RMs, we know little about CO toxicity towards microorganisms, despite CO being a stable gas that might find application in antimicrobial therapies. Indeed, recent studies in inflammatory models of disease, such as endotoxin exposure, suggest that HO-1 and its products exert beneficial effects in mice (6-8). Although biliverdin (9) generated by HO activity and CO significantly suppress the inflammatory response (10), it is now clear that HO-1-derived CO plays an important role in the antimicrobial process without inhibiting the inflammatory response (11). HO-deficient mice suffered exaggerated lethality from polymicrobial sepsis, whereas HO-1 over-expression improved survival from *Enterococcus faecalis*-induced sepsis (11). Direct evidence for CO involvement came from injection of CORM-2 (tricarbonyldichlororuthenium (II) dimer $[Ru(CO)_3Cl_2]_2$) into wild-type mice, which increased phagocytosis and rescued HO-1-deficient mice from sepsis-induced lethality. CO-RM-derived CO also inhibits bacterial growth and viability *in vitro* (12). The effectiveness of CORM-2 and CORM-3 was greater in near-anaerobic environments, suggesting competitive inhibition of oxygen utilisation, but no link between the established hemoprotein reactivity of CO and the antibacterial activity of CO-RMs has been demonstrated.

Exploiting the antimicrobial utility of CO and CO-RMs requires an understanding of the growth inhibitory effects and elucidation of the stress responses elicited. Here, we demonstrate that CORM-3 possesses potent antimicrobial activity against *E. coli*, particularly aerobically and that, in such cells, the compound is internalised and delivers CO to terminal oxidases. Moreover, the growth-inhibitory effects correlate with the results of a transcriptomic study and application of a probabilistic systems model, which reveal for the first time that bacteria mount a global response to CORM-3 involving aerobically eight transcription factors. We anticipate that understanding the molecular and biochemical mechanisms of CO delivery and toxicity, and the cognate bacterial stress responses, will contribute towards utilisation of CO-RMs in sepsis and other clinical applications.

EXPERIMENTAL PROCEDURES

E. coli strains and growth conditions - All strains used were *E. coli* K-12 derivatives: the wild-type was MG1655. Strains carrying *lacZ* transcriptional fusions were ECL933 [Φ(*cyo-lac*) *bla*$^+$ *cyo*$^+$] (13), MC4100 λRS88 [*spy-lacZ*] (14) and RKP2910 [Φ(*zntA-lacZ*)] (15). The *cyo* mutant was RKP4544 (*cyo::kan*; laboratory collection). Cells were grown in defined media with glycerol (54 mM) as sole carbon source, as before (16). For anaerobic growth, fumarate was added as terminal electron acceptor (50 mM), in addition to LB (5% v/v) and casamino acids (0.1% w/v) to give a growth rate comparable to aerobic cultures. For aerobic growth, cells were grown in 30 ml defined medium in 250 ml baffled flasks fitted with side-arms for measurements of optical density with a Klett meter (red filter) during shaking at 200 rpm and 37^{o}C. Anaerobic growth was in Klett-compatible 8.5 ml screw-cap tubes, filled to the brim with defined medium, at 37^{o}C in a water bath. For viability assays, anaerobic cells were grown in 175 ml batch cultures in defined medium in stirred (200 rpm) mini-fermenter vessels (17) modified for anoxic sampling by sparging with N_2. Starter cultures were grown in defined media (anaerobic) or 10 ml LB (aerobic); for the latter, cells were harvested and resuspended

in defined medium before inoculation. Viability was determined after dilution of samples in water by plating 10 µl drops on nutrient agar. Protein concentrations were measured using the Markwell assay (18).

CORM-3 and CO treatments - CORM-3 (Hemocorm) was prepared as a 100 mM stock solution in distilled water freshly for each experiment. $RuCl_2(DMSO)_4$ was prepared similarly. CO-saturated solutions were made by bubbling CO gas (CP grade) from a cylinder (BOC, Guildford, GU2 5XY) through buffer for 20 min, resulting in a 1.8 mM stock solution. CORM-3, $RuCl_2(DMSO)_4$ and CO-saturated solution were added directly to growing cultures in log phase, determined by a Klett OD of ~40 (when grown in side-arm flasks or screw cap tubes) or an OD_{600} of ~ 0.5 (when grown in mini-fermenter vessels).

Uptake of CORM-3 by growing cells - CORM-3 was added during logarithmic phase to aerobic (final concentration 30 µM) and anaerobic (final concentration 100 µM) cultures. After 15 min, cells were harvested by centrifugation at 5000 g for 5 min (Sigma 4K15) in polypropylene tubes (Sarstedt 62.547.004 (50 ml) or 62.554.001 (15 ml)). Culture supernatants were retained for analysis. Cell pellets were washed thrice in 0.5 % HNO_3 (0.5 ml, Aristar nitric acid, 69 % v/v) to remove loosely bound elements. Supernatants collected from the washes were also retained for analysis. Pellets were resuspended in HNO_3 (0.5 ml 69%) and placed in an ultrasonic bath for 30 min to break the cells. The resultant digest was then quantitatively transferred to a calibrated 15 ml tube and made up to 5 ml with 1% HNO_3. Samples were analysed using a Spectro Ciros^{CCD} (Spectro Analytical) inductively coupled plasma-atomic emission spectrometer (ICP-AES) using background correction. Calibration curves were created for each element to be tested using multi-element standard solutions containing 0.1, 0.2, 1, 5 and 10 mg L^{-1}. The wavelengths (nm) for elements reported here were Zn (213.856) and Ru (240.272). A 1% nitric acid solution in MilliQ water was used as a blank and to dilute cell digests before ICP-AES analysis. Elemental recoveries were calculated from these samples.

Calculation of dry cell weight and intracellular ruthenium concentrations – Dry weight was determined by filtering known volumes of culture (usually 10 ml, 20 ml and 30 ml) through pre-weighed cellulose nitrate filters, 47 mm diameter and pore size 0.2 µm (Millipore). The filters had previously been dried at 105 ^oC for 18-24 h to constant weight. Filters were again dried at 105 ^oC until a constant weight was attained, which was recorded. Alternatively, bacteria were sedimented in pre-weighed, dried centrifuge tubes, which were reweighed after drying to constant weight. Both methods gave similar results. To calculate intracellular Ru concentrations, we adopted published values for individual cell dry mass and volume (19). Values in Table 1 are means of two growth experiments, with each sample being assayed in triplicate by ICP-AES.

Cytochrome and respiration assays - Difference spectra (CO plus reduced *minus* reduced) of whole cells were recorded in a dual-wavelength spectrophotometer (20). Cells were reduced with dithionite; after treatment with CORM-3, CO or $RuCl_2(DMSO)_4$, spectra were recorded in triplicate (10 mm path length) and averaged. For myoglobin competition analysis, 8 µM CORM-3 was added to cells or buffer with dithionite. Myoglobin (10 µM) was added at 0, 10 and 20 min and spectra recorded using a baseline of either reduced cells and myoglobin, or reduced buffer. The contribution of cytochrome *o* to CO difference spectra was determined by a deconvolution method. From the CO difference spectrum (mean of 3 scans) of *cyo::kan* cells was subtracted the spectrum of purified cytochrome *bd* (21), such that the distinctive signals in the red region (575-675 nm) were brought to zero. The resultant residual and the cytochrome *bd* spectrum were then subtracted from a scan of wild-type cells, leaving only the cytochrome *bo'* spectrum.

Samples were taken from cultures after exposure to CORM-3, and the harvested cells washed and resuspended in 50 mM Tris buffer (pH 7.5); respiration was measured using a Clark-type polarographic oxygen electrode (Rank Bros) at 37^oC (22).

β-galactosidase assays – Assays were carried out as described before (23) in $CHCl_3$- and sodium dodecyl sulfate-permeabilised cells by monitoring *o*-nitrophenyl-β–D-galactopyranoside hydrolysis. Activities are expressed in terms of the OD_{600} of cell suspensions using the formula of

Miller (23). Each suspension was assayed in triplicate and results were confirmed in at least two independent experiments. For assays of Φ(*zntA-lacZ*) activity (15), we included where shown 0.5 mM Zn(II) to elevate the low basal levels of expression, as described in (24).

Microarray Analysis - Cells were grown aerobically in 30 ml defined medium in 250 ml baffled side arm flasks or anaerobically in fermenter vessels as above. At log phase (Klett OD~40 or OD_{600}~ 0.5), CORM-3 (30 μM for aerobic cells, 100 μM for anaerobic cells) was added and, after 15 min, 30 ml culture was added to chilled phenol (188 μl)/ethanol (3.6 ml), and flash-frozen in liquid nitrogen. RNA was prepared using an RNeasy Minikit from Qiagen, from four biological repeats of control and CORM-3 exposed cells. Other procedures were as described before (16,17). The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE13048 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13048) (25).

Modelling transcription factor activities - Transcriptomic data were analysed using a probabilistic model (26), which adopts a log-linear approximation to the transcriptional reaction to changes in transcription factor activity. Changes in gene expression as a weighted linear combination of changes in transcription factor activity are given by

$$y_n = \sum_m X_{nm} b_{nm} c_m + \varepsilon_n .$$

Here, y_n is the log-fold change for the n-th gene, X_{nm} a binary matrix encoding the structure of the regulatory network (obtained from the literature), b_{nm} the unknown rate constants for activation/ repression, c_m the (log) change in transcription factor activity and ε_n an error term. Both the rate constants and the transcription factor activity changes are given zero mean normal priors. Standard statistical inference techniques provide estimates of the changes in activity of regulators from an analysis of the behaviour of their targets. This technique has been applied to reconstructing the regulatory response in *E. coli* to the transition between aerobic and microaerobic conditions (27).

RESULTS

CORM-3, but not a solution of CO gas, targets both aerobically and anaerobically growing E. coli - To establish conditions for CO-RM toxicity, we first defined the growth inhibitory effects of CORM-3, a proprietary water-soluble CO-RM (28). We first tested CORM-3 against an anaerobic culture with glycerol as carbon source and fumarate as terminal electron acceptor, expecting the absence of oxygen, with which CO competes, to reveal maximal CORM-3 toxicity. Addition of 100 μM CORM-3 slowed growth but higher concentrations (200 μM) were required to inhibit growth completely (Supplementary Fig. S1A). However, under these aerobic conditions (Supplementary Fig. S1B), CORM-3 at only 30 μM inhibited growth and 100 μM CORM-3 totally prevented further growth. Inoculation of cells into media containing 250 μM CORM-3 also prevented growth and killed a significant proportion of cells within 2 h (not shown). However, the same concentrations of CO added as a CO-saturated solution did not prevent anaerobic or aerobic growth, nor did $RuCl_2(DMSO)_4$, a compound with similar structure to CORM-3 but where the carbonyl groups have been replaced with DMSO (28) (Supplementary Fig. S1A and B, insets).

The toxicity of CORM-3 was also investigated by measuring cell viability under our growth conditions. Anaerobically, 100 μM CORM-3 had no effect on viable counts within 2 h (Supplementary Fig. S2A). Aerobically, 30 μM CORM-3 was without effect on viability within 10 min but caused a 10-fold reduction in counts after 2 h. However, 125 μM CORM-3 reduced the viable count by 10^4-fold within 30 min; the marked increase in toxicity between 30 and 125 μM CORM (Supplementary Fig. S2B) may reflect the level of accumulation of CORM-3 (see below). The toxicity of CORM-3, but not $RuCl_2(DMSO)_4$, to aerobic cells was also confirmed by fluorescent live/dead staining (Invitrogen): SYTO 9 stains all bacteria green, whereas propidium iodide penetrates only bacteria with damaged membranes and stains them red whilst reducing the amount of SYTO 9 when both are present. Aerobically, addition of CORM-3 caused a 48% reduction in the percentage of live cells (only green) and an

increase in the percentage of dead and unhealthy cells (red or red/green) but, anaerobically, CORM-3 did not reduce the percentage of live cells. $RuCl_2(DMSO)_4$ was without effect on the proportions of live and dead cells (data not shown). Thus, CORM-3 is a potent antibacterial molecule even under aerobic conditions.

Uptake of CORM-3 by growing cells – To demonstrate uptake of CORM-3 by both aerobic and anaerobic cells, we assayed for the presence of Ru from the $Ru(CO)_3Cl(glycinate)$, since the radiolabelled compound is unavailable. Assays by ICP-AES of cells from CORM-3-treated cultures, after extensive washing and digestion to solubilize biomass, are presented in Table 1. The levels of Ru detected in control cells not treated with CORM-3 were insignificant as expected, since Ru has no known biological function. The majority of CORM-3 added was recovered in the culture supernatant and small amounts were found in the washings after treating cells with dilute HNO_3. Although the absolute amounts of Ru recovered in digested cells was also small, these levels equated with significant intracellular concentrations assuming literature values (19) for the mass and volumes of individual cells. For aerobic cells, the estimated intracellular Ru concentration (209 μM) exceeds by 7-fold the concentration in the culture, whilst for anaerobic cells, the accumulation is 2.1-fold. Recoveries in the procedure of over 80% testify to the reliability of the analytical data. In a further pair of experiments on aerobic cells, the recovery was lower (63%) but again a high level of intracellular uptake of Ru (168 μM) was demonstrated (results not shown). Thus, CORM-3 (and the CO released, 1 CO per CORM molecule) are taken up to achieve concentrations significantly greater than those in the external medium, and the capacity of aerobically grown cells to accumulate CORM-3 appears greater than for anaerobic cells, given the higher external CORM-3 concentrations used for the latter. Thus, in terms of intracellular uptake of Ru and the observed effects on cell viability (Supplementary Fig. S2), 30 μM CORM-3 for aerobic cells equates with 100 μM CORM for anaerobic cells. The uptake of Ru after treatment of aerobic cells with 125 μM CORM was not investigated due to the extreme toxicity observed under these conditions.

CO is delivered intracellularly by CORM-3 - Since CORM-3 is taken up by growing cells and is an effective inhibitor of growth at concentrations where dissolved CO gas is not, we investigated further CO delivery. The high affinity of ferrous sperm whale myoglobin for CO (k_{on}/k_{off} = 2 x 10^7 M^{-1}) and the impermeability of bacterial cells to myoglobin (M_r 16.7 kDa) allows distinction between extracellular and intracellular CO. When CORM-3 and myoglobin at near equimolar concentrations were mixed in the absence of bacteria, there was immediate formation of the carbonmonoxy adduct, characterised by the appearance in CO difference spectra (reduced myoglobin + CO *minus* reduced myoglobin) (Fig. 1) of a Soret maximum at 426 nm and minimum at 442 nm (29). In contrast, myoglobin addition to bacteria supplemented with CORM-3 detected reduced levels of CO; within 10 min of CORM-3 addition to bacteria, only 28% of the initial CO was found by the myoglobin competition assay, and after 20 min only 15% (Fig. 1). Thus, in support of the ICP-AES assays, CORM-3 rapidly enters bacterial cells and renders CO inaccessible to exogenous myoglobin.

CORM-3 inhibits bacterial respiration and reacts with terminal oxidases - We next investigated the nature of the intracellular targets. Although CO is a classical inhibitor of respiration, high partial pressures of CO relative to O_2 (typically 9:1) are required for significant competitive inhibition (1). Surprisingly, CORM-3 (125 or 250 μM) was an effective inhibitor of bacterial cell respiration when added to vigorously aerated cultures at concentrations equimolar with dissolved oxygen, i.e. approx 200 μM (Fig. 2A). Neither CO added as a gaseous solution nor $RuCl_2(DMSO)_4$ were effective as inhibitors under these conditions. Inhibition of respiration by 125 μM CORM-3 was rapid, 50% inhibition being achieved within 10 min of CORM-3 addition (Fig. 2B).

Inhibition of respiration by CO was accompanied by rapid formation of carbonmonoxy adducts of terminal oxidases within intact cells when CORM-3 was added exogenously (Fig. 3). Immediately after CORM-3 addition to *E. coli* cells, the distinctive α-band at 648 nm of the CO compound of cytochrome *d* was formed, with accompanying change in the Soret region near 448

nm. Subsequently, the Soret features intensified and a trough formed at 560 nm, without change at 648 nm, suggesting CO reactivity with a cytochrome of lower CO affinity. This component is probably the oxidase cytochrome *bo'* based on the appearance of the Soret band at 422 nm (the CO-ligated ferrous species) and a trough at 434 nm (the loss of the ferrous signal) (29,30). In contrast, addition of a similar concentration of $RuCl_2(DMSO)_4$ did not result in reaction with cytochrome *d* over 60 min (Supplementary Fig. S3) but absorbance minima of lower magnitude centred at 432 and 562 nm indicated cytochrome oxidation perhaps due to the DMSO moiety of $RuCl_2(DMSO)_4$. We conclude that CORM-3, but not $RuCl_2(DMSO)_4$, or CO administered as a gas, is an effective respiratory inhibitor, an activity that is attributable to CO release and reaction with terminal oxidases.

CO delivered by CORM-3 elicits global transcriptional changes - To identify bacterial genes that are differentially expressed during exposure to CORM-3, we performed transcriptomic profiling of cultures exposed to CORM-3. In both aerobic and anaerobic cultures, the CORM-3 concentrations used (30 and 100 μM, respectively) were without significant effect on viability over 15 min (Supplementary Fig. S2) and growth rates were slowed to similar extents (Supplementary Fig. S1). The gene expression profiles were reproducible and revealed marked differences between the aerobic and anaerobic consequences of CORM-3 exposure (Figs. 4, 5). Microarray analysis is presented in full in Supplementary Tables S1-4. Under aerobic conditions, 63 genes were up-regulated by more than 2-fold ($P<0.05$) and 183 were down-regulated using the same criteria. Under anaerobic conditions, 29 genes were up-regulated by more than 2-fold ($P<0.05$) and 41 were down-regulated using the same criteria. The major functional categories of genes affected are described below.

CORM-3 targets bacterial respiration - In the defined medium used here, aerobic growth is dependent on respiratory energy conservation with oxygen as terminal electron acceptor. The effects of CO from CORM-3 on respiration (Fig. 2) and oxidases (Fig. 3) were reflected in dramatic down-regulation of many genes encoding components of the aerobic respiratory chain (Fig. 4). Most prominent were 10- to 22-fold reductions in transcript levels for the entire *cyo* operon (*cyoABCDE*) that encodes the cytochrome *bo'* heme-copper terminal oxidase and the *sdh* operon (*sdhABCD*) that encodes the membrane succinate dehydrogenase, which couples the Krebs cycle to the respiratory electron transport system. Down-regulation of *cyo* genes was accompanied by modest up-regulation of the *cydAB* operon that encodes the alternative terminal oxidase, cytochrome *bd*. We also observed down-regulation of the *nuo* operon (11 genes) encoding the major NADH dehydrogenase, of *fdo* genes encoding three subunits of the membrane-bound formate dehydrogenase, and of *glp* genes encoding aerobic and anaerobic glycerol 3-phosphate dehydrogenases, and glycerol/glycerol 3-phosphate transporters. There were smaller decreases in expression from genes in the *atp* operon (previously *unc*) encoding the F_1F_o ATP synthase.

To confirm the differential regulation of oxidase genes revealed by transcriptome profiling, we first recorded absorbance difference spectra of intact cells from control and CORM-3-treated aerobic cultures of a *cyo* mutant, in which the sole CO-reactive oxidase is cytochrome *bd*. In reduced *minus* oxidised difference spectra, an elevated level of cytochrome *d* in the CORM-3-treated cells was evident from the characteristic red band at 633 nm (Supplementary Fig. S4A). Interestingly, in CO difference spectra of this strain (Supplementary Fig. S4B), cytochrome *d* was readily detectable in control cells but not in cultures treated with CORM-3, a result that we attribute to reaction of the oxidase with CORM-derived CO during growth, so that the baseline spectrum is not further perturbed by CO. These data confirm up-regulation of cytochrome *bd* by CORM-3 and reveal reaction of CORM-derived CO with the oxidase *in vivo* during growth. Second, we assayed cytochrome *bo'* in wild-type cells by CO difference spectra using a spectral deconvolution method to eliminate cytochrome *bd* (Supplementary Fig. S4C). The distinctive Soret bands of cytochrome *o* (415, 429 nm) were detected in control, but not CO-RM-treated, cells. Third, we measured β-galactosidase activity in a Φ(*cyo-lacZ*) strain and demonstrated down-regulation of *cyo-lacZ* activity by CORM-3, but

not by $RuCl_2(DMSO)_4$ (results not shown). Collectively, these data support a marked decrease in *cyo* operon transcription elicited by CORM-3 and a modest up-regulation of the alternative oxidase, cytochrome *bd* (Fig. 4).

There was also evidence of a downshift in respiratory and bioenergetic gene expression anaerobically, also shown in Fig. 4. Under these conditions, fumarate is provided as alternative terminal electron acceptor. A much smaller set of genes was differentially regulated, but these included genes of the *cyo* and *cyd* operons, formate dehydrogenase, glycerol 3-phosphate dehydrogenase and succinate dehydrogenase.

CORM-3 perturbs metal biochemistry - In biology, CO binds preferentially and almost exclusively to transition metals, especially Fe(II) (2), but also cobalt (31,32). Transcriptomics revealed many genes known to be involved in metal metabolism, homeostasis or transport (Fig. 5). Aerobically, CORM-3 elicited a 26-fold up-regulation of *spy*, encoding a periplasmic protein that is induced by zinc and copper via the CpxR and BaeR sensor-response regulator systems (33). Other genes implicated in zinc homeostasis and regulation observed in this study are *yodA* (16-fold up-regulated) and *znuA* (5-fold up-regulated). The former encodes a periplasmic Zn- and Cd-binding protein (34-36). The *znuA* gene also encodes a periplasmic Zn(II)-binding protein (37,38) associated with the high-affinity ATP-binding cassette ZnuABC transporter (39). Genes encoding ferritin-like proteins were differentially regulated by CORM-3 in aerobically grown cells with *ftnB* being up-regulated and *bfr* down-regulated. Several genes in the membrane transport class are also implicated in metal homeostasis (see below).

In anaerobically grown cells, *spy* was up-regulated >100-fold and is thus the gene most dramatically regulated in response to CORM-3 administration in our studies. Confirmation of the microarray data was obtained by measuring β-galactosidase activity in anaerobic cultures of a strain harbouring a *spy-lacZ* transcriptional fusion; 100 μM CORM-3 added to an exponentially growing culture elicited an increase from 381 to 2960 Miller units over 60 min, whereas untreated cultures or those treated with 100 μM $RuCl_2(DMSO)_4$ showed no change (Supplementary Fig. S5). β-galactosidase was not measured at zero time, but the basal level is very low (< 36 Miller Units), similar to that measured for the untreated control at later times. Addition of $CuSO_4$ (200 mM) elicited only a 6-fold increase in *spy* transcription over 60 min (Supplementary Fig. S5), as reported recently (33).

Other zinc-regulated genes exhibiting increased transcript levels on CORM-3 treatment were *yodA* and *znuA* (as in aerobic cells) but also *zraP*, encoding a periplasmic zinc-binding protein. We therefore considered whether CORM-3 liberates free intracellular zinc. Zn was detectable in cell digests by ICP-AES at levels of 0.01-0.02 μg per mg, but was not markedly affected on CORM-3 treatment of cells. Since these elemental analyses cannot distinguish between free and bound metal, we exploited the zinc-responsive expression of Φ(*zntA-lacZ*). This fusion is sensitive to intracellular zinc since *zntA* encodes a P-type ATPase that exports excess zinc and contributes to zinc homeostasis (40). We showed previously (24) that NO elicits intracellular zinc release that is detectable by this fusion and that the response is dependent on zinc sensing by *zntR*. In the absence of additional zinc in the medium, modest or insignificant changes in β-galactosidase activities were measured in both aerobic and anaerobic cultures treated with CORM-3 (Supplementary Fig. S6). Therefore, to elevate expression of Φ(*zntA-lacZ*) from very low basal levels, zinc (0.5 mM) was added to cultures (24); under these conditions, significant increases in Φ(*zntA-lacZ*) expression were recorded (Supplementary Fig. S6). Thus, zinc release and metal homeostasis are casualties of CORM-3 treatment.

Differential expression of genes in other functional classes on CORM-3 treatment - Amongst the most dramatically down-regulated genes are several linked to acetate and acetyl group metabolism. Chief among these is *acs*, which was down-regulated 18-fold in aerobic cells. This gene encodes acetyl Co-A synthetase (ACS); intriguingly, this metalloenzyme, together with CO dehydrogenase (CODH), plays a pivotal role in CO metabolism (41). CODH oxidizes CO to CO_2, providing low-potential electrons for the cell, or alternatively reduces CO_2 to CO. The latter reaction, when coupled to ACS, generates acetyl-CoA from CO_2 for cell carbon synthesis. Also

highly down-regulated were the acetate transporter gene *actP* (11-fold), and three genes in the *ace* operon (anaerobically) encoding isocitrate lyase, malate synthase and pyruvate dehydrogenase. Genes implicated in diverse membrane transport processes were strongly represented in the aerobic gene lists (Supplementary Tables S1-2). Notable are the down-regulated genes encoding transporters for methyl-galactoside (*mglABC*), and the highly up-regulated (8- to 13-fold) *mdtABC* operon implicated in a multidrug export system, which is also up-regulated in response to supraoptimal zinc levels (17). The *cus* operon was highly up-regulated anaerobically and this too is up-regulated in response to high zinc levels (17).

Statistical analysis of CO-RM data - In modelling the transcriptome data, we focused on a subset of eight key regulators: ArcA, CRP, Fis, FNR, Fur, BaeR, CpxR and IHF. A list of genes populating the (overlapping) regulons of these transcription factors, together with information on the sign of the regulation where available, was obtained from regulonDB (http://regulondb.ccg.unam.mx/). The estimated change in transcription factor activity (with associated uncertainty) for these regulators is shown in Fig. 6. In aerobic conditions, all transcription factors investigated exhibit a significant change in activity after 15 min exposure to CORM-3. In all cases, the signal to noise ratio is very high, giving estimated *p*-values of less than 10^{-6}. Interestingly, the reaction of the transcriptional regulators in anaerobic conditions seems to be very different. In particular, no significant change in activity was predicted for CRP, Fis and FNR under anaerobic conditions. In order to assess the statistical significance of these results, we ran the model on the same transcriptomic data, but reshuffling at random the regulatory network. This was repeated for 100 different random reshuffles. Strikingly, in the vast majority of cases (84%), the model identified only one significantly changing regulator in these random networks; in 12 cases it explained the data with two significantly changing regulators, and in only 4 cases it predicted three regulators to significantly change. Based on these simulations, we can estimate that the probability of obtaining such a rich regulatory response purely from random data is less than 10^{-6}.

DISCUSSION

CO is a classical respiratory poison but also a critical endogenous 'gasotransmitter' (42, 43). Its potential impact in microbiology is considerable: contrary to expectations that endogenously generated CO might suppress the inflammatory response, HO-derived or CO-RM-derived CO limits sepsis (11), suggesting new intervention approaches in microbial pathogenesis and disease. Although CO is also toxic *in vitro* to gram-positive and -negative bacteria (12), the mode of action has remained obscure. It is remarkable that, in the present study, CORM-3 is effective as an antimicrobial agent (whether assayed by growth rates or viability counts or live/dead staining) even under aerobic conditions, and at ambient oxygen concentrations higher than CO. Critically, the control molecule $RuCl_2(DMSO)_4$ is ineffective as an inhibitor of growth or respiration, and does not elicit increased *spy* gene expression, suggesting that the effects observed here can be directly attributed to released CO. Based on (i) direct assays of the accumulation in cells of Ru from $Ru(CO)_3Cl(glycinate)$ (CORM-3) by bacteria (Table 1), (ii) our use of myoglobin to demonstrate loss of accessible CO from the culture media (Fig. 1) and (iii) direct demonstration of the formation of the carbonmonoxy forms of terminal oxidases (Fig. 3), we propose that CORM-3 effectively delivers CO to intracellular targets. We are unaware of other reports of the accumulation of a Ru-containing CO-RM by bacterial or higher cells, but Mo is accumulated by bacteria after administration of the CO-RM tetraethylammonium Mo pentacarbonyl bromide (ALF062) (12). It should be noted that although only 1 CO is promptly released from CORM-3, 2 residual CO moieties remain coordinated to the Ru. Intracellular catabolism may result in release of >1 CO per CORM-3.

Toxicity of CORM-3 is observed even in competition with oxygen, perhaps effecting a rise in the CO/O_2 ratio in critical micro-environments of the cell, membrane or oxidase active site. The importance of respiration as a target for CO-RMs is highlighted by the recent report that CORM-2 or CO gas inhibits L-type Ca^{2+} channels in rat ventricular cardiac myocytes (44). The inhibition can be attributed to inhibition by CO of electron

transfer to cytochrome *c* oxidase, consequent generation of reactive oxygen species, and redox modulation of critical cysteine residues in the channel. Although the idea that regulatory effects might be due in part to part-reduced oxygen species appears inconsistent with the presented modelling outcome, Sox has been implicated in additional preliminary modelling (G. Sanguinetti, unpublished) which requires extension in a time-course study. Critically, direct experimental assays of superoxide and peroxide generation are required to test further this hypothesis but are outside the scope of the present report.

Transcriptomics and modelling reveal the involvement of several CO-RM-responsive transcription factors, but the mechanism for only one of these, ArcAB, can be readily explained with current information. This two-component signal transduction system of *E. coli* mediates adaptive responses to the prevailing respiratory conditions. Oxidised forms of ubiquinone in the cytoplasmic membrane act as direct negative signals that inhibit autophosphorylation of ArcB and consequently of formation of ArcA-P (45), which binds to DNA targets. Thus, inhibition of respiration by CO, for example, results in ubiquinone reduction; under these conditions, the kinase activity of ArcB is unchecked, the level of ArcA-P rises and repression of respiratory chain gene expression ensues. The widespread occurrence of the ArcAB system in diverse bacteria, including *Salmonella*, *Vibrio*, *Shigella*, *Yersinia* and *Erwinia* (46) suggests that pathogens might sense host-derived CO by such a mechanism.

The involvement of CRP activity in aerobic cells, revealed in the transcriptomics and their mathematical modelling, may be a consequence of changing cAMP availability, in turn driven by the effects of CO on DOSEc, a heme-regulated phosphodiesterase that is expressed especially in aerobic cells (47). It is well known that CO combines only with ferrous, not ferric, heme (1) but interaction of CO with other intracellular metal targets might explain the participation in the cellular responses to CORM-3 of Fnr and Fur, both of which contain Fe centers. Fnr contains, in its active form, a [4Fe-4S] cluster that is reactive with O_2 (48) and NO (49). Although hydrogenases and nitrogenases possessing [4Fe-4S] clusters are inhibited by CO, rapid interaction of a synthetic cluster with CO occurred only when the highly reduced state is accessed, corresponding to a physiologically unusual reduction level (50). Future work might test Fnr *in vitro* with CO-RMs. A less likely explanation is that ferrous iron binds to, say two cysteine and two lysine residues, and CO then binds to iron (51). Even bleomycin will coordinate Fe(II), which then coordinates CO, but the binding is weak (52). We are unaware of any reports on the reactivity of CO with the metal centers of Fur.

Other effects may reflect not CO reactivity in vivo but global disturbance by the ruthenium content of CORM-3. Previous work has shown the effects of dimeric, mixed-valence Ru compounds (53) on growth of *E. coli* at micromolar concentrations, but ruthenium nitrosyl complexes are less inhibitory because of their inability to enter cells (54). The effects of CORM-3 on intracellular free (but not total) zinc pools cannot currently be explained.

The up-regulation of ferritin-like proteins is of interest since FtnB in *Salmonella* is implicated in repair of oxidatively damaged proteins (55), which could arise from inhibition by CO of terminal respiratory pathways and consequent radical formation. Indeed, CORM-2 increases oxidant production by mitochondria (56) and NO, by arresting respiration of *Salmonella*, triggers an adaptive response to oxidative stress (57).

Also implicated both aerobically and anaerobically by the modelling data are CpxR and BaeR. The Cpx stress response is activated by misfolded envelope proteins and, in the present study, *cpxP* (encoding the periplasmic inhibitor) is highly up-regulated both aerobically (24-fold; Supplementary Table S2) and anaerobically (8-fold; Supplementary Table S4), suggesting that the Cpx response is important in maintaining the integrity of envelope proteins in the face of CO-related insults. The most dramatic up-regulation in this study is that of *spy*, which encodes a periplasmic protein of obscure function, inducible by exposure to copper and zinc ions (33) and by envelope stress in general (58). The *spy* gene is regulated by both BaeSR and CpxAR (33,58).

In conclusion, we demonstrate the effectiveness of a ruthenium-based carbonyl

compound as an inhibitor of bacterial growth and respiration and the rapid uptake of this compound into bacterial cells where heme proteins are targeted. Moreover, the effects on growth and viability are evident in aerobic cultures, in apparent contradiction to the conventional view that CO acts by competing with oxygen for binding to ferrous heme proteins. Understanding the delivery of CO-RMs and/or CO release to intracellular targets, some of which are revealed by global transcript profiling, will be critical for future antimicrobial applications of CO-RMs. Although physiological side-effects of the rapid release of CO (as measured by formation of carbonmonoxymyoglobin *in vitro*) might be anticipated, increases in the levels of the CO adduct of hemoglobin in vivo are modest. Thus, intravenous injections of CORM-3 in mice do not lead to substantial increases in carbonmonoxyhemoglobin (0.5% increase per 100 μM CORM-3) (59). Since the first symptoms of CO poisoning in humans (headache, dilation of cutaneous blood vessels) is manifest at carbonmonoxyhemoglobin concentrations exceeding 15-20%, there is reason to predict that side-effects of CO-RM therapy may be minimal. Surprisingly, the predominantly aerobic environment of the vasculature and macrophage may prove to be a suitable milieu for antimicrobial CO-RM activity.

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TABLE 1

Uptake of CORM-3 by aerobically and anaerobically growing *E. coli*

CORM-3 was added to aerobic (final concentration 30 μM) and anaerobic (final concentration 100 μM) cultures. Cells recovered by centrifugation were subjected to successive washings as detailed in Experimental Procedures. Ru was assayed in all fractions including the culture supernatant and acid-digested cell pellet by ICP-AES then used to calculate intracellular Ru concentrations, using the values for cell dry mass and volume detailed in Experimental Procedures. The values are means of two biological replicates (growths) for each condition, and each sample was assayed in triplicate by ICP-AES.

	Ru (μg)					
	Added as CORM-3	Culture supernatant	Pellet washes[1]	Cell pellet[2]	Ru in cell (μM)	Recovery (%)[3]
Aerobic	26.4	19.4	0.067	0.40	209	81.0
Anaerobic	85.9	63.2	0.72	0.28	214	80.7

[1]Only given where determinations exceed limits of detection (LOD)

[2]Ru levels in control cells were less than the LOD, defined as 3x s.d. of a blank sample

[3]Expressed as the sum of Ru assayed in the culture supernatant, washes and cell pellet as a percentage of the added Ru in the form of CORM-3

FIGURE 1. **Myoglobin competition assays reveal CORM-3 uptake by bacteria.** CORM-3 (8 μM) and myoglobin (10 μM) were added to buffer only (A) or buffer plus cells (B). CORM-3 was added at t = 0, and myoglobin added at 0 (−), 10 (− −), or 20 min (. . .). Difference absorbance spectra are shown: the reference spectrum is Fe(II) myoglobin with cells or buffer, reduced with dithionite.

FIGURE 2. **CORM-3 inhibits bacterial aerobic respiration.** In A, cultures were grown to mid-log phase, then exposed for 2 h to 125 or 250 μM CORM-3 (black bars), $RuCl_2(DMSO)_4$ (dark hatched) or CO gas-saturated solution (light hatched); controls (no addition) are shown by white bars. Protein concentration of the suspensions was 4.5 mg ml^{-1}. Asterisks denote significant differences from control: *, $P<0.01$, **, $P<0.002$, ***, $P<0.001$. B shows the time course of inhibition by 125 μM CORM-3 of cell respiration (2.6 mg protein ml^{-1}).

FIGURE 3. **Reaction of terminal oxidases *in vivo* with CORM-3 (2 mM) added to intact cells in a dual wavelength spectrophotometer.** CO difference spectra (reduced + CO *minus* reduced) were taken at times (shown in min) after addition of CORM-3.

FIGURE 4. **Differential expression of genes involved in respiratory electron transfer and energy conservation.** The mean fold increase or decrease in individual gene expression after exposure to CORM-3 compared to controls lacking the compound both aerobically (30 μM CORM-3) and anaerobically (100 μM CORM-3) is indicated by the colour scale bar. Genes encoding terminal oxidases are highlighted in pale grey; genes encoding dehydrogenases are highlighted in darker grey. Relevant regulatory proteins were taken from the EcoCyc and EchoBase websites. SU = subunit.

FIGURE 5. **Differential expression of genes involved in metal biochemistry.** The mean fold increase or decrease in individual gene expression after exposure to CORM-3 compared to controls lacking the compound both aerobically (30 μM CORM-3) and anaerobically (100 μM CORM-3) is indicated by the colour scale bar. Genes implicated in metabolism of, or regulation by, zinc ions are highlighted in pale grey. Relevant regulatory proteins were taken from the EcoCyc and EchoBase websites. SU = subunit.

FIGURE 6. **Probabilistic modelling of the transcriptomic data for the effects of CORM-3 treatment.** Predicted activities (which may be positive or negative) under aerobic (black) and anaerobic (grey) conditions are shown for ArcA, CRP, Fis, FNR, Fur, BaeR, CpxR and IHF. CORM-3 concentrations were 30 μM (aerobic) and 100 μM (anaerobic).