Prokaryotic Real-Time Gene Expression Profiling for Toxicity Assessment

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Examining global effects of toxins on gene expression profiles is proving to be a powerful method for toxicity assessment and for investigating mechanisms of toxicity. This study demonstrated the application of prokaryotic real-time gene expression profiling in *Escherichia coli* for toxicity assessment of environmental pollutants in water samples, by use of a cell-array library of 93 *E. coli* K12 strains with transcriptional green fluorescent protein (GFP) fusions covering most known stress response genes. The high-temporal-resolution gene expression data, for the first time, revealed complex and time-dependent transcriptional activities of various stress-associated genes in response to mercury and mitomycin (MMC) exposure and allowed for gene clustering analysis based on temporal response patterns. Compound-specific and distinctive gene expression profiles were obtained for MMC and mercury at different concentrations. MMC (genotoxin) induced not only the SOS response, which regulates DNA damage and repair, but also many other stress genes associated with drug resistance/sensitivity and chemical detoxification. A number of genes belonging to the P-type ATPase family and the MerR family were identified to be related to mercury resistance, among which *zntA* was found to be up-regulated at an increasing level as the mercury concentration increased. A mechanism-based evaluation of toxins based on real-time gene expression profiles promises to be an efficient and informative method for toxicity assessment in environmental samples.

Introduction

Over the past century, water quality monitoring has relied almost exclusively on chemical detection of acutely toxic priority pollutants. The rapidly increasing number of recognized emerging contaminants, such as pharmaceuticals and personal care products (PPCPs), and endocrine-disrupting chemicals (EDCs), makes it neither feasible nor economical to analyze for every single compound of concern in the water. In addition, the synergistic toxicity manifested by

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a mixture of compounds cannot be inferred directly from the concentrations of single compounds alone. Application of the few existing toxicity evaluation methods, such as whole effluent toxicity testing (WET) and toxicity identification evaluation (TIE), has been limited by the high cost, laborious and sophisticated analytical procedure, and long test durations (weeks to months). There is a pressing need for more sophisticated and informative, yet feasible and reliable, toxic-responsive assessment methods to detect and evaluate the presence and toxicity effects of contaminants in water.

Recent advancements in biotechnology have led to the emerging field of toxicogenomics, in which genomic techniques such as gene expression profiling are used to examine the signaling pathways and the response of multiple genes to toxins; it provides a significant advance in toxin evaluation and understanding toxic mechanisms (*1–3*). A number of recent studies have demonstrated the successful application of gene expression profiling for evaluating toxicity effects of environmental pollutants by use of either microarrays (*1, 2, 4–6*) or cell arrays with green fluorescent protein (GFP), galactosidase (lacZ), or luciferase (Lux) fusions (*7–16*). Several drawbacks associated with the microarray technology limit its wide application in environmental monitoring; they include (a) complex protocol that involves RNA extraction, PCR amplification, and labeling and hybridization; (b) high cost for microarray chip design and manufacture, which is not reusable; (c) results are condition-sensitive due to possible artifacts that can arise during RNA isolation or from cross hybridization processes (*4, 6*); and (d) lack of temporal resolution. It can only produce a snapshot profile at an arbitrarily selected time point and therefore it does not reflect the full picture of the state of genes in response to toxins.

Although gene expressions of genes fused with Lux or GFP has been reported (*7–9, 11–16*), extensive time-series gene expression profiling of a large number of genes using GFP-fused prokaryotic cells has not yet been investigated for environmental toxicity evaluation because of the limited number of recombinant strains available. It was not until very recently that a method for constructing a large number of fluorescent recombinants of prokaryotic strains has become available (*17*). In this study, we have explored a new prokaryotic *real-time* gene expression profiling method for toxicity assessment, employing a comprehensive cell array of transcriptional fusions of GFP to each of 93 different gene promoters in *Escherichia coli* K12, covering most of the known stress response genes. Compound-specific and concentration-sensitive two-dimensional (genes and time) gene expression profiling for MMC and for mercury at various concentrations were obtained. The gene expression alterations as the result of exposure to these two contaminants were revealed and discussed, which provided insights into the underlying toxic mechanisms of these two compounds.

Materials and Methods

Prokaryotic Stress Cell Library with Transcriptional GFP Fusions. A library of transcriptional fusions of GFP (Open Biosystem, Huntsville, AL) that include different promoters in *E. coli* K12 MG1655 was employed in this study. Each promoter fusion is expressed from a low-copy plasmid, pUA66 or pUA139, which contain a kanamycin resistance gene and a fast-folding gfpmut2; this enables measurement of gene expression at a resolution of minutes with high accuracy and reproducibility (*18*). For this particular study, 93 different promoters were selected that control the expression of genes associated with the known stress responses and other specific functions in *E. coli* (Table 1). Our "stress

TABLE 1. Genes Included in the Stress Cell Library for Chemical-Induced Real-Time Gene Expression Profiling Analysis[a]

Category	Genes selected	Known Functions	References	Color code
General stress	*uspA, otsB, ydgL, bolA, rpoE,*	Disturbance of the biochemical and biophysical homeostasis of the cell	[9, 16, 21]	🟨
SOS response/ DNA repair	*uvrA, recX, dinG, ybfE, sbmC, ftsK, recN, yebG, sulA, polB, uvrD, lexA, recA, umuD, ssb, ada, mutT, dinB, nfo*	DNA strand breaks and cross-linking, alter superhelicity, oxidative DNA damage, DNA alkylation, inhibition of DNA synthesis and replication	[4, 9, 21, 22]	🟩
Protein stress	*clpB, ycgE, cueR, entC, grpE, dnaK, fepB, dnaJ, rpoD, lon*	Denaturation, misfolding, cross-linking and alkylation of proteins, oxidation of individual amino acids and protein damage (due to heat and chemicals)	[8, 9, 15, 21, 23]	🟧
Redox stress	*soxS, soxR, oxyR, inaA, dps, ahpF, katG, sodA, ahpC, katE*	Increased levels of superoxides, increased levels of peroxides, any other conditions which alter the redox potential of the cell	[7-9, 21]	🟪
Energy stress/ Electron transport	*sdhC, cyoA*	Perturbations of electron transport and exposure to uncoupling agents, which affect ATP levels in the cell	[24]	⬜
cold shock	*cspA, cspB*	Temperature downshift	[9]	🟪
drug resistance /sensitivity	*amiC, yedW, dacA, dacB, cls, marR, sbmA, bacA, yhjX, emrE, sanA, emrA, marC, mdtK, yajR, fsr, cmr, mrcB, pbpG, ssrA*		[9, 12, 24, 25]	🟩
detoxification	*sodB, nor, fpr, tam, yeiG, yniC, uspB, sodC, gst, zntA, yeaE*	Related to compound /chemical induced killing or stress (ex. response to antibiotic and drugs)		🟦
cell killing	*dinJ, slyA, yeeV, yfjG, relB*			⬜
general function	*phoB, crp, cdaR, ydeO, ybgI, gadX, ompC*		[24]	⬜

[a] See refs *4, 7−9, 12, 15, 16, and 21−25*.

library" can be compared to a low-density cDNA microarray (*19*), having, however, the advantage to record real-time change in gene expression level that can be used to understand and classify toxicants based on their mechanism of action upon the cell. Each category of stress genes and their main functions are briefly described in Table 1.

Environmental Pollutants Evaluated. This study evaluated the toxicity of mercury (Hg^{2+}) and mitomycin (MMC) (Fisher Scientific, Pittsburgh, PA) at concentrations of 0.05, 0.5, and 5 μM for mercury and 1 μM for MMC. Mercury was selected as a representative toxic heavy metal and MMC as a model genotoxin. These two compounds were chosen because their toxic effects are relatively well-known and for both of which some specific genes involved in the toxin resistance have been identified (*1, 20*), therefore allowing us to compare and validate our results with previous findings.

Measuring Temporal Gene Expression under Various Conditions. We inoculated 93 different reporter strains from frozen stocks into LB medium supplemented with 25 μg/mL kanamycin and incubated the cultures for 16 h. The cells were then diluted 1:100 into fresh LB medium supplemented with 25 μg/mL kanamycin to a total volume of 200 μL into individual wells of black 96-well plates (Costar). Cells were grown for about 2 h at 37 °C with shaking in a Microplate Reader (Synergy HT Multi-Mode, Biotech, Winooski, VT) with continuous measurement of the optical density at 600 nm (OD600) every 5 min. When the cultures reached early exponential growth (OD600 about 0.05−0.1), 10 μL of the specific chemical was added per well; the plate was then returned to the microplate reader for absorbance (OD600)

measurements and fluorescence readings (filters 485 and 535 nm) at time intervals of 4 min.

Data Processing and Analysis. *Data Preprocessing.* OD and GFP raw data were smoothed by calculating the simple moving average of every five successive measurements. Both plate background OD and the background GFP expression produced by the promoterless plasmids pUA66 and pUA139 were averaged and subtracted from the GFP measurement for each gene at the corresponding time point in both experimental and control tests. To filter the noise, the GFP measurement was set to zero if it was less than 2 times the standard deviation of the background GFP expression.

Gene Expression Data Preparation. The GFP expression level (P) at each time point for each gene in both control and experimental sets was calculated as the GFP measurement divided by the corresponding OD. The alteration in gene expression, also called induction factor (I), for a given gene and at each time point due to the chemical exposure was represented by the ratio ($I = P_e/P_c$) of the P_e value in the experimental condition to the P_c value in the control condition. Then the natural log of the I value (ln I) at all time points were compiled for further clustering analysis. A more detailed description of the algorithm used for data processing and analysis is presented in the Supporting Information.

Clustering Analysis. For clustering analysis and visualization, we used the microarray software suite MeV (MultiExperiment View) version 4.1 (*26*). The methods of HCL (hierarchical clustering) and SOM (self-organizing maps) were employed. In HCL analysis, the distance metric was set as Euclidean distance and the linkage method was set as average
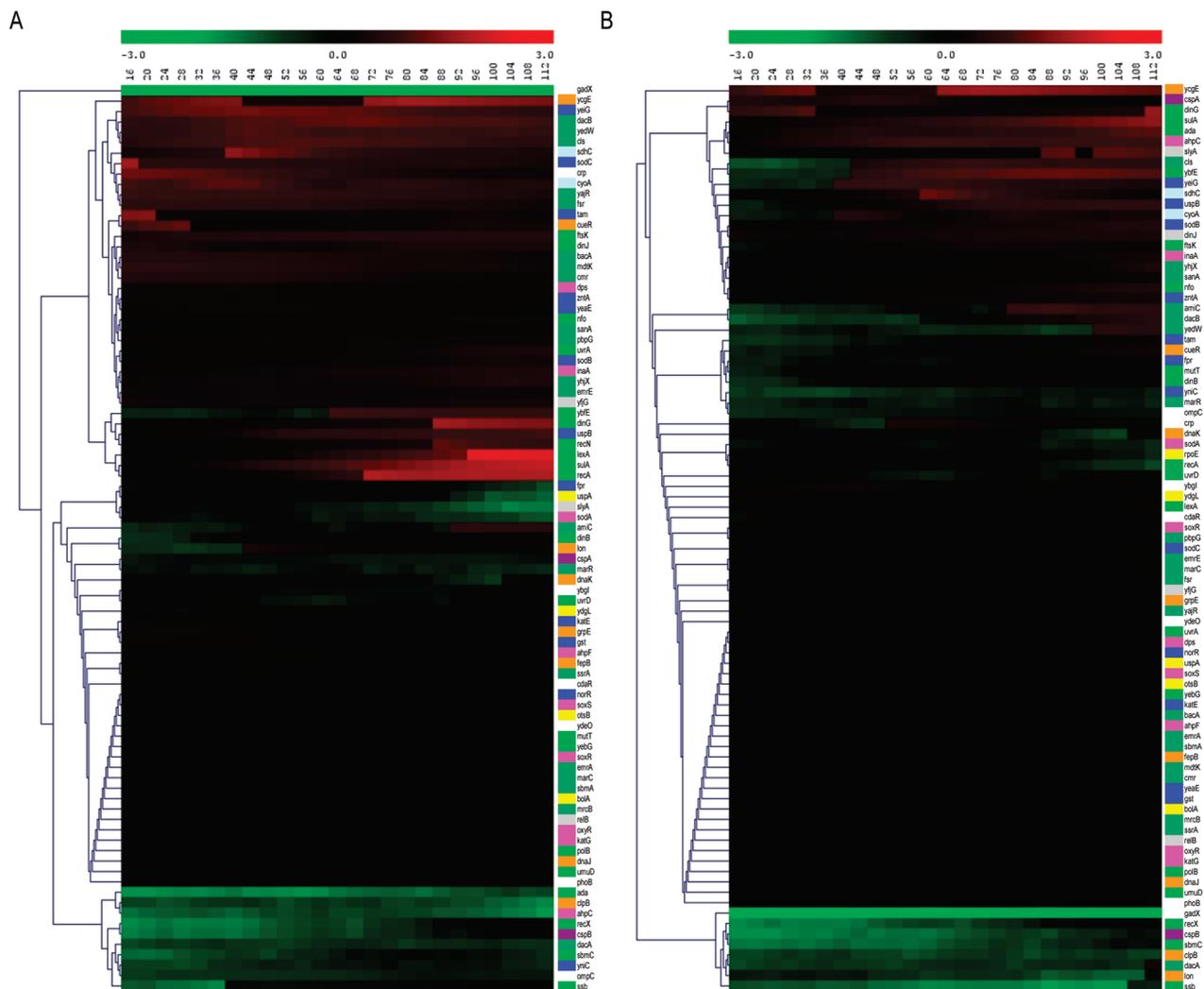
FIGURE 1. Real-time (temporal) gene expression profiles of 93 stress genes in *E. coli* in exposure to (A) MMC (1 μM) and (B) mercury (0.5 μM). (*x*-axis) Natural log of induction factor (ln *I*) (red spectrum colors indicate up-regulation, green spectrum colors indicate down-regulation) and time in minutes (the first data point shown is at 16 min after exposure due to moving average). (*y*-axis left) Clustering of the profiles. (*y*-axis-right) List of genes color-coded on the basis of functional categorization (Table 1).

linkage clustering. The cluster diagram was generated in a hierarchical way. In SOM analysis, the distance metric was set as Euclidean distance, α was set to 0.05, and radius was set to 3.0. Random genes were initiated in clustering and Gaussian neighborhoods, and hexagonal topology were selected. The cluster centroid and expression graph were generated. These two methods used completely independent and unrelated algorithms to classify gene expression data, which allows the results to be compared to find the common gene classification and remove bias specific to each.

Results and Discussion

Complexity and Dynamics of Time-Dependent Gene-Expression Profiles. We evaluated the real-time gene expression of the 93 stress genes in *E. coli* over a period of 2 h of exposure to MMC and mercury. Figure 1 shows the results obtained with (A) 1 μM MMC and (B) 0.5 μM mercury. The data are clustered on the basis of their similarity in the temporal expression pattern via a hierarchical clustering computation method. Toxin-induced real-time gene expressions were very dynamic and complex, with most of the genes exhibiting different patterns and varying magnitudes of expression activities over time. Genes could be clustered into different groups based on their temporal expression patterns, which depend on the roles and time sequence in which they are involved in the stress-response mechanism. From the

time profile, we observed the transient response of some genes and the delayed reaction of some others, in addition to the information that reveals the toxin-specific genes that are either up- or down-regulated in stress compared to the control. The temporal gene expression profiles are compound-specific and they have high resolution for distinguishing different compounds due to the slight differences in the molecular alterations that the compounds cause at various time points. This validates and highlights the advantage of our approach using real-time profiling to gain temporal resolution, in contrast to a possibly biased "snapshot" of the dynamic profiles, such as in DNA microarrays.

Time-Dependent Gene Expression Profiles on Exposure to MMC and Mercury. MMC is a known DNA-damaging agent and has been used in previous studies as a model genotoxic compound (*11, 27, 28*). It is known to induce the SOS regulatory response, which is activated after DNA damage. Our results confirmed the involvement of genes in SOS system, and more importantly, they highlighted the dynamics and complexity of the temporal response patterns of the SOS genes (Figure 1 and Figure S1 in Supporting Information).

The results also revealed a large number of other stress genes besides those associated with SOS system that were induced upon contact with this compound. Involvement of over 1000 genes upon contact with MMC was recently shown
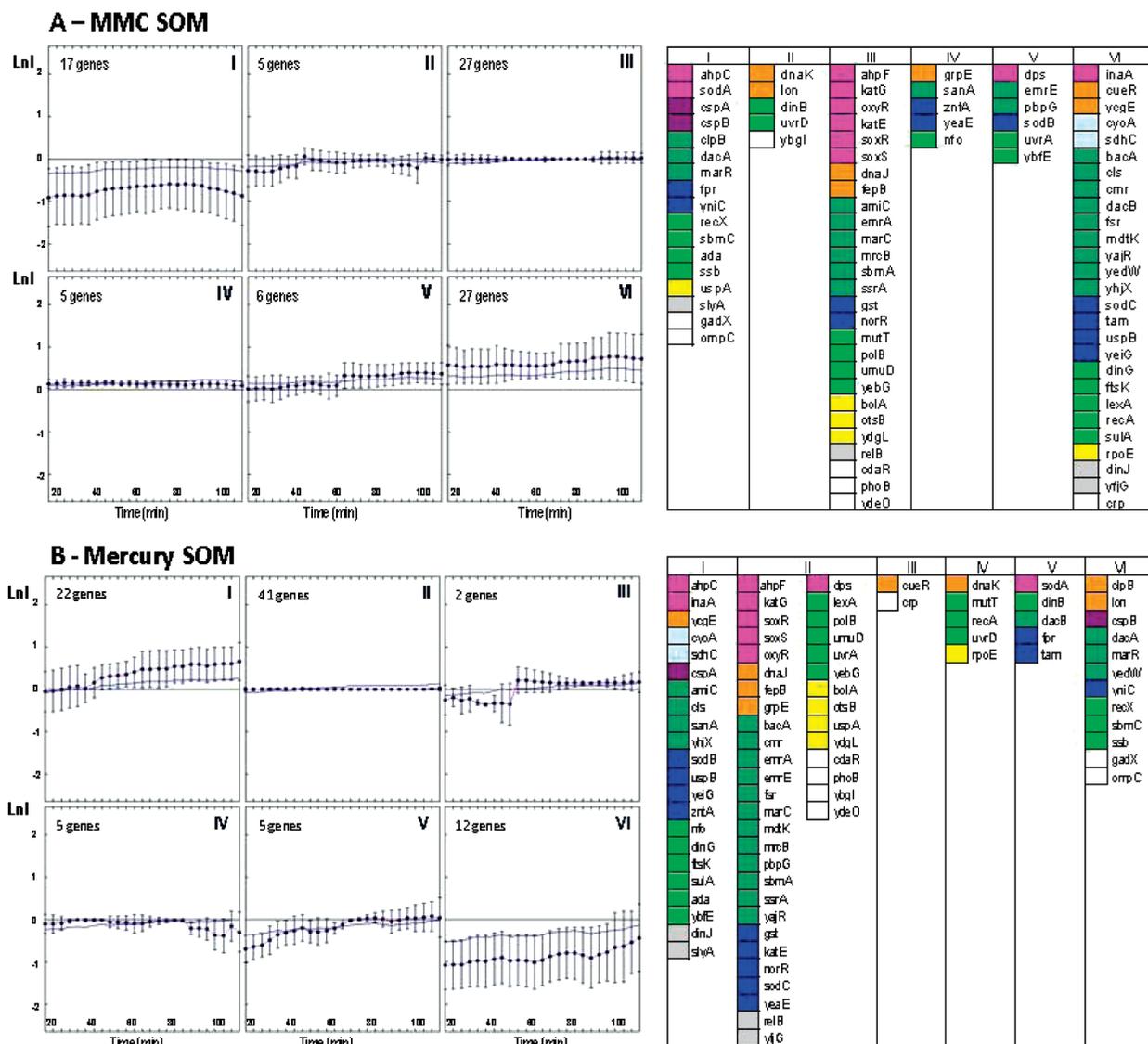
FIGURE 2. Temporal gene expression patterns observed upon exposure to (A) MMC and (B) mercury, determined via self-organizing map (SOM). (x-axis) Time in minutes. (y-axis) Dotted lines represent the average natural log value of gene expression induction factor (ln *I*) for all the genes in each cluster, with standard deviation shown as vertical bars. Values: 0, neutral; >0, up-regulated; <0, down-regulated. Right-hand table lists all the genes in each cluster.

by Khil and Camerini-Otero (*28*) using microarray. With microarray, however, the results are snapshots at arbitrary selected time points, whereas, as Figure 1 and Figure S1 in Supporting Information clearly show, there is a distinct time-dependent response of some genes, which would not have been captured with arbitrarily selected time points. By use of the SOM method, six clusters of genes were identified on the basis of their temporal expression patterns (Figure 2A). Genes in cluster I, for example, are down-regulated, while genes in cluster II transitioned from down-regulated into "neutral" state after about 40 min. In contrast, genes in cluster VI, covering mostly those involved in drug resistance/ sensitivity, SOS DNA repair, and detoxification, remained up-regulated; and genes in cluster V transitioned from neutral to up-regulated. The temporal change in gene expression level reflects the dynamic of the cellular response system and the time sequence for a particular set of gene expression to be altered, which may depend on the system-level multiple gene activation and signaling pathways in an organism.

The mechanisms that living cells have developed to overcome stress induced by the presence of mercury are not completely understood; however, mercury is known to cause modification of thiol groups of proteins, often leading to

inhibition of enzyme function (*29*). It can also alter sulfhydryl groups in cell membranes, causing changes in membrane permeability and transport (*29*). Analyses of the expression profiles of specific genes can hence potentially give some indications on these protective mechanisms, or at least point out the possible stress response pathways that this toxic compound induces in the cell.

For mercury, six clusters of genes were identified (Figure 2B): cluster I include 22 genes that switch from neutral to up-regulated; the genes in cluster II, which comprises nearly 40% of all genes, did not respond to mercury or had expression levels similar to the control; cluster V includes five genes whose profiles show a transition from neutral to down-regulated; and cluster VI, which includes genes of the SOS response as well as functional genes, comprises down-regulated genes.

Toxicity Mechanism of MMC As Revealed by Real-Time Gene Expression. As expected, many of the genes in the SOS system, including *sulA, lexA, yebG, uvrA, dinB, dinG, recA, ftsK, ybfE,* and *nfo,* were up-regulated in the treatment with MMC. The *recX, ssb,* and *sbmC* genes, which are also part of the SOS response, exhibited lower expression levels compared to the control and therefore were classified as down-
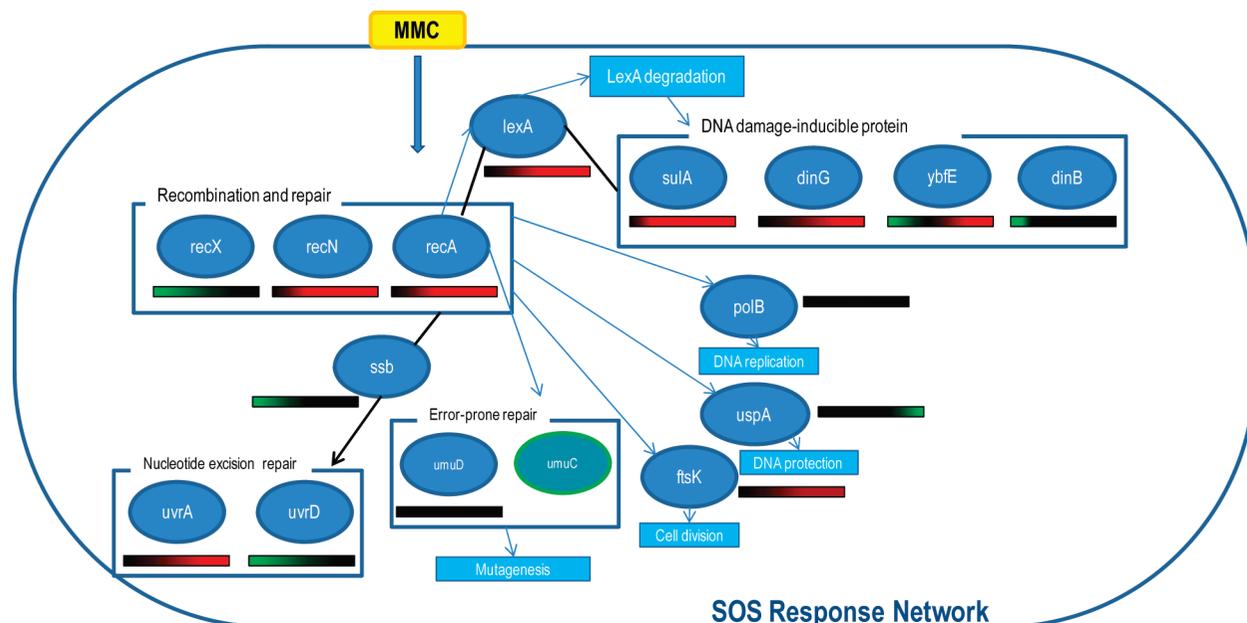
FIGURE 3. Temporally dynamic gene expression in SOS DNA damage repair regulatory network upon exposure to MMC. Black lines indicate pathways in the normal repair process, and blue lines with arrows show activation/induction due to exposure to damaging agents (MMC). For each gene, the temporal gene expression level upon exposure to MMC for a duration of 2 h is shown as a colored bar. Red, overexpressed; green, underexpressed; transitional color, neutral compared to control.

regulated. Down-regulation of the *ssb* gene in the presence of MMC was also found by use of microarray (*28*), while another study observed no change in its expression following UV irradiation (*22*), which also causes DNA damage. The *umuD* gene, which is involved in DNA error-prone repair, was not expressed in exposure to MMC, and this is consistent with observation from Khil and Camerini-Otero (*28*).

Figure 3 shows the interrelationship among key SOS genes and their temporally dynamic gene expression in the SOS DNA damage repair regulatory network upon exposure to MMC. This network map was constructed on the basis of a previous framework (*3*) with the addition of our findings, which revealed variations in the gene expression initiation time and the temporal expression level over time for exemplary SOS response genes. Detailed examination of temporal transcriptional activity of genes involved in the SOS response, including *sulA, recA, lexA, dinG, recX, uvrA,* and *ybfE* (Figure S1 in Supporting Information), revealed not only the expression levels of various genes but also the sequential gene activation and change in the rate of transcription (expression) over time. DNA damage induced by genotoxins (e.g., MMC) initiates the SOS response system by forming RecA (the product of *recA*) and single-strained DNA combinant (RecA/ssDNA), which then stimulates the degradation of LexA (the products of *lexA*), which is a repressor of RecA in the normal repair process. The inactivation of LexA led to the overexpression of genes regulated by *lexA*, including *uvrA, dinG,* and *recN* (*30, 31*). Our results showed that *recA* and *lexA* were overexpressed immediately upon exposure to MMC, while activation of other genes that are regulated by *lexA* were initiated at much later times (16−40 min) (Figure 3 and Figure S1 in Supporting Information). The *uvrA* gene was expressed at less magnitude than the *sulA* gene, as reported by Walker (*30*), and more noticeably, it was activated at a much later time (56 min) than *sulA* (Figure S1 in Supporting Information).

Although we do not yet have enough knowledge to fully explain the interrelationship, roles, and time sequence in which all the genes are involved in the SOS system, the information obtained here will surely contribute to our further understanding of the complex and dynamic regulatory mechanism of the SOS system. Moreover, the high temporal-

resolution measurements can be used to identify new genetic regulatory networks and help quantify the kinetics as previously shown by Ronen et al. (*32*), provided with appropriate algorithms for data analysis.

In addition to SOS response genes, 18 genes of the drug resistance/sensitivity and of the detoxification categories were up-regulated in the presence of MMC. Four drug resistance genes, namely, *cmr, fsr, yajR,* and *yhjX,* produce proteins as members of the major facilitator superfamily (MFS) of transporters. Particularly, the *cmr* gene, also known as *mdfA,* is important for antibiotic resistance, and its product is a multidrug efflux protein whose overexpression confers resistance to a broad spectrum of chemical compounds, including MMC (*33*). The genes associated with drug sensitivity that were down-regulated include the *dacA* and the *marR* genes; the latter, as part of the *marRAB* operon, negatively autoregulates its own expression when associated with multiple antibiotic resistance. Although previous studies have explored the mechanisms that confer multidrug resistance to the *mar* regulon, in those studies MMC was not one of the antibiotics analyzed. Our finding suggests that this gene is likely involved in resistance to MMC as well. The presence of MMC may also affects the ATP level of the cells, as suggested by the overexpression of the two genes associated with energy stress: *cyoA* and *sdhC*.

Other genes up-regulated in exposure to MMC included the *inaA* and *dps* genes of the redox stress category. The Dps protein is usually associated with entrance in stationary phase and it was also found to be involved in the protection of bacterial DNA against oxidative stress (*24*) in presence of MMC (*28*). With a microarray experiment, Khil and Camerini-Otero (*28*) showed varying levels of change in the expressions of the *katG* and *katE* genes (0 to 2-fold) in response to oxidative stress by MMC in different treatments with varying exposure time and concentrations. Another study by Mitchell and Gu (*34*) using Lux biosensor showed no alteration in *katG* gene expression. In our study, we showed unaltered transcription level of the *katG* gene [and also of the *oxyR* gene, which regulates *katG* (*7*)] and a slight temporary overexpression (about 1.2-fold) of the *katE* gene. The differences observed among these studies could be partially due to the different experimental methods and conditions

applied, but it is also possible that temporal variation in the gene expression level contributed to the varying results observed. Functional genes that were down-regulated include transcription (*gadX*, *slyA*), transport (*ompC*), and cell division (*dacA*), indicating an overall suppressed metabolic state due to the toxicity of MMC.

In summary, our findings are in general consistent with MMC toxicity assessment performed by Khil and Camerini-Otero (*28*) using microarray, which validates our methods and confirms that the dominant toxic mechanism of MMC involves the DNA damage repair SOS system and oxidative stress. We showed time-dependent gene expression dynamics among genes, and the results provide insights into the signaling pathways and sequential involvement of genes in a given regulatory network such as the SOS system.

Toxicity Mechanism of Mercury As Reveled by Real-Time Gene Expression. Preventing ion accumulation by active cation efflux and sequestering by small binding proteins are some of the mechanisms that bacteria have developed to tolerate high concentrations of metals (*35*). Out of the 22 genes that were overexpressed in the presence of mercury, eight belong to the drug resistance/sensitivity and detoxification category. In *E. coli*, the gene encoding an ATPase involved in the transport of divalent soft metals, including mercury, is the *zntA* gene (*36*). In our experiment the *zntA* gene was up-regulated upon treatment with mercury, and the activation of this gene was concentration-sensitive (see next section), indicating the possible involvement of the *zntA* gene for detoxifying mercury, as already shown for cadmium and lead (*36*).

Two protein stress (also known as heat-shock) genes, *cueR* and *ycgE*, showed a temporal pattern that ranged from neutral to overexpression. Both of these genes belong to the MerR family, whose regulators are activated in response to stress signals in bacteria, such as that caused by oxygen radicals, heavy metals, or antibiotics (*37*). CueR is a metal-dependent regulator that regulates the mercury resistance (mer) genes, and it has been used with a fluorescent reporter as a biosensor for the detection of a broad range of metal ions, including mercury (*38*). Based on the full genome sequence of *E. coli* K12, YcgE is one of the MerR homologues, but the nature of the inducing signal of the *ycgE* gene has not yet been fully characterized; our results suggest that the presence of mercury induces the expression of this gene, and it might be involved in the process of detoxification of this metal.

Among the SOS response genes, *dinG*, *ftsK*, *sulA*, *ybfE*, and *nfo* genes were overexpressed upon mercury exposure, whereas *recX*, *smbC*, and *ssb* were underexpressed, similarly to that observed with MMC treatment. Also similar to the results with exposure to MMC, the two energy stress genes, *cyoA* and *sdhC*, were up-regulated. This indicates that both MMC and mercury directly or indirectly cause DNA damage and the genes listed above are common to both mercury and MMC toxic response. Particularly, the *nfo* gene is known to respond to oxidative agents such as hydrogen peroxide and other reactive oxygen species (ROS) (*39*). The presence of mercury and other heavy metals is known to induce oxidative stress (*1*), causing toxic effects through the production of peroxides and free radicals that can damage all components of the cell.

The promoters known to respond to redox stress are part of the two regulons *soxRS* and *oxyR*, and both are inactivated in "unstressed" cells (*40*). SoxS facilitates the binding of RNA polymerase to some promoters and it also activates the transcription of several genes, including *nfo* and *ina* (*24*), both of which were overexpressed in our experiment. Of all the genes regulated by the *oxyR* regulon, the *ahpC* gene was found to be up-regulated in our experiment with mercury, suggesting possible production of alkyl hydroperoxide by mercury. The *katG* gene, which remained neutral in our study,
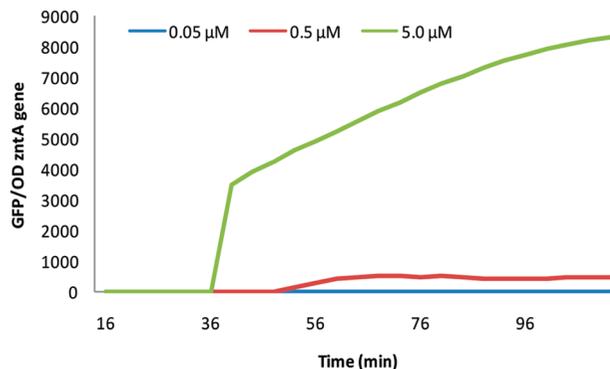

FIGURE 4. Temporal gene expression profiles of *zntA* gene upon exposure to mercury at different concentrations. *y*-axis, GFP fluorescence reading normalized to OD. The expression levels of *zntA* gene for controls were zero for all concentrations examined.

can be induced by H_2O_2 and by other compounds such as phenols and some heavy metals; however, it does not respond to superoxide-generating compounds (*7, 8, 34*). This fact suggests that the oxidative stress induced by mercury is probably caused by the formation of superoxide compounds. Another supporting validation of this hypothesis is the overexpression in our experiment of the *sodB* gene, whose expression is indeed essential for defense against toxic superoxide radicals (*24*).

In summary, examination of the states of various stress genes upon exposure to mercury suggests that DNA damage caused by mercury is likely associated with generation of reactive oxygen species (ROS), such as alkyl hydroperoxide, which in turn lead to redox stress and DNA damage in the cells. Production of superoxide radicals has previously been proposed as one of the mechanisms by which heavy metals, including mercury, cause cell damage (*41*). Genes that are known to be involved in metal toxicity resistance mechanisms via active cation efflux and metal ion sequestering, including *zntA* of P-type ATPase family and genes in the MerR family, were expressed upon exposure to mercury, indicating the activation of these known defense mechanisms.

Sensitivity of Gene Expression Profiles in Exposure to Mercury at Different Concentrations. To evaluate the sensitivity of the stress gene expression profiling for differentiating the toxic response to toxin at different concentrations, three concentrations were tested for mercury (0.05, 0.5, and 5 μM). Distinctive profiles (Figure 1; data for 0.05 and for 5 μM not shown) were obtained with mercury at three different concentrations, indicating that the real-time gene expression profiles are very sensitive and even the slightest changes in the cellular-system response as a result of variation in concentrations of the same toxin, were captured and reflected. At the lowest concentration (0.05 μM) examined, a majority (about 85%) of the stress genes were expressed similarly to those for the control (data not shown); however, as the concentration increased, the number of genes that had alterations in their transcription level increased as well.

As an example, Figure 4 shows time-dependent expression of gene *zntA*, in which both the lagging time before the onset of gene activation and the magnitude of gene expression corresponded with the increase in mercury concentrations. Interestingly, there was a very rapid initial increase in the *zntA* gene expression level that did not occur until approximately 35 min after exposure to mercury, followed by continuous increase in the expression level at a much lower rate. As previously discussed, the *zntA* gene belongs to the P-type ATPase family that is involved in metal homeostasis and confers resistance to toxic concentrations of several heavy metals. In our experiment, expression of *zntA* was marginal

with the lowest concentration ($0.05\,\mu$M), indicating a limited sensitivity in the nanomolar range, as also found by Riether et al. (*12*), who applied a *zntA*-luminescent biosensor to detect mercury as low as 300 nM.

In summary, our results show that prokaryotic real-time gene expression profiling provides multiple layers of information related to cellular metabolic response to chemicals: elucidation of genes that have chemical-induced expression alteration (up- or down-regulation) and the toxic mechanism specific to each compound; revelation of temporal gene expression patterns, such as no change-to-overexpress, overexpress-to-neutral, associated with the time sequence of the gene involvement depending on the response and signaling pathways; and identification of potential markers for response to a specific compound and demonstration of the highly sensitive system-level transcriptional alterations in response to the same compound at different concentration levels. To our knowledge, we are first to show high temporal-resolution measurements of promoter activities of a large number of stress genes in response to environmental toxins. Our results highlight the fact that toxin-induced gene expressions are very dynamic; therefore, selecting an arbitrary time point to obtain a gene expression profile associated with toxin exposure is probably not the best approach to fully understand and identify genes that are involved in the toxicity mechanisms. This was also recognized and pointed out by Kim and Gu (*19*), who found that the gene expression levels were different when two arbitrary end points were selected to examined microarray data, due to the faster or slower response of the various genes to a specific compound.

Detailed and quantitative information on temporal transcriptive activities among a large number of genes could potentially help us recognize previously unknown signaling pathways in a regulatory system, gain further understanding of known regulation networks, and evaluate kinetics and elucidate interactions between different regulons. The sequential activation of key SOS response genes shown in our study more clearly illustrated the previously recognized initial regulatory mechanism of SOS system. A library of limited GFP-infused *E. coli* promoters has already been successfully used to quantify the kinetics of the SOS system (*32*) and some metabolic pathways (*42*).

Our results seem to indicate that prokaryotic real-time gene expression profiling is compound-specific and concentration-sensitive, which could potentially produce multidimensional "fingerprints" that consider gene, time, and concentration as three dimensions. Large amounts of multidimensional gene expression data obtained also pointed out the challenges for data processing, analysis, and interpretation. Further research will include establishing a comprehensive database of gene expression profiling (fingerprinting) over nearly 2000 promoter−fusion genes of *E. coli* (covering most of the genome) in response to a large number of various environmentally relevant pollutants, as well as exploring new computation algorithms and improving data analysis methods to allow for future evaluation, classification, and potentially identification of toxic contaminants on the basis of their underlying toxic mechanisms in water and wastewater samples.

Acknowledgments

Supporting Information Available

Detailed description of data processing method and algorithm for GFP data gene expression induction factor calculation, and a figure showing temporal gene expression profiles for exemplary genes involved in the SOS response upon exposure to MMC. This information is available free of charge via the Internet at http://pubs.acs.org.

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