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Profiling Early Osmostress-Dependent Gene Expression in *Escherichia coli* Using DNA Macroarrays

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DNA macroarray technology was used to monitor early transcriptional alterations of *Escherichia coli* in response to an osmotic upshift imposed by the addition of 0.4 M NaCl. Altered mRNA levels of 152 genes were detected; 45 genes showed increased expression while the expression of the remaining 107 genes was reduced. Northern blot analysis of several selected genes differing in their relative expression values confirmed the results obtained by the array technology.

Mechanisms of adaptation to environments of high osmolality have been investigated by genetic, physiological, and biochemical methods (for reviews, see references 17 and 18). Sudden exposure of *Escherichia coli* to an environment of high osmolality causes rapid loss of water (plasmolysis), loss of turgor, and shrinkage of the cell. Within the first minutes, respiration ceases (46), whereas both the intracellular ATP concentration (51) and the cytoplasmic pH increase (20). Among the first adaptive responses to a hyperosmotic upshift there is a large increase in the rate of uptake and the amount of cytosolic K^+ (20, 23, 47, 53). A number of secondary adaptive mechanisms occur after the onset of increased accumulation of K^+, including the accumulation of glutamate (44), the synthesis of trehalose (12, 57), and the release of putrescine (56). A number of so-called osmoprotectants (e.g., betaine and proline) are taken up by *E. coli* when available externally. These solutes are able to increase the internal osmotic pressure without interfering with vital cellular protein functions (18, 24–26, 48).

There are a few studies in which the general response of *E. coli* to osmotic upshift has been investigated (14, 29). The analysis of two-dimensional gel electrophoresis patterns of radiolabeled total cellular proteins (9, 34) as well as the analysis of global transcription patterns (13) indicated that an increase in osmolality has a global effect on gene expression. It has been shown that in response to osmotic stress, *E. coli* expresses a broad set of normally stationary phase-specific genes whose expression depends widely on RpoS (σ^S), an alternative transcription factor (31, 34, 49).

DNA macroarray measurements. DNA macroarrays were used to profile early osmostress-dependent gene expression. *E. coli* MC4100 (11) was grown at 37°C in phosphate-buffered minimal medium supplemented with 0.4% (wt/vol) glucose (22) containing 10 mM K^+ until the mid-logarithmic phase. Cells were then transferred in fresh prewarmed medium (control cells) or in medium containing 0.4 M NaCl (stressed cells) (37), and after 9 min, total RNA was isolated according to the method of Aiba et al. (1). The obtained RNA samples were treated with RNase-free DNase I (Qiagen). ^{33}P-labeled cDNA was synthesized by using open reading frame (ORF)-specific *E. coli* primers and hybridized to DNA macroarrays (Sigma-Genosys) according to the manufacturer's instructions. Exposed PhosphorImager screens were scanned on a PhosphorImager SI (Molecular Dynamics), and quantification of all 4,290 PCR-amplified ORFs of the *E. coli* K-12 (strain MG1655) genome was performed with the Array-Vision, version 5.1, software (Imaging Research, Inc). Expression signals from each spot were expressed as the percentages of total pixels contributed by all of the gene spots in the array (except the spot signal from the genomic DNA). The background value was determined by averaging 294 individual background spots randomly selected from the entire array membrane. Signals with pixel values that were three or more times greater than the pixel intensity of the background were shown by 72% ± 3% of the genes in the control array and 75% ± 8% of the genes in the stress array.

The criteria used to determine whether a gene was considered to be affected by an osmotic upshift or not were as follows. (i) Only those genes whose average pixel intensities showed the same regulatory trend (up-regulated or down-regulated) in at least two of the three independent experiments and whose values were clearly deviating from the background were considered to be influenced by an elevated NaCl concentration. The calculated expression ratio between stressed cells and control cells resulted in the change. A gene (*i*) was considered to be significantly changed when the change was $\geq \pm 1.4$-fold. This is below the threshold set by the manufacturer, Sigma-Genosys, which is twofold. A lack of correlation between differences (*n*-fold) and significance has been described, and it has been asserted that gene expression measurements cannot be assessed simply by the magnitude of the difference (*n*-fold) between two experimental conditions (4). Furthermore, it is generally important to apply statistical methods to eliminate false-positive or -negative signals that can occur due to differences in RNA preparations or by chance when experiments are only replicated at nominal levels (41). (ii) For these reasons, we performed a significance analysis by using the statistical program SAM (59). Permutations are used to estimate a percentage of genes identified by chance, called the false discovery rate (FDR). The FDR is defined as the percentage of falsely significant genes compared to the genes called significant (for

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TABLE 1. Functional classification of genes affected by osmotic upshift

Functional group[a]	Total no. of genes	No. of genes with expression after osmotic upshift vs control		Gene(s)[b]
		Increased	Decreased	
Complete genome	4,290	45	107	
Amino acid biosynthesis and metabolism	97	2	12	*aroD*, *asd*, *cysK*, *cysM*, *dapB*, *gdhA*, *hisC*, leuD, ***leuC***, ***metC***, *metE*, *proA*, *trpB*, *thrC*
Biosynthesis of cofactors, prosthetic groups, and carriers	106	3	1	*folE*, ***moaC***, ***moeA***, ***ribE***
Carbon compound catabolism	124	0	1	*gatY*
Cell processes (including adaptation and protection)	170	7	10	*acrB*, *ahpC*, *cspA*, ***div***, ***dps***, *ftsK*, *ftsN*, *ftsZ*, ***osmC***, ***osmY***, ***otsA***, ***otsB***, ***proX***, *sufI*, *tig*, *tpx*, *uspA*[c]
Cell structure	85	1	9	*glgA*, *lpp*, ***lpxA***, *kdsB*, *mrcA*, *mrdA*, *murA*, *ompF*, *ompT*, *yccZ*
Central intermediary metabolism	149	3	8	***aceK***, ***cynT***, *cysD*, *cysH*, *cysI*, *cysJ*, *gcvP*, *gltB*, *gltD*, ***metF***, *ydiD*
DNA replication, recombination, modification, and repair	105	4	4	***dfp***, ***himD***, *hupA*, *hupB*, *priB*, ***rnt***, *topA*, ***xerD***,
Energy metabolism	136	0	18	*aldA*, *appC*, *atpC*, *atpF*, *atpH*, *atpI*, *ycdN*, *cyoC*, *fdoI*, *fumB*, *gapA*, *gpmA*, *hyaF*, *nuoF*, *sdhC*, *sdhD*, *sucD*, *yjcU*
Fatty acid and phospholipid metabolism	41	1	0	***arp***
Hypothetical, unclassified, or unknown	1,428	13	5	***bax***, *lomR*, ***ybaD***, ***ybdM***, ***ybdQ***, *ybfN*, *ycgO*, ***yciA***, ***yddX***, ***ydfS***, ***ydiH***, ***yfhJ***, *yfhN*, ***ygaY***, *yhcB*, ***yjbM***, ***yjgH***, ***ymfS***
Nucleotide biosynthesis and metabolism	66	1	4	*carA*, *carB*, *purF*, *pyrB*, ***tmk***
Phage, transposon, or plasmid	91	1	2	*nfrB*, *pspB*, ***ybcC***
Putative cell structure	43	0	0	
Putative enzymes	453	0	1	*ysgA*
Putative factors	67	0	1	*ycbF*
Putative membrane proteins	54	0	1	*yiaT*
Putative regulatory proteins	167	2	1	***ybgS***, ***yegW***, *yohI*
Putative transport proteins	291	0	1	*prlA*
Regulatory function	208	0	3	*fnr*, *phoU*, *relB*
Transcription, RNA processing, and degradation	28	2	0	***rpoS***, ***sbcB***
Translation and posttranslational modification	128	0	21	*ileS*, *prfB*, *rplC*, *rplD*, *rplE*, *rplF*, *rplI*, *rplU*, *rplW*, *rplX*, *rpmI*, *rpsA*, *rpsI*, *rpsJ*, *rpsN*, *rpsR*, *rpsS*, *rpsT*, *slyD*, *thrS*, *trmD*
Transport and binding proteins	254	5	4	***copA***, ***crr***, *cysW*, *fruB*, ***proP***, ***proV***, ***proW***, *ptsN*, *rbsB*

[a] According to references 8, 54, and 58.
[b] Boldface type indicates genes with increased transcriptional levels due to an osmotic upshift.
[c] See Table 2 for a contradictory result.

further details, see reference 59). The whole set of all 4,290 ORFs has passed the SAM test with an FDR of 12% and a SAM threshold tuning parameter of $\Delta = 1.3$, which is set as the threshold of the distance between observed and expected relative differences (59). Genes detected by this analysis and whose expression change was $\geq \pm 1.4$-fold were again analyzed by SAM, with the SAM FDR set to 0%, indicating a high confidence of significance ($\Delta = 2.8$). The complete data set for the genome-wide expression ratios is available online (http://www.biologie.uni-osnabrueck.de/Mikrobiologie/Kdp/Stimulus.html).

This combined approach identified significant changes in gene expression resulting from an osmotic upshift for 152 genes in which the expression of 45 genes was induced and the expression of 107 genes was repressed. Table 1 demonstrates that the genes differently expressed under the investigated condition encode proteins that are distributed over a broad range of cellular function. Fifteen of the genes showing an increased expression value are yet unclassified, of unknown cellular function, or hypothetical.

Northern blot measurements. Out of the 152 genes identified by the macroarray analysis, 14 genes with differing relative difference scores, $d(i)$, *ompC*, and three genes of the *kdpFABC* operon (whose expressions should be induced under the conditions used [37, 52] but were not picked up by the array analysis) were tested by Northern blot analysis (Table 2) (see also http://www.biologie.uni-osnabrueck.de/Mikrobiologie/Kdp/Stimulus.html for complementary material). The same RNA samples (5 μg) were used, and slot blot analysis was performed according to the method described in reference 37. The intensity of each signal was measured by phosphorimaging, and the expression ratio (stressed/control cell ratio) was calculated (Table 2). For the majority of the genes, the results of the two methods are similar. Contradictory results were obtained for only one gene (*uspA*) (see below). Furthermore, induction of genes of the *kdpFABC* operon was only detected by Northern blot analysis. In general, values for induction or repression were much higher in the case of the Northern blots. Although we do not have an explanation for this phenomenon, it might be related to the black box problem that can occur

TABLE 2. Induction of transcripts in response to osmotic upshift as determined by DNA macroarray and Northern blot analyses

Gene	Blattner no.[a]	Fold change determined by:	
		Northern blot analysis[b]	Macroarray analysis[c]
cspA	b3556	−1.7	−1.9
dps	b0812	8.3	1.8
hupA	b4000	−2.0	−1.8
kdpA	b0698	6.1	1.1
kdpB	b0697	2.7	1.1
kdpC	b0696	6.1	1.3
metE	b3829	−5.0	−5.1
ompC	b2215	1.1	1.1
ompF	b0929	−4.2	−6.9
ompT	b0565	−2.0	−3.1
osmY	b4376	6.0	3.0
otsA	b1896	8.1	1.5
proX	b2679	19.9	3.7
rpoS	b2741	3.3	1.4
uspA	b3495	2.1	−1.9
ybdQ	b0607	1.7	1.5
ybgS	b0753	8.1	1.4
yddX	b1481	8.5	1.5

[a] See reference 8.
[b] Northern blots were quantified by PhosphorImager analysis. Changes are intensity values obtained from experimental RNA samples divided by intensity values obtained from control RNA samples. Values are the averages of three independent experiments.
[c] Change values are means of values obtained from two independent experiments.

with reverse transcription. Unsolved RNA secondary structures might prevent equal cDNA synthesis of the transcripts. As discussed earlier, there are also some problems related to the use of ORF-specific primers (4). To be sure that the genes presented here are induced or repressed due to an osmotic upshift rather than resulting from salt-specific effects, we also used sorbitol to increase osmolality and obtained comparable results (data not shown).

Genes known to be involved in osmoadaptation. Significant hyperosmolal transcriptional regulation of *proP*, which encodes a permease for osmoprotectants like glycine, betaine, and proline (25, 26, 39, 45), was found with the macroarray technique.

Cells exposed to hyperosmolal conditions are characterized by an altered ratio of the porins OmpF and OmpC, whereby the expression of *ompF* is repressed and that of *ompC* is induced (52). Whereas repression of *ompF* was found by gene array and Northern blot analyses, induction of *ompC* was not detectable (Table 2). There is probably a delayed *ompC* induction, a phenomenon which has been observed earlier (36).

kdpFABC encoding the K^+ uptake system KdpFABC is known to be induced after an osmotic upshift (21, 42). Transcripts have already been detected in cells that were exposed to an osmotic upshift for 10 min (37). We confirmed this for three (*kdpA*, *kdpB*, and *kdpC*) of the four genes of the operon by Northern blot analysis (Table 2). However, the gene array analysis failed to identify these genes. It also has to be mentioned that a reverse transcription-PCR approach failed to determine alterations of *kdpFABC* expression (data not shown). Because both methods rely on cDNA synthesis, secondary RNA structures might be a problem in case of the *kdpFABC* operon. Cross-hybridization with cDNA transcripts other than *kdp* seems to be relevant, too, because high signal intensities, especially for *kdpA*, have been reported for gene arrays applied to various cultivation conditions which do not induce *kdp* at all (6, 58).

σ^S-dependent genes. A number of genes whose expression was found to be up-regulated are regulated by σ^S (RpoS). This confirms the earlier observation that some, but not all, σ^S-dependent genes are induced by changes in osmolality in exponentially growing cells (31). Our analysis revealed significant induction of the genes *otsA* and *otsB*, which are responsible for the de novo synthesis of trehalose (33), a compatible solute for *E. coli* to cope with variations in changes of osmolality (Tables 1 and 2).

The *proU* operon, consisting of the genes *proV*, *proW*, and *proX*, encodes a multicomponent ABC transport system involved in the uptake of glycine, betaine, and proline, which are important as compatible solutes during osmotic stress (16). The macroarray analysis revealed an increased expression of the complete *proU* locus with an induction between 1.8- and 3.7-fold, as demonstrated before (7). The enhancement of *proX* transcripts was confirmed by Northern blot analysis, for which the highest change was determined (Table 2).

The *dps* gene encodes a nonspecific DNA-binding protein which is directly responsible for the protection of DNA against oxidative stress (43), nucleases, and other stressful conditions (2). Under our test conditions, induction of *dps* (*pexB*) was found with the macroarray analysis and the Northern blot technique (Tables 1 and 2). Osmotic induction of *dps* transcription has been described as a rapid process, as previously reported for several other σ^S-dependent genes (34). Positive transcriptional regulation by σ^S has been reported for *dps*, which is part of the *oxyR* regulon and is activated in the stationary phase by σ^S and the integration host factor, representing a global regulator encoded by *himA* and *himD* (3, 5, 40). The latter gene was also found to be up-regulated after the osmotic upshift (Table 1).

We found a significant induction of *osmC*, an osmotically inducible gene that is a member of the *rpoS* regulon (10, 15, 30). Although the exact biochemical function of the envelope protein OsmC remains unclear, recent data indicate that it participates, directly or indirectly, in the defense against oxidative compounds (15). Interestingly, we observed an increased expression of an ORF (*yddX*, Blattner no. b1481) that directly maps at 33.5 min on the *E. coli* genetic map between *osmC* and *rpsV*. *rpsV*, which encodes a small ribosomal protein, has recently been described as stationary phase inducible and is partly under the control of σ^S and the integration host factor (35). Under our test conditions, the expression of *rpsV* was unchanged.

Promoter activity of *osmY* (*csi-5*), which encodes a periplasmic protein, was previously shown to be stimulated by growth-phase or starvation signals or by increased osmolality (34, 60, 62). *osmY* expression was increased 3.0-fold on the macroarrays, and a 6.0-fold increase was observed on Northern blots (Table 2).

RpoS. Although it has been previously stated that the transcriptional level of *rpoS* remains the same for at least 90 min in response to the presence of 0.3 M NaCl, as demonstrated by a chromosomal single-copy *rpoS*::*lacZ* fusion (34), we found a

1.4-fold change and the highest SAM score of $d(i) = 43$ in our macroarray experiments. Northern blot analysis confirmed these results (Table 2). The apparent contradiction of our results and the earlier observations could be due to the selected time point of the measurement (9 min after the upshift in our experiments and 15 min after the upshift in the earlier experiments) and the higher osmotic stress (0.4 M NaCl compared to 0.3 M NaCl). It seems likely that in addition to the posttranscriptional regulation and the regulation of σ^S stability (49), *rpoS* transcription is increased shortly after osmotic upshift.

UspA. A significantly increased transcriptional level of *uspA* was found by Northern blot analysis (2.1-fold), whereas the macroarray analysis revealed a decreased expression (1.9-fold) (Table 2). The reasons for this discrepancy are unknown; however, *uspA* expression is probably one example of falsely identified genes by the macroarray method. *uspA*, which encodes the small, cytoplasmic protein UspA (universal stress protein A), is induced to survive prolonged periods of complete growth inhibition caused by a variety of diverse stresses, including $CdCl_2$, H_2O_2, 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone exposure, and osmotic shock (50). Although growth is not completely inhibited at moderate NaCl concentrations (0.4 M), it is conceivable that *uspA* is already induced.

Other induced genes. Other genes whose expression were increased under the tested conditions (*aceK*, *arp*, *copA*, *crr*, *cynT*, *dfp*, *div*, *leuC*, *lpxA*, *metC*, *metF*, *moaC*, *moeA*, *ribE*, *rnt*, *sbcB*, *tmk*, and *xerD*) are distributed over the entire *E. coli* chromosome. They seem to be unrelated to osmoadaptation thus far and belong to various functional groups, 15 genes are of yet unknown function (Table 1). An online database search for common transcriptional units with RegulonDB (55) gave no result. The finding that not all genes of common operons (e.g., *cynTSX*, *leuABCD*, *moaABCDE*, and *moeAB*) are induced or regulated in opposite directions (*leuC* and *leuD* of the *leuABCD* operon) could probably be explained by the use of ORF-specific primers instead of random hexamers, as the latter prevent large signal differences (4). A new BLAST search revealed homologies of gene *ymfS* (Blattner no. b1155) to a protein family carrying a conserved domain of unknown function (DUF144 domain). Interestingly, this gene is also induced by autoinducer 2-stimulated quorum sensing in *E. coli* (19). Moreover, the YbdQ protein belongs to the universal stress protein domain family whose members are induced by a wide range of stress conditions (28).

Gene repression. According to the data known so far, examination of global regulation of gene expression has revealed only a narrow relationship between the stationary-phase expression profile and the osmotic stress response. It has been proposed that osmotic shock may mimic cells entering the stationary phase (13). Adaptation to high osmolality of the environment occurs stepwise. The early phase is characterized by growth arrest. Cell division is restored after about 1 h (61). Since our studies investigated the early response, the pattern of repressed genes reflects more or less a general down-regulation of central metabolic pathways combined with a decreased transcriptional gene expression encoding ribosomal proteins (Table 1). It is known that faster-growing cells synthesize protein faster and that the cellular content of ribosomes correlates to the growth rate (27, 38). Several genes encoding components of the 50S and 30S ribosomal subunits show significant down-regulation (Table 1). This has already been observed in the expression analysis of *E. coli* growing in minimal media compared to that growing in rich media (58). The intracellular concentration of σ^S strongly increases under several tested starvation conditions, e.g., the lack of amino acids (32). Several genes (11% of total decreased genes) encoding amino acid biosynthesis enzymes are significantly repressed under conditions of high osmolality (Table 1), which could be an additional effect triggering *rpoS* expression and probably underlines the slow growth rates of *E. coli* under osmotic stress. This concerns genes involved in methionine (*metE*), leucine (*leuD*), proline (*proA*), threonine (*thrC*), tryptophan (*trpB*), lysine (*asd* and *dapB*), cysteine (*cysK* and *cysM*), glutamate (*gdhA*), and histidine (*hisC*) biosynthesis. The fact that transcription of the tRNA synthases (*ileS* and *thrS*) is significantly decreased under osmotic stress is consistent with the notion that synthesis of tRNA synthetases is coupled to the synthesis of other ribosomal components (27). Furthermore, a decrease in cell growth probably goes hand in hand with down-regulation of genes of the cell division apparatus (*ftsK*, *ftsN*, and *ftsZ*) (Table 1), implying delayed cell division. Decreased transcription of genes encoding the F_1/F_0-ATP synthase (*atpC*, *atpF*, *atpH*, and *atpI*) presumably explains the severe inhibition of respiration as a consequence of osmotic stress (46).

Conclusions. The osmotic upshift of *E. coli* evokes a highly complex regulatory process involving genome-wide expression changes of functionally different groups of genes. These genes are part of global adaptive response processes in which expression of anabolic genes and transport systems responsible for de novo synthesis or uptake of compatible solutes participate. The rapid induction and repression of a multiplicity of genes obtained within only a few minutes of osmoadaptation demonstrates well the rapid and complex adaptive process of *E. coli* exposed to osmotic stress. The observation that several genes of central metabolic pathways combined with a set of genes encoding protein components of the protein biosynthesis apparatus are repressed parallels the reduced growth rate of *E. coli* under hyperosmotic stress conditions.

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