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Time-Dependent Proteome Alterations under Osmotic Stress during Aerobic and Anaerobic Growth in *Escherichia coli*

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Escherichia coli **lives in the mammalian gastrointestinal tract anaerobically at high osmolarity as well as in the soil aerobically at varying osmolarities. Adaptation to these varying environmental conditions is crucial for growth and survival of *E. coli*. Two-dimensional protein gels were used to visualize global time-dependent changes (10 to 60 min) in the proteome of cells responding to osmotic stress (0.4 M NaCl or 0.7 M sorbitol) under aerobic or anaerobic conditions. The protein profiles revealed an induction of 12 proteins (Dps, HchA, HdhA, InfB, OsmC, OsmY, ProX, KatE, PspA, TalA, TktB, and TreF) under osmotic stress in an aerobic milieu. Eleven additional proteins (OtsB, YceI, YciE, YciF, YgaU, YjbJ, AcnA, MetL, PoxB, Ssb, and YhbO) were induced by osmotic stress imposed by NaCl. Most of the accumulated proteins were cross-protecting proteins (e.g., OsmY, OsmC, Dps, and KatE) which are regulated at the transcriptional level predominantly by RpoS and other regulators (e.g., integration host factor, OxyR, H-NS, LRP, and FIS). Comparative analysis of the proteome of *E. coli* grown under aerobic or anaerobic conditions under osmotic stress (NaCl) revealed an overlap of the up-regulated proteins of more than 50%. Ten proteins (PoxB, AcnA, TalA, TktB, KatE, PspA, Ssb, TreF, MetL, and YhbO) were detectable only under aerobic, high-osmolality conditions. Time-dependent alterations of the proteome were monitored, allowing classification of the up-regulated proteins into early, middle, and long-term phases of adaptation. Only a few proteins were found to be down-regulated upon osmotic stress.**

Bacteria such as *Escherichia coli* are remarkably adaptable organisms. They are able to survive and multiply in diverse and sometimes hostile environments. In the gastrointestinal tracts of mammals, bacteria are faced with high osmolality and anaerobic conditions (50). Although *E. coli* is a common gut inhabitant and is found in the gastrointestinal tracts of virtually all mammals, it is usually outnumbered 100-fold or more by strictly anaerobic gut bacteria (13, 61, 64). *E. coli* is a facultative anaerobic bacterium which can use alternative electron acceptors, such as nitrate or fumarate, for anaerobic respiration. When these acceptors are absent, *E. coli* switches to mixed-acid fermentation (18). In the soil, *E. coli* grows aerobically and faces varying osmolarities.

Mechanisms of adaptation to environments of hyperosmolarity have been studied intensively by genetic, physiological, and biochemical methods (11, 64). Exposure of *E. coli* to high osmolality results in rapid loss of water (plasmolysis), loss of turgor, and shrinkage of the cell. Within the first minutes, respiration ceases (42), whereas both the intracellular ATP concentration (47) and the cytoplasmic pH (12) increase. Among the first adaptive responses to hyperosmolarity is a large increase in the uptake rate and the amount of cytosolic potassium (12, 15, 43, 49). After the initial accumulation of K^+, several secondary adaptive mechanisms occur, including the accumulation of glutamate (41), the synthesis of trehalose (7, 60), and the release of putrescine (54). When available externally, a number of osmoprotectants (e.g., glycine betaine and proline) are taken up mainly via two osmotically regulated permeases, ProP and ProU, to increase the internal pressure in *E. coli* (19–21, 44).

There have been several studies in which the general response of *E. coli* to osmotic upshift was investigated. Clark and Parker (10) identified only three major osmotic-upshift-induced proteins by two-dimensional gel electrophoresis. Using Tn*phoA* insertion mutagenesis, 37 genes coding for proteins of the cell envelope were found (22). Among them was *ompC*, whose expression is under control of the sensor kinase/response regulator system EnvZ/OmpR (25). The analysis of two-dimensional gel electrophoresis patterns of radiolabeled total cellular proteins (5, 28), as well as the analysis of global transcription patterns (9, 63, 64), indicated that an increase in osmolality has a global effect on gene expression. During osmoadaptation, *E. coli* expresses a broad set of overlapping stationary-phase or acid stress-induced genes whose expression depends widely on RpoS (σ^S), an alternative sigma factor (26, 28, 46, 64). All of these studies were done exclusively with aerobically grown *E. coli*.

To understand the regulatory network involved in osmoadaptation, we studied time-dependent global alterations in the proteome of *E. coli* under osmotic stress imposed by a neutral solute (sorbitol) or a salt (NaCl). We further investigated whether there are differences in the protein pattern when *E. coli* faces osmotic stress under aerobic or anaerobic conditions. Anaerobically grown cells clearly face energy limitation. Therefore, their osmotic response might be different; for example, synthesis and/or transport of compatible solutes is an energy-consuming process.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* MG1655 (3) was grown aerobically in a 5-liter fermentor at 37°C in phosphate-buffered minimal medium supplemented with 0.4% (wt/vol) glucose (14) containing 10 mM K^+ until the mid-exponential phase (optical density at 600 nm [OD_{600}] of 0.6). At this point NaCl or sorbitol was added to the medium, reaching a final concentration of 0.4 M or 0.7 M, respectively (stressed cells; 0.95 osmol/kg), or the cells were left untreated (unstressed control cells; 0.26 osmol/kg). The hyperosmotic stress response under anaerobic growth conditions was monitored in cells which were cultivated in the same medium containing methylene blue (2 mg · $liter^{-1}$) as a redox indicator and which were preadapted overnight to anaerobic conditions. Before the fermentor was inoculated with *E. coli*, the medium was treated with nitrogen for 5 min. The partial pressure of oxygen and the pH of the medium were followed during the whole experiment by using oxygen and pH electrodes. Hyperosmotic stress was induced as described above at the exponential growth phase (OD_{600} of 0.3), whereas control cultures were left untreated. All experiments were done in duplicate.

Measurement of osmolality. The osmolality of the cultivation medium was measured by freezing point depression (Gonotec Osmomat 030) according to the manufacturer's instructions.

Preparation of crude cell extract. Samples (200 ml) were taken directly before and 10, 30, and 60 min after hyperosmotic stress (under aerobic or anaerobic growth conditions) and were treated with chloramphenicol (1 mg · ml^{-1}), harvested by centrifugation (10 min at 4,500 × *g* and 4°C), and washed with 1/3 volume of buffer W (100 mM Tris-HCl, pH 7.5; 0.1 mg · ml^{-1} chloramphenicol). Cells were disrupted by sonication on ice in buffer D (10 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 50 μg · ml^{-1} RNase; 50 μg · ml^{-1} DNase; 100 μg · ml^{-1} lysozyme; 1.39 mM phenylmethylsulfonyl fluoride), and the crude protein extract was separated from unbroken cells or cell debris waste by centrifugation (10 min at 11,000 × *g* and 4°C) and stored at −80°C. Protein was determined according to a modified Lowry method (48), using bovine serum albumin as standard.

Resolution of whole cell extracts by two-dimensional (2D) polyacrylamide gel electrophoresis. Isoelectric focusing was carried out using immobilized pH gradient strips with a linear pH gradient between 4 and 7 (13 cm) on a IPGphor system (GE Healthcare). Three hundred micrograms of protein of the whole-cell extract was rehydrated for 19 h (20°C and 50 μA [constant]) in 250 μl of buffer R {8 M urea, 2 M thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1.25% (vol/vol) immobilized pH gradient buffer (pH 4 to 7); 28.4 mM dithiothreitol} and was focused for 19,650 V · h. Equilibration of the strips was carried out two times at room temperature (15 min) in buffer E (50 mM Tris-HCl, pH 6.8; 6 M urea; 30% [vol/vol] glycerol; 4% [wt/vol] sodium dodecyl sulfate; 18.2 mM dithiothreitol), followed by second-dimension analysis using 13% resolving gels with 7% stacking gels as described previously (35). Gels were run overnight at 5 mA (constant) and 15°C. Gels were Coomassie blue stained for 1 h with CS solution (40% [vol/vol] ethanol, 10% [vol/vol] acetic acid, 0.2% [wt/vol] Coomassie brilliant blue R250) and destained for 30 min with CD solution (40% [vol/vol] ethanol, 10% [vol/vol] acetic acid), followed by an incubation in 10% (vol/vol) acetic acid.

Stained gels were scanned with an Epson Expression 1689 Pro scanner in transmission mode with a resolution of 300 dpi. 2D protein patterns were analyzed using the PDQuest V7.3.1 (Bio-Rad) software. Only those protein spots exhibiting at least a twofold higher or lower level under osmotic stress compared to the control cultures in two independent experiments were considered to be altered under the tested conditions.

Protein identification using MALDI-TOF MS. Proteins with altered amounts in comparison to the control were cut out of the stained 2D gels, washed in distilled water (four times for 30 min each at 37°C) and then treated with 50% (vol/vol) acetonitrile (two times for 15 min each at 37°C). Tryptic digestion of the proteins was done in 40 mM ammonium bicarbonate solution overnight at 37°C. After desalting of the tryptic peptides with ZipTip μ-C18 columns (Millipore), the peptides were directly eluted with 1 μl matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% [vol/vol] acetonitrile–0.6% [vol/vol] trifluoroacetic acid) and analyzed on a matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS) system (Voyager DE STR; Applied Biosystems) using the reverse mode with a range of 700 to 3,500 Da. Calibration of the peptides was done using the known internal autolysed products of trypsin. The MASCOT database search algorithm was used for identification of the proteins.

RESULTS AND DISCUSSION

We investigated time-dependent changes in the proteome of *E. coli* K-12 strain MG1655 during adaptation to hyperosmolality under aerobic and anaerobic growth conditions. Bacteria were cultivated in minimal medium, and external osmolality was rapidly increased by the addition of iso-osmolal amounts of sorbitol (0.7 M) or NaCl (0.4 M) during mid-logarithmic growth. Samples were taken before the stress and 10, 30, and 60 min after setting the stress. Other growth parameters, such as oxygen pressure, temperature, and pH, were monitored and held constant during the investigated time.

With our gel system we were able to separate and detect about 600 proteins of *E. coli*, with a pI range of 4 to 6.5 and a molecular mass range of 5 to 100 kDa (Fig. 1). Due to methodology restrictions, the number of membrane-attached or membrane-integrated proteins was rather small. Proteins were considered to be involved in osmoadaptation when their mean spot intensities were at least two times higher than those in the untreated control and when a continuous regulatory trend was observed within the tested time. Protein spots of interest were cut out, digested with trypsin, and analyzed by MALDI-TOF MS.

Proteins involved in adaptation to osmotic stress under aerobic growth conditions. Twelve identified proteins were found to be accumulated in response to osmotic stress (Table 1; Fig. 1), regardless of the chemical nature of the osmolyte used to increase osmolality. Two of these proteins typically accumulate in cells exposed to high external osmolality, as indicated by their names OsmY and OsmC, although their function is still unknown (23, 63, 69). Transcription of both genes is regulated by the alternative sigma factor RpoS (σ^S) (17, 68). Although the exact cellular role of OsmC needs to be explored, it was reported that this protein might be important for the cross-protection against different stress conditions (64). The resolved crystal structure shows similarities to peroxiredoxines (57). Peroxiredoxines provide protection against reactive oxygen derivatives. Similarly to plants (56), these derivatives might accumulate in *E. coli* under osmotic stress.

Dps, another protein with a protecting function, was found to be up-regulated after an increase of extracellular osmolality. Dps, a ferritin-like protein, unspecifically binds to DNA and protects it against oxidative stress (39), nucleases, and other stressful conditions (1). Interestingly, Dps was found either in one protein spot (with sorbitol) or in three separate protein spots (with NaCl) on the 2D gels. According to the predicted pI and relative molecular size, Dps isoform 1 (Fig. 1) represents the unmodified protein, while additional Dps isoforms accumulated that had an altered pI (isoform 2) or a lower molecular mass (isoform 3). Isoforms 2 and 3 might represent the phosphorylated (16) and proteolytically modified (59) forms of Dps, respectively.

Synthesis or uptake of compatible solutes is one of the most important responses of cells against osmotic stress (66). ProX is the periplasmic binding protein of the ABC transporter ProU, an uptake system for glycine betaine and proline, two major osmoprotectants in *E. coli* (24). ProX was identified by MALDI-TOF MS in two separate spots distinguishable by a shift in the theoretical isoelectric point of the protein (Fig. 1, ProX isoform 2).

HchA or Hsp31 (YedU), a chaperone, was significantly in-

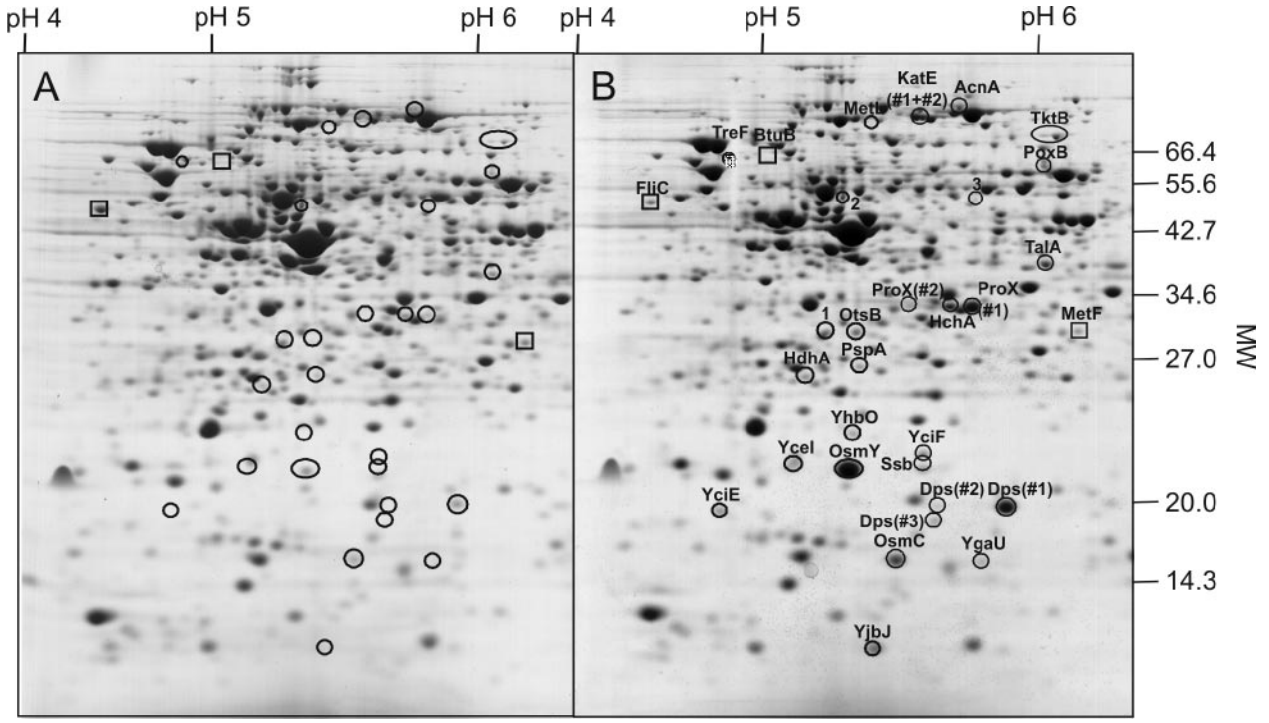


FIG. 1. Protein profiles of *E. coli* MG1655 immediately before (A) and 30 min after (B) the addition of NaCl (0.4 M) under aerobic growth conditions. Crude cell extract prepared as described in Materials and Methods was separated in a pH range of 4 to 7. Proteins with at least twofold changes in amount were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry. Proteins with increased concentrations after NaCl upshock are circled. Proteins with decreased concentration are marked by boxes. Spots that represent protein mixtures are numbered. MW, molecular weight, in thousands.

creased (3-to 11-fold) in stressed cells. At elevated temperatures Hsp31 interacts with partially and fully denatured proteins. Upon a decrease in temperature, Hsp31 dissociates from renatured proteins (38, 52). A similar function can be assumed for this protein under conditions of increased osmolality. Recently, a direct or indirect regulation of the corresponding gene by RpoS was reported (64).

The protein HdhA, a NAD^+-dependent α-hydroxysteroid

TABLE 1. Proteins accumulating in response to both 0.7 M sorbitol and 0.4 M NaCl under aerobic and anaerobic growth conditions

Protein	Description	Induction ratio (stressed cells/unstressed cells) after 60 min			Regulation at the transcriptional level[a]
		Aerobic growth		Anaerobic growth with 0.4 M NaCl	
		With 0.4 M NaCl	With 0.7 M Sorbitol		
OsmY	Periplasmic protein	16.6 ± 2.9	4.6 ± 0.6	5.6 ± 2.0	RpoS (+), Lrp (−), Crp (−), IHF (−)
OsmC	Osmotically inducible protein	13.0 ± 0.7	15.9 ± 2.0	3.1 ± 0.3	RpoS (+), Lrp (+), RcsB (+), NhaR(+)
Dps (isoform 3)	Stress response DNA-binding protein with ferritin-like domain	6.6 ± 1.6	2.7 ± 0.4	2.9 ± 0.1	RpoS (+), OxyR (+), IHF (+)
ProX (isoform 1)	Glycine-betaine-binding periplasmic protein	9.3 ± 0.1	10.0 ± 0.4	15.6 ± 1.4	H-NS (−)
HchA	Hsp31	10.9 ± 1.1	6.4 ± 3.5	3.1 ± 0.1	**RpoS (+)**
HdhA	Alpha-hydroxysteroid dehydrogenase, NAD dependent	5.3 ± 1.4	3.3 ± 1.3	3.2 ± 1.0	**RpoS (+)**
InfB	Protein chain initiation factor IF-2	2.8 ± 0.1	2.9 ± 0.5	3.7 ± 0.1	Fis (+), CRP (−), ArgR (−)
PspA	Phage shock protein A	3.3 ± 0.5	2.4 ± 0.2	1.2 ± 0.1	PspF (+), IHF (+)
KatE	Hydroxyperoxidase II	4.0 ± 1.7	3.8 ± 2.0	0.6 ± 0.1	RpoS (+), Lrp (+)
TreF	Cytoplasmic trehalase	20.3 ± 2.1	25.3 ± 0.9	1.0 ± 0.1	**RpoS (+)**
TalA	Transaldolase A	3.5 ± 0.5	2.1 ± 0.4	1.5 ± 0.3	RpoS (+), CreBC (+), **FNR (−), ArcA (−)**
TktB	Transketolase II	4.1 ± 0.6	6.8 ± 1.9	1.1 ± 0.1	**RpoS (+)**

[a] Data are from references 34, 61, and 64. Boldface indicates indirect regulation. IHF, integration host factor.

TABLE 2. Proteins accumulating only in response to salt stress (0.95 osmol/kg) under aerobic and anaerobic growth conditions

Protein	Description	Induction ratio (stressed cells/unstressed cells) after 60 min, with 0.4 M NaCl		Regulation at the transcriptional level[a]
		Aerobic growth	Anaerobic growth	
YciE	Conserved protein	220.4 ± 58.2	100.2 ± 28.7	ND[b]
YciF	Conserved protein	166.4 ± 21.4	71.7 ± 30.7	**RpoS (+)**
YjbJ	Highly abundant protein	380.5 ± 28.2	270.6 ± 25.9	RpoS (+)
YgaU	Conserved hypothetical protein	3.4 ± 0.3	2.8 ± 0.2	RpoS (+), Lrp (+)
YceI	Periplasmic protein, possibly secreted	184.7 ± 73.1	110.7 ± 32.2	ND
OtsB	Trehalose-6-phosphate phosphatase	4.5 ± 0.8	2.3 ± 0.4	RpoS (+), Lrp (+)
AcnA	Aconitate hydratase I	4.0 ± 0.4	1.4 ± 0.4	FNR (−), ArcA (−), CRP (+)
PoxB	Pyruvate dehydrogenase/oxidase	2.8 ± 0.3	1.1 ± 0.4	RpoS (+), SoxS (+), MarA (+)
MetL	Aspartokinase and homoserine dehydrogenase II	14.7 ± 2.1	1.0 ± 0.2	MetJ (−)
YhbO	Putative intracellular proteinase	101.7 ± 10.5	1.8 ± 0.0	**RpoS (+)**
Ssb	Single-stranded binding protein	36.0 ± 7.0	1.0 ± 0.3	LexA (−)
Mixture 1, MinD/OtsB	Septum side-determining protein/trehalose-6-phosphate phosphatase	2.7 ± 0.5	1.0 ± 0.1	ND/RpoS (+), Lrp (+)
Mixture 2, RfaE/GadA/GadB	Putative kinase/glutamate decarboxylase A and B	3.3 ± 0.9	1.0 ± 0.2	ND/GadW (−), HNS (−), CRP (−), GadX (+), GadE (+)/GadW (−), GadX (+)
Mixture 3, HflX/GorA	Putative kinase, possible regulator of HflKC/glutathione reductase	17.3 ± 2.5	1.0 ± 0.1	ND/ND
AhpC	Alkyl hydroperoxide reductase	1.4 ± 0.8	3.7 ± 0.6	MetJ (−), OxyR (+)
OmpC	Outer membrane porin C	ND	2.8 ± 0.8	Lrp (−), EnvZ (+), OmpR (+)
Mixture 4, HemL/FruB	Glutamate-1-semialdehyde aminotransferase/phosphotransferase system, fructose-specific IIA/FPr component	1.9 ± 0.5	2.2 ± 0.3	ND/FruR (−)

[a] Data are from references 34, 61, and 64. Boldface indicates indirect regulation.
[b] ND, not determined.

dehydrogenase, also accumulated after osmotic upshock. HdhA catalyzes the dehydroxylation of bile acids (70). By dehydroxylation, bile acids loose their detergent properties. Increased osmolality might serve as a general signal for a preadaptation of enterobacteria to the conditions of the gut.

In response to external osmolality, InfB, the initiation factor 2 of protein synthesis, was raised and found in two isoforms (Fig. 1). A comparable accumulation of this factor was observed after shifting cells to lower temperature (31). Taking these results together, accumulation of InfB seems to be correlated with a stop of protein synthesis in *E. coli*.

The phage shock protein A, PspA, is a peripheral inner membrane protein that negatively regulates the *psp* operon (65). PspA plays a role in maintenance of the proton motive force under certain conditions of cellular stress, such as heat and ethanol exposure (6), or osmotic stress as demonstrated in this study.

The hydroxyperoxidase II, KatE, accumulated in two isoforms, which were quantified together because of their marginal differences in pI.

One of the proteins found to be up-regulated, the cytoplasmic trehalase TreF, might play an indirect role in osmoadaptation by adjusting a constant intracellular level of the compatible solute trehalose and removal or utilization of internal trehalose after the cells return to low osmolality (29).

Transaldolase A (TalA) and transketolase B (TktB) are involved in the nonoxidative branch of the pentose phosphate pathway. The genes coding for TktB and the isozyme TktA are regulated in opposite ways by RpoS. Under conditions of increased levels of RpoS, *tktB* is induced, while *tktA* is repressed (30, 64).

All of these proteins seem to be important for the general osmoadaptive response of *E. coli*. The fold changes of the described proteins were found to be in the same range irrespective of the osmolyte used, with the exception of OsmY (Table 1). OsmY accumulated to a significant higher level in aerobically grown cells stressed by NaCl in comparison to the other tested conditions.

Salt stress induces an additional set of proteins under aerobic growth conditions. Seventeen additional proteins accumulated in cells which were exposed to the same osmolality but where the osmolality was increased by salt (NaCl) (Table 2; Fig. 1). These include a number of proteins with unknown function: YciE, YciF, YjbJ, YgaU and YceI. With the exception of YgaU, all of these proteins were detectable only in NaCl-stressed cells, and they were absent in nonstressed cells. YciE and YciF are single-domain proteins which contain the same domain of unknown function (DUF892). The corresponding genes are located side by side on the *E. coli* chromosome. YjbJ resembles the general stress response protein CsbD of *Bacillus subtilis*. Neither the function of CsbD nor the

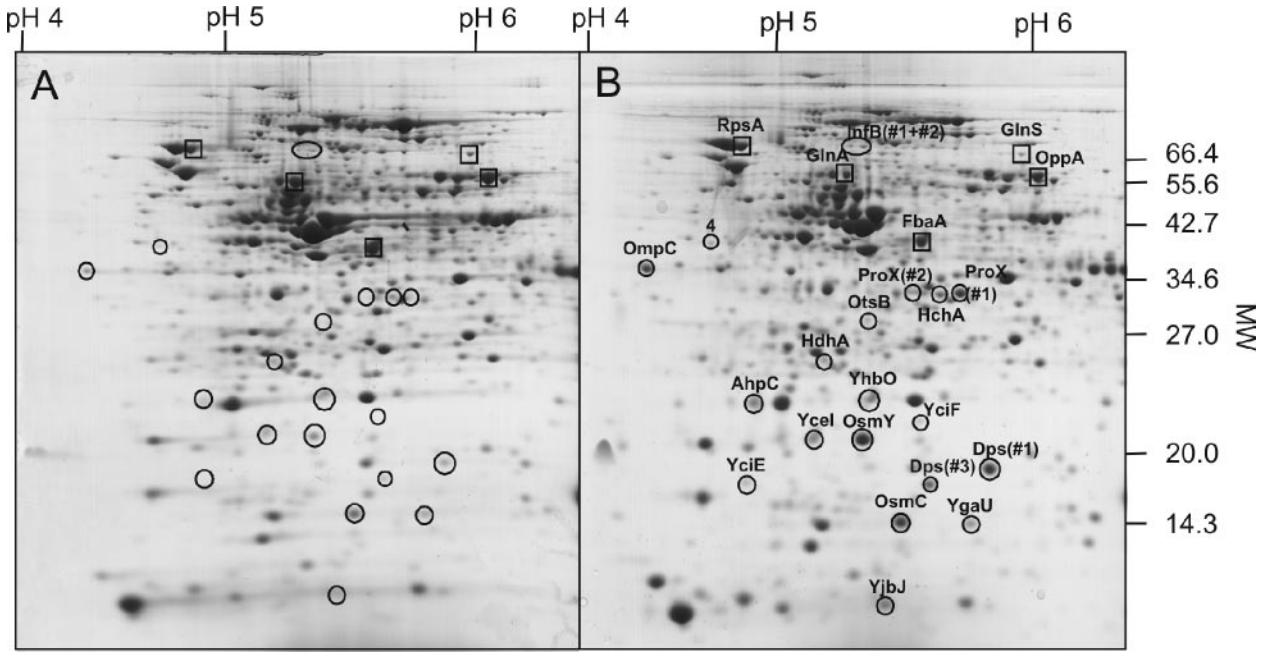


FIG. 2. Protein profiles of *E. coli* MG1655 immediately before (A) and 30 min after (B) the addition of NaCl (0.4 M) under anaerobic growth conditions. For details, see the legend to Fig. 1. MW, molecular weight, in thousands.

function of YjbJ is known. Moreover, YjbJ is dispensable in *E. coli* cells grown in rich or minimal medium (37).

The protein YgaU consists of a BON domain and a LysM domain. The above-mentioned OsmY contains two copies of the BON domain as well. This domain presumably functions as a membrane-binding domain, comprises about 60 amino acids, and has a predicted α/β-fold. It contains a conserved glycine residue and several hydrophobic regions. This amino acid pattern suggests a function of this protein in membrane binding or structural maintenance rather than in catalysis (67). The LysM domain is found in diverse enzymes involved in bacterial cell wall degradation. YceI, a potential periplasmic protein, was also up-regulated after NaCl upshock, although the abundance of this protein was also shown to be increased during growth at high pH (58). Recently, an RpoS-dependent regulation was demonstrated for *yciF*, *yjbJ*, and *ygaU* (61, 64).

Production of the compatible solute trehalose is one of the typical adaptation mechanisms of *E. coli* grown in minimal medium to react to an osmotic upshift. OtsB, the trehalose-6-phosphate phosphatase, catalyzes the last step in the synthesis of trehalose from glucose (27). An accumulation of this enzyme was also observed in this study. OtsB accumulated to a higher level in NaCl-stressed cells (4.3-fold) than in sorbitol-stressed cells (1.5-fold), reflecting the additional effect of using salt as the osmolyte.

Furthermore, accumulation of a set of metabolic enzymes was observed. These enzymes are isoforms of the constitutively produced enzymes. Aconitase A (AcnA), like its isozyme AcnB, catalyzes the isomerization of citrate to isocitrate but was shown to be more stable and to have a higher affinity for citrate. AcnA seems to be a survival enzyme during stress conditions (32). The identified pyruvate oxidase PoxB catalyzes an alternative route of pyruvate metabolism without pyruvate deydrogenase. MetL, the aspartate kinase II/homoserine dehydrogenase II of *E. coli*, might be an isozyme to ThrA. However, nothing is known about the regulation of these isoenzymes thus far.

yhbO is under control of RpoS (64), and the corresponding protein YhbO belongs to the DJ-1/PfpI protein family (Pfam accession no. PF01965). This signature defines a diverse group of protein families which include proteins involved in regulation of RNA/protein interaction, Ras-related signal transduction, thiamine biosynthesis, and proteases. The above-mentioned chaperone HchA also belongs to this protein family.

The single-stranded-DNA-binding protein Ssb of *E. coli* accumulated in cells after an increase of the extracellular NaCl concentration. Since Ssb was found to be involved in the acid tolerance of *Streptococcus mutans*, it is postulated that Ssb, like Dps, plays a role in the protection or repair of chromosomal DNA under cellular stress (36).

Three protein spots (spots 1 to 3) (Fig. 1; Table 1) analyzed by MALDI-TOF MS corresponded to protein mixtures of two or more proteins, among them glutamate decarboxylase isozyme A (GadA) and isozyme B (GadB), which are important during the acid stress response in *E. coli* and whose genes are regulated directly by the activator GadX and indirectly by RpoS (62). The up-regulation of the potential GTP-binding protein HflX (Pfam accession no. PF01926), which was found in a protein mixture together with the glutathione reductase GorA, was not found before during salt stress adaptation.

Osmotic stress response under aerobic and anaerobic growth conditions. In the gut *Escherichia coli* faces not only osmotic stress but also anaerobic conditions. The influence of oxygen availability was tested with cells exposed to an osmotic upshift with salt as the osmolyte, because, as shown above, it

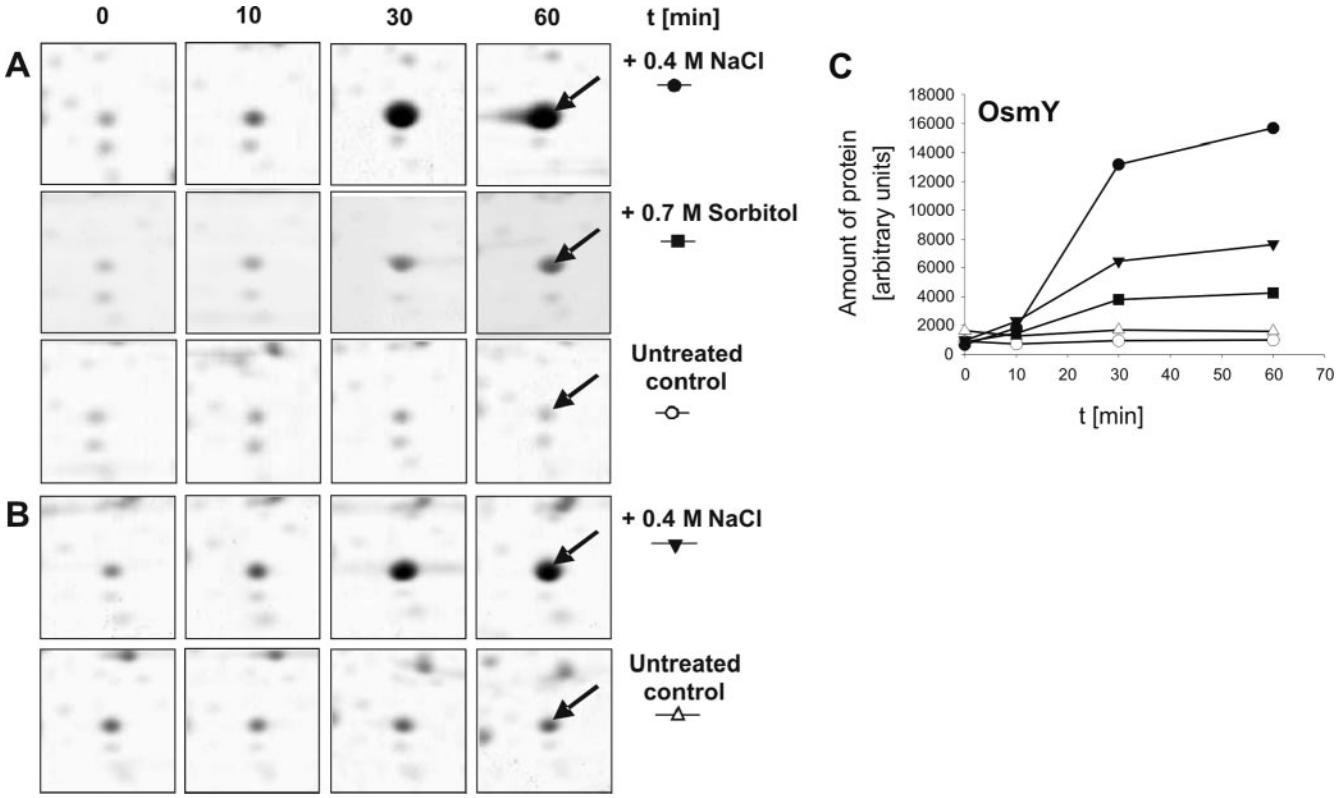


FIG. 3. Time-dependent accumulation of the periplasmic protein OsmY. Cells of *E. coli* MG1655 were grown in a fermentor at 37°C in K10 minimal medium with 0.4% (wt/vol) of glucose as a carbon source to mid-exponential growth phase. Before and 10, 30, and 60 min after addition of 0.4 M NaCl or 0.7 M sorbitol, samples were taken and analyzed as described in Materials and Methods. Corresponding sections of 2D gels illustrate the time-dependent accumulation of OsmY (arrows) under aerobic (A) and anaerobic (B) growth conditions. The spot intensities of OsmY were quantified, and mean values from two independent experiments were plotted against the time (C).

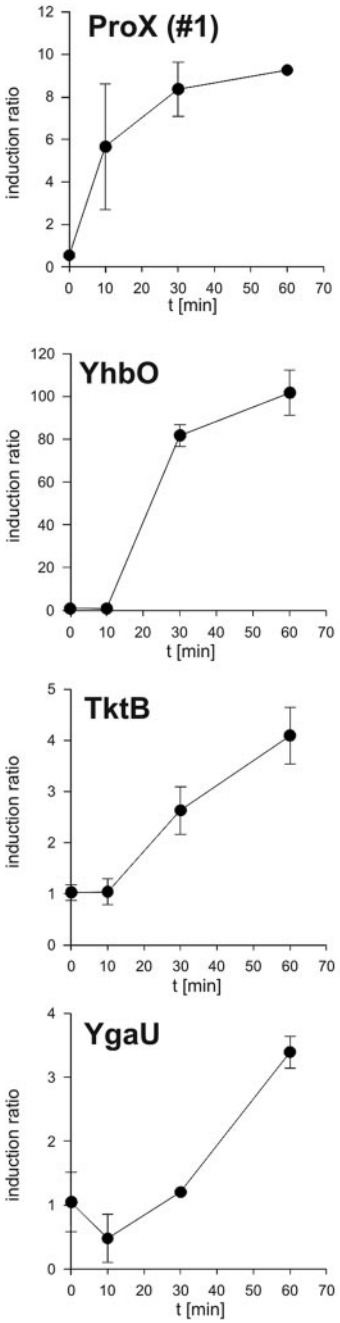
caused more dramatic changes of the proteome than sorbitol. Cells preadapted to anaerobic growth were treated with 0.4 M of NaCl in mid-exponential growth phase (OD_{600} of 0.3), and alterations of the proteome were visualized with 2D gels (Fig. 2). The following proteins were found to be accumulated regardless of oxygen availability (Tables 1 and 2; Fig. 2B): OsmY, OsmC, Dps (isoform 3), ProX (two isoforms) HchA, HdhA, InfB, YciE, YciF, YjbJ, YgaU, YceI, and OtsB. In contrast, 11 proteins (PspA, KatE, TreF, TalA, TktB, AcnA, PoxB, MetL, YhbO, Ssb, and Dps isoform 2) and the three protein mixtures identified in aerobically salt-stressed cells did not increase under anaerobic growth conditions. For some of them an oxygen-dependent regulation was previously reported: TalA and AcnA are negatively regulated by ArcA (51). KatE, the hydroxyperoxidase, is produced mainly under aerobic conditions (53). Moreover, a decreased expression of *poxB* under anaerobic growth conditions has been reported (8).

We found only two proteins and one protein mixture that accumulated solely in osmotically stressed, anaerobically grown cells (Table 2). The outer membrane protein OmpC represents one of these proteins. It is well known that 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 (33). Transcription of *ompC* and *ompF* is regulated by the two-component regulatory system EnvZ/OmpR (25, 45). Earlier data indicated changes of the expression profiles of OmpC and OmpF in response to external osmolality and oxygen availability (2). Due to a cross-regulation by the anaerobiosis-responsive sensor kinase ArcB, *ompC* is induced under anaerobic conditions even in low-osmolarity media, and therefore the increase of *ompC* transcription after osmotic stress is less distinct than that under aerobic growth conditions (40). The calculated increase in the amount of OmpC was only 2.5-fold after 60 min of osmotic stress.

AhpC was the second protein that accumulated specifically under anaerobic, osmotic stress conditions. AhpC, the alkyl hydroxyperoxide reductase, functions besides hydroxyperoxidases HP I and HP II as a scavenger of H_2O_2 in *E. coli* (55). Under anaerobic conditions, AhpC might compensate the activity of HP II, which was found to be up-regulated under aerobic conditions (Fig. 1, KatE). Consistently, *ahpC* transcription is repressed by hyperosmolality under aerobic growth conditions (63).

In addition, one protein mixture consisting of the two metabolic enzymes HemL (glutamate-1-semialdehyde aminotransferase) and FruB, a component of the fructose phosphotransferase system, was identified in cells exposed to these stress conditions (Table 2). The role of these proteins in adaptation is unknown thus far.

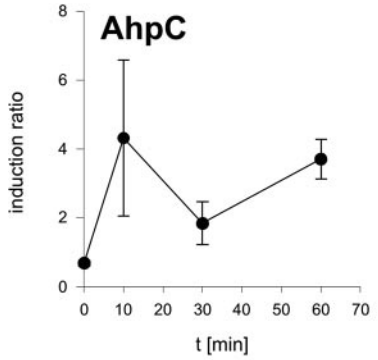
Time-dependent stress response. The protein spots were quantified as described in Materials and Methods. A typical

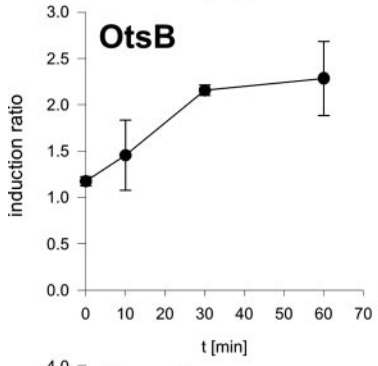


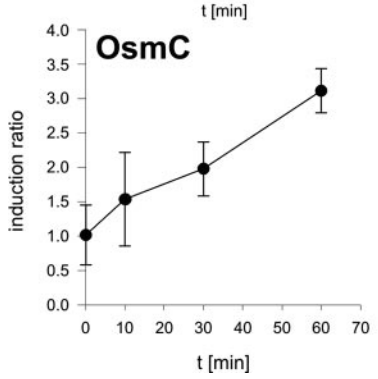
Phase of adaptation	Other proteins following this pattern
early and middle phase	InfB and OsmC protein mixtures 1 and 3
middle phase	Dps(#1,#2), KatE, OsmY, OtsB, PoxB, ProX(#2), Ssb, TalA, YceI, YciE, YciF and YjbJ
middle and long phase	AcnA, Dps(#3), HchA, HdhA, MetL, PspA, TreF and protein mixture 2
late phase	

FIG. 4. Classification of salt stress-adapting proteins according to their time-dependent appearance under aerobic growth conditions. The time courses of up-regulation for ProX (isoform 1), YhbO, TktB, and YgaU after osmotic stress (time zero) are shown as representative examples. Mean values of the induction ratios (pixel value of osmotically stressed cells/pixel value of unstressed cells) are shown, and the absolute deviation is presented. Proteins with similar time-dependent accumulation were grouped and are presented in the table.

analysis of the time-dependent changes is presented in Fig. 3. Under aerobic growth conditions (Fig. 3A and C), the amount of OsmY increased significantly between 10 and 30 min after the shift to hyperosmolality. After 60 min, the amount of OsmY was about 16-fold higher in the cells treated with NaCl than in the untreated control cells. When sorbitol was used as the osmolyte, the increase was less pronounced (fivefold). In the absence of osmotic stress, there were no obvious changes in the amount of OsmY. Under anaerobic growth conditions (Fig. 3C), the amount of OsmY increased, but less than in aerobically grown cells. In all cases the amount of OsmY remained at a steady-state level after 30 min of stress.







Phase of adaptation	Other proteins following this pattern
early phase	InfB, OmpC and YgaU, protein mixture 4
middle phase	Dps(#3), HchA, HdhA, ProX(#2), YciE, YciF and YjbJ
middle and long phase	Dps(#1), OsmY, ProX(#1) and YceI

FIG. 5. Classification of salt stress-adapting proteins according to their time-dependent appearance under anaerobic growth conditions. The time courses of up-regulation for AhpC, OtsB, and OsmC after osmotic stress (time zero) are shown as representative examples. Mean values of the induction ratios (pixel value of osmotically stressed cells/pixel value of unstressed cells) are shown, and the absolute deviation is presented. Proteins with similar time-dependent accumulation were grouped and are presented in the table.

In this way we were able to group the osmoresponsive proteins into different phases of adaptation (Fig. 4 and 5). Under aerobic conditions, most of the proteins described above are involved in the middle phase of adaptation to osmotic stress (Fig. 4). A detectable accumulation of these proteins started after 10 min, and their level reached a maximum at 30 min and remained stable afterwards. Only a few proteins, such as ProX, InfB, and OsmC, accumulated significantly within the first 10 min. These proteins seem to be involved in the early and middle phases of osmoadaptation. A third group of proteins accumulated linearly within 10 to 60 min after osmotic upshock. These proteins are involved in middle- and long-term adaptation processes. Only one protein, YgaU, seemed to be unimportant for adaptation within the first 30 min. This protein presumably plays a more pronounced role in the later acclimatization phase (60 min).

Under anaerobic growth conditions most proteins were synthesized in the middle phase of adaptation to osmotic stress (Fig. 5). A few proteins (AhpC, OmpC, InfB, and YgaU) increased in concentration within 10 min and remained more or less stable within the tested time scale. In contrast to the case under aerobic conditions, some proteins, such as Dps (isoform 1), ProX (isoform 1), and OsmY were continuously increasing and seem to be required during all stages of adaptation against hyperosmolality. Under anaerobic conditions, the amount of YgaU increased solely in the first 10 min and then remained at a higher level. This time course was completely different from that under aerobic conditions, where

accumulation started much later. Since the function of YgaU is unknown, the importance of this observation remains unclear. Together, the time dependence studies indicate that the cellular stress response at the protein level is slow. Most of the newly synthesized proteins appear at 30 min and seem to be important for long-term adaptation.

Proteins down-regulated in response to osmotic stress. Regardless of oxygen availability, the abundance of only a few proteins decreased significantly after osmotic stress (Fig. 1 and 2). These were mainly proteins involved in housekeeping processes of the cells. The amount of GlnA, the glutamine synthetase, decreased under all tested conditions. Under aerobic growth conditions, the intracellular concentration of FliC, the subunit flagellin of the flagellar filament, as well as BtuB, an outer membrane receptor for transport of vitamin B_{12}, and MetF, a 5,10-methylene-tetrahydrofolate reductase involved in the biosynthesis of methionine, decreased. Under anaerobic conditions, the following proteins were found to be down-regulated: GlnS, a glutaminyl-tRNA-synthetase; OppA, the binding subunit of an oligopeptide ABC transporter; FbaA, an *E. coli* class II fructose bisphosphate aldolase; and RpsA, the 30S ribosomal subunit protein S1. The last protein plays an essential role during initiation of translation of most mRNA species in *E. coli* (4).

Comparison to other whole-cell osmostress analyses. In general, the data presented here correlate well with the transcriptome data that were published recently (64). Particularly, in our proteome studies we found a multiplicity of RpoS-regulated proteins, whose genes have been described to be transcriptionally induced by salt stress, including *osmC*, *osmY*, *dps*, *treF*, *hchA*, *hdhA*, *katE*, *talA*, *tktB*, *poxB*, *otsB*, *gadA*, *gadB*, *yciF*, *yjbJ*, *ygaU*, and *yhbO*. However, the transcriptome analysis uncovered some genes whose corresponding proteins remained unresolved by the proteomic approach. These include some transport proteins, e.g., the high-affinity K^+ uptake system Kdp, which cannot be solubilized using CHAPS (63; unpublished results). Other proteins simply run outside the pI range used and/or the resolution of the sodium dodecyl sulfate gel system, which explains the discrepancy with earlier studies with respect to OtsA (27), the proline/betaine transporter ProP, the glycine betaine/L-proline transport protein ProW of the *proU* operon (19–21, 44, 63), and the hypothetical protein YciG (64).

Although the regulatory trend for all of the proteins involved in osmoadaptation correlates well with previous investigations, the induction ratios determined for some proteins differ. Specifically, Gutierrez et al. (22) observed induction ratios for ProU, OsmC, and OmpC of 700-fold, 21-fold, and 5-fold, respectively, using Tn*phoA* fusion studies, whereas the values determined for these proteins in our study were much lower (Tables 1 and 2). This discrepancy might be related to differences in the turnover rate at the transcriptional (mRNA degradation) or posttranslational (proteolysis) level for native proteins compared to reporter proteins. Furthermore, it should be mentioned that in this study the induction ratio was determined after 60 min; however, the time-dependent alterations of the protein levels (Fig. 4 and 5) indicate that some proteins did not reach steady-state levels within this time period of osmoadaptation, which might further explain the different values of the induction ratios. In case of OmpC, the protein level did not change in aerobically stressed cells, which is in agreement with the transcriptional analysis of cells grown under the same condition (63).

Conclusions. Adaptation of *E. coli* to an increase of the external osmolality caused several alterations at the protein level. Although the majority of up-regulated proteins are regulated by RpoS at the transcriptional level, different time frames were found. Nevertheless, most of the newly synthesized proteins appeared in the early and middle phases of adaptation (10 to 30 min) and might be involved in long-term osmoadaptation. Some of the identified proteins are known as specific osmoadaptive proteins (e.g., OtsB and ProX). However, most of the proteins play a more general role in cross-protecting the cell against diverse stress conditions, which is well reflected by their direct or indirect transcriptional control through the master stress regulator RpoS. Interestingly, we found a number of up-regulated proteins that are involved in the defense against oxidative stress (e.g., OsmC, Dps, KatE, and AhpC). In this regard it should be noted that one oxidative stress protein, the alkyl hydroperoxide reductase AhpC, appeared exclusively under anaerobic osmotic stress conditions. Despite these observations, the relation between an increase of reactive forms of oxygen and the osmostress response remains unclear and needs further experimentation.

Thus far, none of the global analyses of the osmotic stress response of *E. coli* investigated anaerobically grown cells, although the combination of these two stresses is a common feature of the natural environment of an enterobacterium. Preadapted anaerobic cells responded to osmotic stress by up-regulation or synthesis of proteins already known from studies of aerobic stressed cells, corroborating their indispensability in osmoadaptation. However, the number of up-regulated proteins was significantly lower in anaerobic cells (15 proteins) than in aerobic cells (29 proteins). Most of the additional proteins found in aerobically grown cells concern metabolic enzymes, reflecting the greater metabolic flexibility and the higher energy level of *E. coli* in the presence of oxygen.

Finally, the study unraveled a remarkable difference in the amount of TreF, the cytosolic trehalase, in cells grown under different conditions. Trehalose is one important osmoprotectant of *E. coli* grown in minimal medium. For *E. coli* and other microorganisms, it is still unclear why a trehalose-degrading enzyme is up-regulated at the same time when trehalose is needed as an osmoprotectant. Importantly, TreF was highly induced under aerobic high-osmolality conditions but not under anaerobic high-osmolality conditions. This result gives reason to analyze the metabolite pattern of anaerobically grown cells exposed to osmotic stress in future studies.

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