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Impact of Nutritional Factors on the Proteome of Intestinal *Escherichia coli*: Induction of OxyR-Dependent Proteins AhpF and Dps by a Lactose-Rich Diet

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To study the impact of nutritional factors on protein expression of intestinal bacteria, gnotobiotic mice monoassociated with *Escherichia coli* K-12 were fed three different diets: a diet rich in starch, a diet rich in nondigestible lactose, and a diet rich in casein. Two-dimensional gel electrophoresis and electrospray-tandem mass spectrometry were used to identify differentially expressed proteins of bacteria recovered from small intestine and cecum. Oxidative stress response proteins such as AhpF, Dps, and Fur, all of which belong to the oxyR regulon, were upregulated in *E. coli* isolates from mice fed the lactose-rich diet. Luciferase reporter gene assays demonstrated that osmotic stress caused by carbohydrates led to the expression of *ahpCF* and *dps*, which was not observed in an *E. coli* Δ*oxyR* mutant. Growth of *ahpCF* and *oxyR* deletion mutants was strongly impaired when nondigestible sucrose was present in the medium. The wild-type phenotype could be restored by complementation of the deletions with plasmids containing the corresponding genes and promoters. The results indicate that some OxyR-dependent proteins play a major role in the adaptation of *E. coli* to osmotic stress. We conclude that there is an overlap of osmotic and oxidative stress responses. Mice fed the lactose-rich diet possibly had a higher intestinal osmolality, leading to the upregulation of OxyR-dependent proteins, which enable intestinal *E. coli* to better cope with diet-induced osmotic stress.

The intestinal microbiota influences the mammalian host in many ways: it acts as a barrier against colonization by pathogens, modulates the immune system, and has an immense catalytic potential (19, 22, 32). Diet is one of the most important factors influencing microbiota composition. Over 60% of species variation is due to perturbations in diet composition (13, 36). Studies in mice colonized with human fecal microbial communities revealed a rapid change of microbiota composition following a switch from a low-fat, dietary fiber-rich diet to a high-fat, high-sugar Western diet (36).

However, how bacterial cells adapt their metabolism to changing substrate availability in the host environment is poorly understood. Metagenomic analyses of the humanized mouse gut microbiome indicated changes in metabolic pathways such as amino acid and nucleotide sugar metabolism, pentose and glucuronate interconversion, and carbohydrate metabolism as well as in ABC transporters and the phosphotransferase system in response to dietary shifts (36). Nevertheless, the possibility cannot be excluded that many of the observed changes were due to shifts in microbial populations. Therefore, we used a simplified gnotobiotic mouse model associated with only one well-examined bacterial species. In a previous study, *Escherichia coli* K-12 strain MG1655 was shown to be capable of adapting to the intestinal environment (37).

We used this simplified model to gain insights into the mechanisms that enable intestinal bacteria to adapt to nutritional factors. Mice monoassociated with *E. coli* were fed different diets: a diet rich in starch, which undergoes almost complete digestion in the mouse intestine, is supposed to mimic bacterial growth on host endogenous substrates, while a diet rich in nondigestible lactose would make this carbohydrate completely available for *E. coli*. Another important dietary factor is protein. We expected that feeding a diet rich in casein would result in incomplete digestion and absorption of dietary proteins by the host and provide degradable products of casein for the growth of *E. coli*. Intestinal bacterial proteins that were differentially expressed in response to these diets were identified, and selected proteins were tested *in vitro* for their possible role in bacterial adaptation to the various diets. Our results indicate that oxidative stress-related genes support the successful survival of intestinal bacteria under osmotic stress conditions caused by carbohydrates. These results suggest an overlap between the oxidative and osmotic stress responses in *E. coli*.

MATERIALS AND METHODS

Mouse experiments and sample preparation. Three groups of germfree C3H mice (Charles River), 9 to 12 weeks of age, were kept in separate cages within a sterile Trexler type isolator for the duration of the experiment. To confirm the germfree status of the animals prior to the initiation of the experiment, fecal samples were collected to perform Gram staining and cultivation under aerobic and anaerobic conditions on thioglycolate broth (SIFIN, Berlin, Germany) and Wilkins-Chalgren broth (Oxoid, Hampshire, United Kingdom). Each mouse was orogastrically inoculated with 1×10^7 *E. coli* K-12 MG1655 cells. The mice had free access to any of the three sterilized semisynthetic diets given in Table 1 and to autoclaved water. The mice were killed on day 21 after inoculation by cervical dislocation, and the intestinal contents of small intestine, cecum, and colon were collected, weighed, and diluted 1:10 (wt/vol) with phosphate-buffered saline (PBS) (Na_2HPO_4 [80 g/liter], KCl [2 g/liter], Na_2HPO_4 [14.4 g/liter], KH_2PO_4 [2.4 g/liter], pH 7.4) containing a 1:100-diluted 100×

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TABLE 1 Composition of semisynthetic diets

Substrate	% (wt/wt) in indicated diet		
	Starch	Lactose	Casein
Sucrose	20	20	20
Starch	43	33	3
Lactose	0	10	0
Cellulose	5	5	5
Casein	20	20	60
Sunflower oil	5	5	5
Vitamins	2	2	2
Minerals	5	5	5

protease inhibitor mix (GE Healthcare, Munich, Germany). Intestinal contents were homogenized by agitation with a Uniprep 24 gyrator (Uniequip) (speed 2) in the presence of glass beads (diameter, 2.85 to 3.33 mm) and centrifuged (300 × *g* at 4°C for 3 min) to remove coarse particles originating from the diet. Counts of viable cells from the supernatants were determined by serial dilution in PBS and plating 100-μl aliquots on LB-Lennox agar (Roth, Karlsruhe, Germany). Plates were incubated under aerobic conditions at 37°C for 24 h. Dry weight was determined by lyophilization of coarse particles in a Christ Alpha 2-4 lyophilization apparatus (Christ Gefriertrocknungsanlagen, Osterode, Germany) for 24 h.

Supernatants were centrifuged (10,000 × *g* at 4°C for 3 min), and bacterial pellets were resuspended in washing buffer (10 mM Tris [pH 8], 5 mM magnesium acetate, chloramphenicol [30 μg/μl], 100× protease inhibitor mix, diluted 1:100). Bacterial cells were isolated by Nycodenz (Axis-shield PoC, Oslo, Norway) gradient centrifugation as follows: a 0.5-ml cell suspension was layered on top of a 0.5-ml Nycodenz solution (40% [wt/vol]) and centrifuged for 15 min at 186,000 × *g* and 4°C. The interphase volume containing the *E. coli* cells was collected and washed 4 times with washing buffer at 4°C. The isolated *E. coli* cells were stored at −80°C.

To ensure that no contamination occurred during the animal experiment, DNA of representative samples was isolated (RTP Bacteria DNA minikit; Invitek, Berlin, Germany). Bacterial 16S rRNA genes were amplified by PCR with primers 27-f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492-r (5′-TAC CTT GTT ACG ACT T-3′) (21) and checked by sequencing (Eurofins MWG Operon, Ebersberg, Germany). Since all mice were housed in the same isolator, we consider the analysis of a subset of animals to be representative of the microbial status of all animals in the experiment.

Preparation of bacterial proteins and 2D difference in-gel electrophoresis (2D-DIGE). Frozen cells were thawed on ice, resuspended in 0.8 ml of lysis buffer consisting of 8 M urea, 30 mM Tris, and 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (pH 8.5), and incubated for 5 min. Cells were disrupted in an FP120 FastPrep cell disruptor (Thermo Scientific, Waltham, MA) using zirconium-silica beads (Roth, Karlsruhe, Germany) (0.1 mm) and three 20-s cycles at a speed of 4.0 m/s. Cell disruption was interrupted by 5-min intervals for cooling of samples on ice. Unbroken cells were removed by centrifugation (14,000 × *g* at 4°C for 20 min). DNA was eliminated by incubation with 125 U of Benzonase (Novagen, Merck KGaA, Darmstadt, Germany) at 37°C for 5 min. Proteins in the supernatant were enriched and purified from components interfering with proteomic analysis by selective precipitation of proteins (two-dimensional [2D] cleanup kit; GE Healthcare, Munich, Germany). The protein solution was adjusted to pH 8.5 with 50 mM NaOH for an optimal reaction with the fluorescent dyes. The concentration of purified proteins was determined with a Bradford assay (Bio-Rad, Madrid, Spain), using bovine serum albumin as a reference protein.

Purified proteins were labeled with CyDyes (GE Healthcare, Munich, Germany) according to the manufacturer's instructions for DIGE. Isoelectric focusing was done on immobilized pH gradient strips (pH range, 4 to 7; length, 24 cm) in an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare, Munich, Germany). Active rehydration (30 V for 10 h) was followed by focusing of the samples at 20°C as follows: 500 V for 1 h, 1,000 V for 1 h, 10,000 V for 3 h, and 10,000 V until a value of 42,500 Vh was reached. The second dimension was run on 12.5% sodium dodecyl sulfate (SDS) gels in an Ettan-Dalt II apparatus (1 W per gel for 45 min, followed by 17 W per gel for 3.5 h). Gels were scanned with a Typhoon Trio laser scanner, and image analysis was done with DeCyder software version 6.5 (both from GE Healthcare, Munich, Germany).

In-gel protein digestion and identification of differentially expressed proteins. Staining of preparative gels, in-gel protein digestion, nano-liquid chromatography, electrospray ionization mass spectrometry (ESI-MS), and tandem mass spectrometry (MS-MS) analysis, data processing, and protein identification were performed as described previously (2, 3, 37).

Determination of dietary substrates in the luminal gut contents. Concentrations of fructose, glucose, and lactose in the luminal gut contents were determined using enzymatic test kits (lactose, D-galactose, sucrose, D-glucose, and D-fructose; R-Biopharm, Darmstadt, Germany). Analyses were done according to the manufacturer's instructions, except that a 96-well plate format was used, which allowed a 20-fold reduction of the volumes of all test components in comparison to the original protocol. Absorption was measured at 340 nm, and concentrations were calculated with the help of standard curves.

Quantification of amino acids and proteins was based on the ninhydrin (1,2,3-indantrione monohydrate; Fluka, Neu-Ulm, Germany) reaction (20). A total of 60 μl of 20 mM ninhydrin in ethanol was added to 300 μl of luminal gut content samples in Eppendorf tubes and mixed carefully. Determination of absorption was done using 96-well plates. Background absorption was measured at 570 nm (E1). The reaction of ninhydrin with free alpha-amino groups was started by heating at 90°C for 7 min and stopped by cooling on ice. The resulting color change to deep purple was measured at 570 nm in duplicate experiments (100 μl each) (E2). Extinction differences were calculated by the following formula: $\Delta E = (E2 - E1)_{sample} - (E2 - E1)_{blank}$. To calculate the concentrations of free alpha-amino groups, a standard curve using hydrolyzed Casamino Acids (Bacto Laboratories, Mount Pritchard, New South Wales, Australia) (0 to 2.5 g/liter) was determined.

Generation of luciferase reporter gene constructs. Promoter regions of *ahpCF* and *dps* were amplified from *E. coli* MG1655 by PCR using primers flanked by the sequences for the restriction enzymes XbaI and EcoRI plus two nucleotides at the 5′ end to improve restriction digestion (primer-*ahpCFp*, primer-*dpsp*). The primers used for the constructions are listed in Table 2. Amplified fragments and the *luxAB*-containing pKEST-MR plasmid were digested with XbaI and EcoRI (FastDigest; Fermentas, St. Leon-Rot, Germany). Plasmids were gel purified, and DNA was extracted with an Innu Prep gel extraction kit (Analytik Jena, Jena, Germany). PCR products were purified using a High Pure PCR product purification kit (Roche Diagnostics GmbH). DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (peqlab, Erlangen, Germany).

Ligation was done using a plasmid/insert ratio of 1:5 and T4-DNA-ligase (New England BioLabs, Beverley, MA). A 50-μl volume of electrocompetent *E. coli* MG1655 was transformed using 5 μl of ligation mixture and a Gene Pulser apparatus (Bio-Rad, Munich, Germany) (11). Transformed cells were selected on LB-Lennox agar containing carbenicillin (Roth, Karlsruhe, Germany) (50 μg/ml). Positive clones (*E. coli* MG1655 p*ahpCFp*::*luxAB* and *E. coli* MG1655 p*dpsp*::*luxAB*) were checked by sequencing (Eurofins MWG Operon, Ebersberg, Germany) using plasmid-specific primers (pKEST control; Table 2).

Luciferase reporter gene assays. *E. coli* clones MG1655 p*ahpCFp*::*luxAB* and *E. coli* MG1655 p*dpsp*::*luxAB* were precultured aerobically or anaerobically in LB-Lennox medium plus carbenicillin (50 μg/ml) and inoculated at 5% into 300 ml of fresh LB-Lennox medium plus carbenicillin (50 μg/ml). Cells were grown to mid-exponential phase under aer-

TABLE 2 Primers used for generation of luciferase reporter gene constructs

Code	Amplified region	Sequence (5′→3′)
primer-*ahpCFp*	Promoter region of *ahpCF*	CGGAATTCTCAGTCAGTGCAAAAGTCGAG[a]
		CGTCTAGAAGGACATCTATACTTCCTCCG[b]
primer-*dpsp*	Promoter region of *dps*	GCTCTAGATAAAGCAGATTG[a]
		GCGAATTCTTGAATCTTTATTAGT[b]
pKESTMR control	Integration site of pKESTMR	AAAGTGCCACCTGACGT[a]
		GGGTTGGTATGTAAGCAA[b]

[a] Forward primer.
[b] Reverse primer.

obic and anaerobic conditions and harvested at an optical density at 600 nm (OD_{600}) of 0.3 to 0.5 (SmartSpec Plus spectrophotometer; Bio-Rad, Munich, Germany). Cell suspensions were centrifuged (5,000 × *g*, 5 min, 4°C), and pelleted cells were resuspended in approximately 30 ml of LB-Lennox medium plus carbenicillin (50 μg/ml). Cell concentrations were adjusted to approximately 5×10^9 cells/ml. To stimulate promoter activity, various substances of interest were applied at different concentrations either to sterile 6-well plates for analysis under aerobic conditions or to sterile Hungate tubes, gassed with 80% nitrogen-20% carbon dioxide, for analysis under anaerobic conditions. Cell suspensions (1.5 ml) were added to each well or Hungate tube and incubated at 37°C for 30 min under conditions of shaking (120 rpm). To stop protein biosynthesis, cell suspensions were transferred to ice and chloramphenicol (30 μg/ml) was added. Luminescence of 2.5×10^8 cells in 50 μl was measured using a Luminocsan Ascent luminometer (Labsystems, Helsinki, Finland) in white 96-well plates (LuminNunc F96 MicroWell plates; VWR, Darmstadt, Germany). Decanal (Sigma-Aldrich, Steinheim, Germany) (100 μl; 2%) in PBS–10% ethanol was added to each well, the reaction mixture was incubated for 3 s, and luminescence was measured for 10 s. Each sample was measured in triplicate. Absolute luminescence values of stimulated cells were divided by the values obtained from cells grown on LB-Lennox medium without stimuli to calculate the relative luminescence.

Determination of medium osmolality. The osmolality of the media used for determination of luciferase activity was measured by freezing-point depression using an automatic osmometer (Knauer, Berlin, Germany), which was calibrated against water and a calibration solution of 400 mosmol/kg (12.687 g of NaCl/kg). Analyses were done in triplicate.

Generation of deletion mutants. Chromosomal sequences internal to the *ahpCF* and *oxyR* genes (see Fig. S2 in the supplemental material) were replaced by a kanamycin resistance cassette according to the technique of Datsenko and Wanner (9). The primers used for constructions are listed in Table 3. Plasmid pKD13 (9) was used as a template for the antibiotic resistance gene. Mutant candidates were tested for the loss of the target genes by PCR with kanamycin (K2 and Kt)-specific and locus-specific (*ahpCF* control and *oxyR* control) primers and sequenced for genotype confirmation (Eurofins MWG Operon, Ebersberg, Germany).

Characterization of deletion mutants. *E. coli* cells were precultured aerobically overnight in LB-Lennox medium and inoculated at 2.5×10^7 cells/ml into LB-Lennox medium or LB-Lennox medium containing sucrose (400 mM or 700 mM). The cultures were incubated aerobically in 100-ml Erlenmeyer flasks (20 ml of medium) or anaerobically in gassed Hungate tubes (5 ml of medium, 80% nitrogen, 20% carbon dioxide) and shaken at 180 rpm at 37°C. Growth was monitored by measuring the optical density at 600 nm every hour for the first 8 h and after 24 h. Growth

TABLE 3 Primers used for generation of deletion mutants and complementing plasmids

Code	Amplified region	Sequence (5′→3′)	Reference or source
Δ*ahpCF*	Flanking region of *ahpCF*	AAAAATTGGTTACCTTACATCTCATCGAAAACACGGAGGAAGTAT AGATGATTCCGGGGATCCGTCGACC[a]	Baba et al. (5)
		AAGCAATTGCAGGTGAATCTTACTTCTTCTTATGCAGTTTTGGTG CGAATTGTAGGCTGGAGCTGCTTCG[b]	Baba et al. (5)
Δ*oxyR*	Flanking region of *oxyR*	CTATTCTACCTATCGCCATGAACTATCGTGGCGATGGAGGATGGAT AATGATTCCGGGGATCCGTCGACC[a]	Baba et al. (5)
		AAGCCTATCGGGTAGCTGCGTTAAACGGTTTAAACCGCC TGTTTTAAAACTGTAGGCTGGAGCTGCTTCG[b]	Baba et al. (5)
K2	Kanamycin cassette of pKD13	GCAGTTCATTCAGGGCACCG[b]	Datsenko and Wanner (9)
Kt	Kanamycin cassette of pKD13	CGGCCACAGTCGATGAATCC[a]	Datsenko and Wanner (9)
ahpCF control	Upstream and downstream of *ahpCF*	CGCATTAGCCGAATCGGC[a]	This work
		ATAAGTATCCCGCCCTGCCC[b]	This work
oxyR control	Upstream and downstream of *oxyR*	GCTGCAATCGTGCCTCGACA[a]	This work
		TCGTCGGCATGAACGTGGG[b]	This work
ahpCF-compl	*ahpCF*	GCAAGCTTGTCGAGTAAAAGGCATAACCT[a]	This work
		TAGGATCCAAAGCCGCCAGGTTTGA[b]	This work
oxyR-compl	*oxyR*	GCAAGCTTGTGCCGCTCCGTTTCTGTGA[a]	This work
		GCGGATCCAACTACCCGACGATGGCGGAA[b]	This work
pSU19 control	Upstream and downstream of multiple-cloning site of pSU19	CCAGGCTTTACACTTTATGC[a]	This work
		AGGCTGCGCAACTGTTG[b]	This work

[a] Forward primer.
[b] Reverse primer.

of anaerobic cultures was additionally measured after 28 and 32 h. For OD readings above 1, appropriate dilutions in LB-Lennox medium were done. To determine the specific growth rate, the logarithm of the optical density was plotted against time. The slope in the exponential growth phase corresponds to the growth rate (μ). The doubling time (t_d) was calculated as follows: $t_d = \ln 2/\mu \times 60$.

Complementation of deletion mutants. To complement the generated mutants, the *ahpCF* and *oxyR* genes, including their corresponding promoters, were amplified from *E. coli* MG1655 by PCR with the primers *ahpCF*-compl and *oxyR*-compl, digested with HindIII and BamHI, and cloned into the low-copy-number plasmid pSU19 (6). Ligation and transformation in an *E. coli* Δ*ahpCF* or *E. coli* Δ*oxyR* mutant were done as described for the luciferase constructs (11). Transformed cells were selected on LB-Lennox agar containing chloramphenicol (Roth, Karlsruhe, Germany) (10 μg/ml). Positive clones (*E. coli* Δ*ahpCF* pSU19*ahpCF* and *E. coli* Δ*oxyR* pSU19*oxyR*) were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany) using plasmid-specific primers (pSU19 control). To characterize aerobic growth, complementing strains containing pSU19 were cultured as described for the deletion mutants but in the presence of chloramphenicol (Roth, Karlsruhe, Germany) (10 μg/ml).

Detection of free radicals by dihydrorhodamine 123. *E. coli* cells were precultured aerobically overnight in LB-Lennox medium and inoculated into 10 ml of fresh medium (10% [vol/vol]). After 45 min, cells were harvested by centrifugation (5,000 × *g* at 4°C for 5 min) and washed twice with 10 ml of PBS, and the cell number was adjusted to 1.25×10^7 per ml. A 2-ml volume of cells were stained with 5 mM dihydrorhodamine 123 (15, 18)–PBS by shaking at 180 rpm for 45 min at 37°C. For negative controls, cells were incubated with PBS only. Treated cells were washed with 2 ml of PBS and resuspended in 2 ml of M9 minimal medium (Na_2HPO_4 [6 g/liter], KH_2PO_4 [3 g/liter], NaCl [0.5 g/liter], NH_4Cl [1 g/liter], uracil [12.5 mg/liter], 1 mM $MgSO_4$, 0.1 mM $CaCl_2$). Test substances were added to 800 μl of cells in 12-well cell culture plates (Sigma-Aldrich, Steinheim, Germany) (approximately 8-ml volume per well), and cells were incubated in the dark by shaking at 150 rpm for 60 min at 37°C. After incubation, 800 μl of cells were washed with 800 μl of PBS and resuspended in 800 μl of PBS. Fluorescence of 100-μl aliquots was measured in triplicate experiments using a Synergy microplate reader (BioTek, Bad Friedrichshall, Germany). The excitation wavelength was set at 485/20 nm, and the emission wavelength was set at 528/20 nm. Fluorescence per cell was calculated by dividing by the viable cell counts.

Statistical analysis. Statistical analyses were done with GraphPad Prism 5 for determination of dietary substrates, luciferase activity, and growth experiments performed with deletion mutants. SPSS 16.0 was used for descriptions of correlations. Data were tested for Gaussian distribution by the D'Agostino and Pearson omnibus normality test and the Kolmogorow-Smirnow test. Non-normally distributed data are given as medians (minimum to maximum).

RESULTS

Establishment of an *in vivo* mouse model for studying nutritional factors. Mice monoassociated with *E. coli* were fed a lactose-rich diet, a casein-rich diet, or a diet rich in starch. None of the diets caused diarrhea. Contamination of mice was excluded by 16S rRNA gene analyses (see Fig. S1 in the supplemental material). Independent of the diet, the *E. coli* counts in the small intestine were approximately 3- to 10-fold lower than in the cecum or colon. The *E. coli* counts in small intestine and cecum of mice fed the lactose diet were 6- to 16-fold higher than in mice fed the starch diet or the casein diet (Fig. 1).

To check whether the various diets affect substrate availability for bacteria in the intestine, the intestinal concentrations of sucrose, fructose, glucose, lactose, and amino acids were determined. The main potential source of carbohydrates was starch. Starch undergoes degradation by host enzymes and results in the formation of malto-oligosaccharides and glucose, which are rapidly absorbed. Since *E. coli* is devoid of starch-degrading enzymes (14), malto-oligosaccharides and glucose are released only during starch digestion in the small intestine. In agreement with this fact, the glucose concentration in the small intestine of mice fed the starch diet was 3-fold higher than that in the small intestine of mice fed the lactose diet (6 mM versus 2 mM). In contrast, mice fed the casein diet had <0.5 mM glucose in their intestinal content (Fig. 2A). Only mice fed the lactose diet had 10 mM lactose in their small intestines. Approximately one-third of this concentration was observed in cecum and colon of these mice (Fig. 2B). The intestinal concentration of fructose on any of the diets was ≤1 mM (data not shown) and was therefore not expected to significantly affect the growth of *E. coli in vivo*.

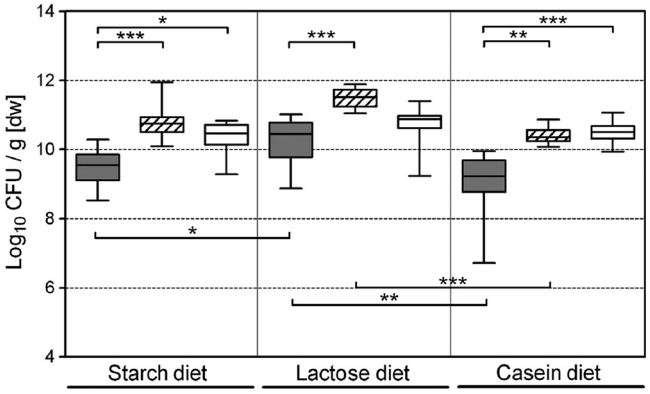

FIG 1 Intestinal cell numbers of *E. coli* after 3 weeks of feeding mice a starch-rich diet, a lactose-rich diet, or a casein-rich diet. Gray bars, small intestine; hatched bars, cecum; white bars, colon. Data are expressed as medians ($n = 18$ to 21/diet). Kruskal-Wallis one-way analysis of variance (ANOVA) and Dunn's multiple-comparison test were used for calculations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Mice fed the starch or the lactose diet (20% casein [wt/wt] each) had 3.4 to 6 mg of protein per g in their intestines. Mice fed the casein diet (60% protein [wt/wt]) displayed only marginally higher protein concentrations (4.2 to 7.7 mg per g) in their intestines (data not shown). In the mammalian gut, dietary proteins are hydrolyzed by host enzymes to peptides and free amino acids and subsequently absorbed. To some extent, they may serve as an energy source for intestinal bacteria. Therefore, we determined the concentration of free amino acids in the intestines of mice. Mice fed the starch or the lactose diet had amino acids at a concentration of 5 to 8.5 mg per g. Mice fed the casein diet had 2-fold-higher intestinal amino acid concentrations (9.5 to 16 mg of amino acids per g) (Fig. 3).

Bacterial adaptation to host diets. To find out which mechanisms enable intestinal *E. coli* to adapt to various diets consumed by the host, bacterial protein expression was analyzed using the 2D-DIGE technique. To prevent proteome alterations, protease inhibitors and chloramphenicol (29) were added before exposure to centrifugation and freezing stress during the preparation of the bacterial proteins. The proteomes of small intestinal and cecal *E. coli* isolates from mice fed the lactose or the casein diet were compared with those from mice fed the starch diet. This analysis revealed 102 differentially (≥2-fold) expressed proteins ($P < 0.05$) (see Table S1 in the supplemental material). For some proteins, different isoforms were identified. Each isoform is indicated by a

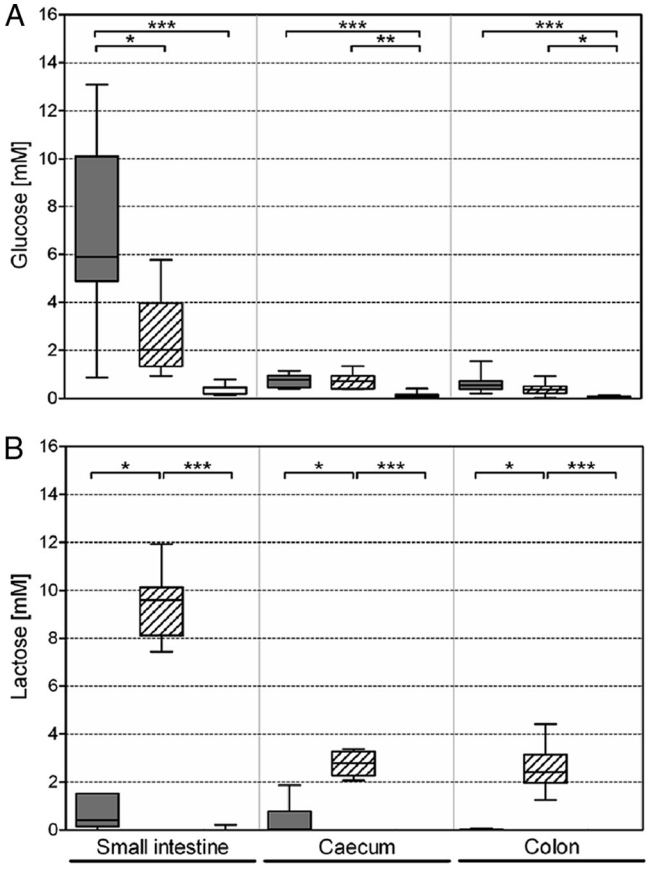

FIG 2 Free dietary carbohydrate concentrations in intestinal contents of mice fed a starch-rich diet, a lactose-rich diet, or a casein-rich diet. Gray bars, starch diet; hatched bars, lactose diet; white bars, casein diet. Data are expressed as medians. (A) For glucose data, $n = 14$ (starch diet), $n = 10$ (lactose diet), and $n = 7$ (casein diet). (B) For lactose data, $n = 7$. Kruskal-Wallis one-way analysis of variance and Dunn's multiple-comparison test were used for calculations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

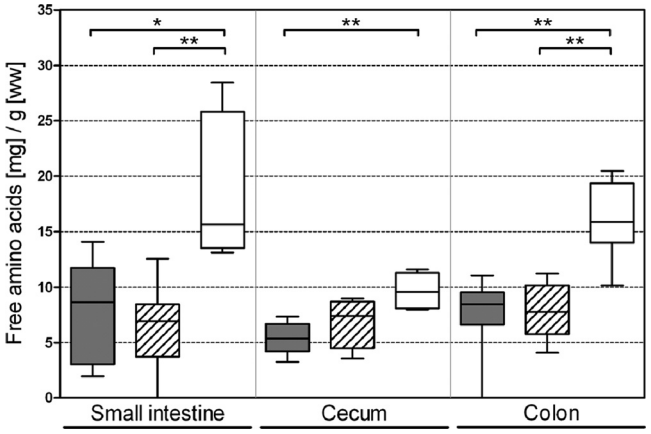

FIG 3 Free amino acid concentrations in the intestinal contents of mice fed a starch-rich diet, a lactose-rich diet, or a casein-rich diet. Gray bars, starch diet; hatched bars, lactose diet; white bars, casein diet. Data are expressed as medians ($n = 8$). Kruskal-Wallis one-way analysis of variance and Dunn's multiple-comparison test were used for calculations. *, $P < 0.05$; **, $P < 0.01$.

separate expression factor. The majority of the identified proteins are involved in central protein and energy metabolism, cellular redox homeostasis, nucleotide metabolism, and gene regulation. We also identified proteins that play roles in substrate uptake or in the degradation of various carbohydrates.

Several of the differentially expressed bacterial proteins reflect the adaptation of the metabolism of *E. coli* to the respective host diet. On the lactose diet, enzymes needed for amino acid biosynthetic processes were upregulated. For example, glutamate dehydrogenase (GdhA) and carbamoyl-phosphate synthase (CarA) were upregulated 2.3-fold and 3-fold on that diet. In contrast, enzymes involved in the degradation of amino acids were upregulated on the casein diet and downregulated on the lactose diet. For instance, glutaminase 1 (GlsA1) was downregulated 3-fold on the lactose diet (see Table S1 in the supplemental material).

The lactose diet also led to the induction of the Leloir pathway enzymes (all upregulated) galactose mutarotase (4.3-fold), galactokinase (2.3- to 6.6-fold), galactose-1-phosphate uridylyltransferase (2.2- to 7.8-fold), and UDP-glucose-4-epimerase (3.6-fold), which are required for the utilization of galactose. Interestingly, proteins involved in the oxidative stress response of *E. coli* were also induced on the lactose diet (Table 4). With few exceptions, most of these oxidative stress-related proteins were upregulated on the lactose diet and downregulated on the casein diet. The genes encoding several of these proteins, including the ferric uptake regulatory protein (Fur), the alkyl hydroperoxide reductase (AhpR) subunits F (AhpF) and C (AhpC), and the DNA protection during starvation protein (Dps), are under the control of the OxyR transcriptional dual regulator. Compared to the results seen with the starch diet, Fur, AhpF, and Dps were upregulated by factors of 2.2 to 3.2 on the lactose diet. On the casein diet, AhpC and AhpF were downregulated by factors of 2.1 to 3.5.

Induction of the *oxyR* regulon by osmolytes. Because of the low oxygen partial pressure in the gut of mice, it is unlikely that oxidative stress triggered the upregulation of stress-related proteins on the lactose diet. We hypothesized that other environmental stimuli caused by the lactose diet were responsible for the OxyR-dependent stress response. The feeding groups differed in the composition of the intestinal contents, with lower amino acid concentrations and the presence of lactose in the lactose-fed mice on the one hand and the absence of carbohydrates in the mice fed the casein diet on the other. To elucidate the mechanism underlying the lactose-induced upregulation of OxyR-dependent gene transcription, the effects of carbohydrates such as glucose, lactose, sucrose, and sorbitol on *ahpCF* and *dps* gene expression were analyzed by luciferase reporter gene assays under aerobic and anaerobic conditions. There was no induction of the luminescence signal when water was added to the medium or when *E. coli* was transferred to the protein-rich SOC medium (2.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). In contrast, when 300 μM H_2O_2 (positive control) was added to the LB medium, the luminescence signal increased 4- to 6-fold under aerobic conditions (Fig. 4) but not under anaerobic conditions (Fig. 5). Increasing concentrations of glucose, lactose, sucrose, or sorbitol led to increased p*ahpCFp*::*luxAB* and p*dpsp*::*luxAB* luminescence signals. Under aerobic conditions, carbohydrate concentrations of 200 mM and 400 mM increased the luminescence signal 2- to 5-fold and 3- to 7-fold, respectively. Under anaerobic conditions, carbohydrate concentrations of 200 mM increased the luminescence signal to a similar extent (2- to 5-fold), while carbo-

TABLE 4 Redox homeostasis and stress response proteins with ≥2-fold expression changes[a]

Swiss-Prot accession no.	Gene	Protein description	Fold change[b]			
			Lactose diet vs. starch diet		Casein diet vs. starch diet	
			SI	C	SI	C
P0A9A9	*fur*	Ferric uptake regulation protein		3.1		
P0ABT2	*dps*	DNA protection during starvation protein		3.2		
P68066	*grcA*	Autonomous glycyl radical cofactor	2.3	−8.8		
P0AFF6	*nusA*	Transcription elongation protein nusA	3.1			
P0A6H5	*hslU*	ATP-dependent protease ATPase subunit HslU			−4.5	
P05055	*pnp*	Polyribonucleotide nucleotidyltransferase		−6.6		
P0A9D2	*gst*	Glutathione S-transferase				−2.7
P35340	*ahpF*	Alkyl hydroperoxide reductase subunit F	3.2	2.2	−3.5	
P0AE08	*ahpC*	Alkyl hydroperoxide reductase subunit C			−2.4	−2.1
P0A862	*tpx*	Thiol peroxidase				2.0
P0ACE0	*hybC*	Hydrogenase-2 large chain	2.6	2.0		
P38489	*nfnB*	Oxygen-insensitive NAD(P)H nitroreductase	2.2			
P39315	*qorB*	Quinone oxidoreductase 2	−4.8			

[a] Comparison of levels in samples obtained from mice fed a lactose or casein diet to the levels in samples obtained from mice fed a starch diet.
[b] Values represent averages of results from 20 biological replicates/feeding group. 2D-DIGE analyses were done in duplicate (small intestine casein diet and cecum starch diet), triplicate (small intestine starch diet and lactose diet), quadruplicate (cecum lactose diet), or quintuplicate (cecum casein diet) using pooled samples, dependent on the available material. $P \leq 0.05$ for all changes. SI, small intestine; C, cecum.

hydrate concentrations of 400 mM resulted in 4- to 12-fold-higher luminescence signals. Addition of 400 mM NaCl increased the luminescence 4-fold for clone p*ahpCFp*::*luxAB* and 6-fold for clone p*dpsp*::*luxAB* under aerobic conditions. Under anaerobic conditions, the NaCl-induced luminescence signal increased 7-fold for clone p*ahpCFp*::*luxAB* and 14-fold for clone p*dpsp*::*luxAB*. The levels of p*ahpCFp*::*luxAB* induction by carbohydrates or NaCl under aerobic and anaerobic conditions were in the same range, while those for p*dpsp*::*luxAB* were higher under anaerobic conditions.

To elucidate the dependence of *ahpCF* and *dps* expression on OxyR, luciferase reporter gene assays were done in mutants lacking the *oxyR* gene. In contrast to the situation seen with the wild type, stimulation with 300 μM H_2O_2 or 400 mM glucose, lactose, sucrose, or NaCl under aerobic and anaerobic conditions did not result in a luminescence signal in the OxyR mutant (Fig. 6). It may therefore be concluded that the induction of *ahpCF* and *dps* by these stimuli in the wild type was directly mediated by OxyR.

A common trait of NaCl and carbohydrates is their osmotic effect. Therefore, the osmolalities of the various media used in the reporter gene assays were determined (Fig. 7). H_2O_2 did not affect the osmolality of LB medium, while addition of water or SOC medium significantly decreased the osmolality. As expected, addition of glucose, lactose, sucrose, or sorbitol increased the medium osmolality. At a given concentration, addition of NaCl resulted in a 2-fold-higher increase in osmolality than addition of nonionic carbohydrates because of its dissociation into its constituent ions. Nevertheless, the luminescence signal intensity observed in *E. coli* MG1655 at a carbohydrate concentration of 400 mM was similar to that observed at 400 mM NaCl. An increase of the osmolality to 1,450 mosmol/kg (corresponding to 700 mM NaCl) did not lead to a further increase in the luminescence signal intensities but was similar to that observed with 400 mM NaCl (data not shown).

To uncover a possible correlation between the activity of the *ahpCF* and *dps* promoters and the medium osmolality, luciferase activities in *E. coli* MG1655 were plotted against medium osmolality. Under aerobic and anaerobic conditions, the luciferase activity correlated positively with increasing medium osmolality for *E. coli* clone *ahpCFp*::*luxAB* as well as *E. coli* clone *dpsp*::*luxAB* (Fig. 8).

OxyR-regulated genes are necessary under osmotic stress conditions. To investigate the biological relevance of the observed effects, mutants lacking the *ahpCF* or *oxyR* gene (see Fig. S2 in the supplemental material) were tested *in vitro*. The upregulation of OxyR-regulated proteins AhpF and Dps in the gut of mice fed the lactose diet suggested that expression was induced by the osmotic effect of carbohydrates. Therefore, nonfermentable sucrose was used as a model to investigate the growth of *ahpCF* and *oxyR* deletion mutants under osmotically constant conditions *in vitro*. Growth on LB medium or LB medium with sucrose was monitored under aerobic and anaerobic conditions (see Fig. S3 in the supplemental material). On LB medium, all strains grew to optical densities of 6.3 to 6.9 under aerobic conditions (Table 5) and 0.78 to 0.85 under anaerobic conditions (Table 6). When 400 mM or 700 mM sucrose was added to the LB medium, the wild type reached maximal optical densities of 4.5 or 2.8 under aerobic conditions and 0.43 or 0.22 under anaerobic conditions, respectively. Under aerobic conditions, the *E. coli* Δ*ahpCF* and *E. coli* Δ*oxyR* mutants grew to optical densities of 2.6 and 2.7 in the presence of 400 mM sucrose and to 1.1 and 1.3 in the presence of 700 mM sucrose (Table 5), respectively. Under anaerobic conditions, the optical densities of the *E. coli* Δ*ahpCF* and *E. coli* Δ*oxyR* mutants reached 0.31 and 0.33 after 24 h in the presence of 400 mM sucrose and 0.11 in the presence of 700 mM sucrose (Table 6). Under both sets of conditions, these values were significantly lower ($P \leq 0.05$) than those observed for the wild type, indicating the importance of these genes for successful growth under osmotic stress conditions.

Furthermore, while the doubling times of the wild type and the mutant on LB medium were similar, in the presence of sucrose the doubling time of the *E. coli* Δ*oxyR* mutant was greater than that of

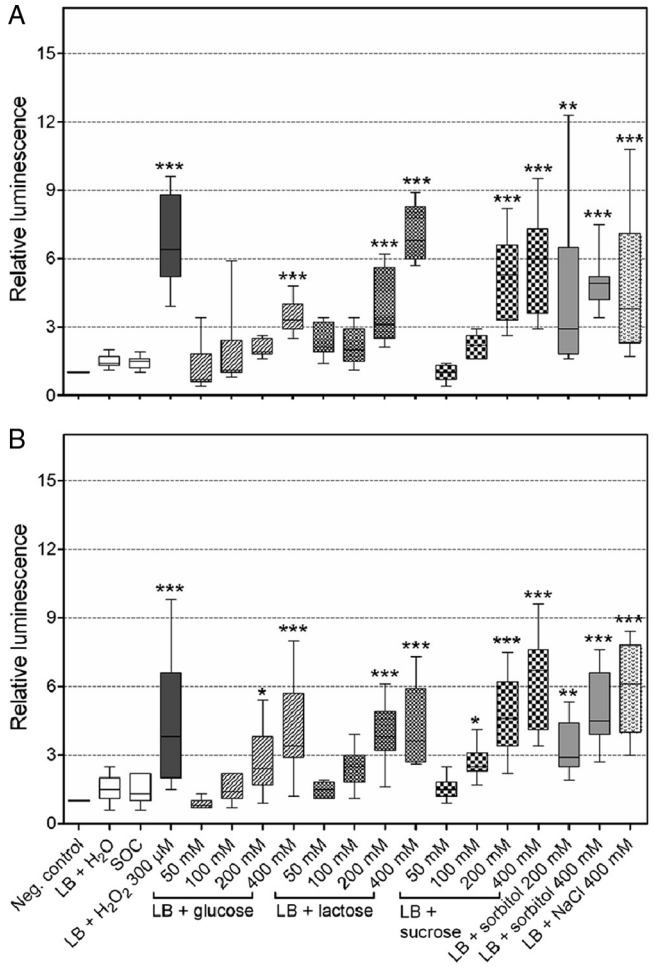

FIG 4 Induction of the *ahpCF* and *dps* promoters in *E. coli* MG1655 by different osmolytes under aerobic conditions after 30 min of incubation. Relative luminescence data for *E. coli* MG1655 carrying either p*ahpCFp*::*luxAB* (A) or p*dpsp*::*luxAB* (B) are shown. Units of luciferase activity were normalized based on values determined for clones grown on LB without the addition of osmolytes. Data are expressed as medians ($n = 11$). Kruskal-Wallis one-way analysis of variance and Dunn's multiple-comparison test were used for calculations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Neg., negative.

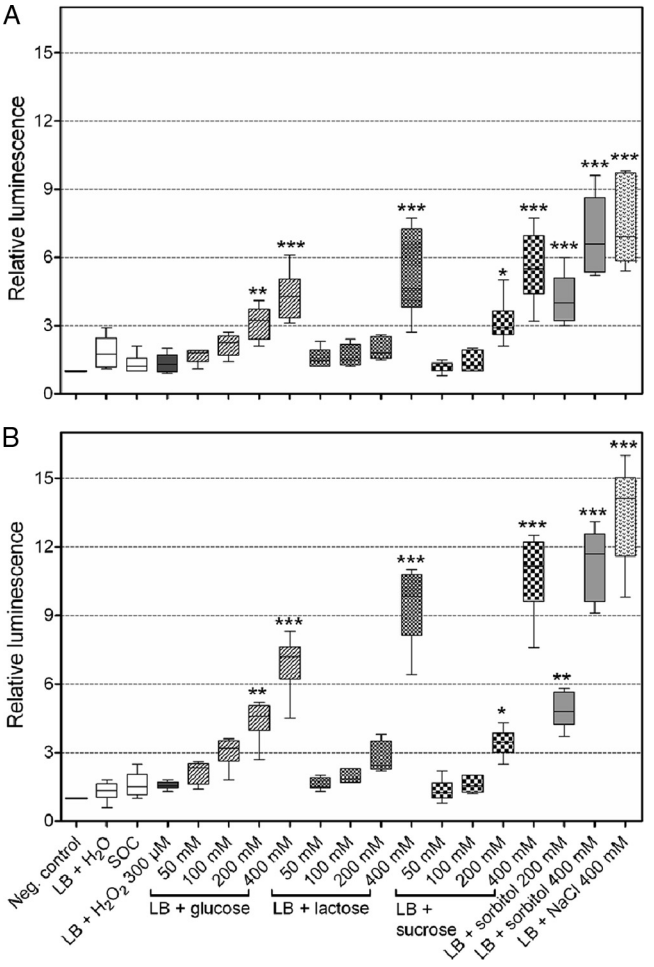

FIG 5 Induction of the *ahpCF* and *dps* promoters in *E. coli* MG1655 by different osmolytes under anaerobic conditions after 30 min of incubation. Relative luminescence data for *E. coli* MG1655 carrying either p*ahpCFp*::*luxAB* (A) or p*dpsp*::*luxAB* (B) are shown. Units of luciferase activity were normalized based on values determined for clones grown on LB medium without the addition of osmolytes. Data are expressed as medians ($n = 6$). Kruskal-Wallis one-way analysis of variance and Dunn's multiple-comparison test were used for calculations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the wild type. In contrast, doubling times did not differ between the *E. coli* Δ*ahpCF* mutant and the wild-type *E. coli* strain (Tables 5 and 6). The significantly longer doubling time seen with the *E. coli* Δ*oxyR* mutant ($P \leq 0.05$) might reflect the loss of the expression of all OxyR-dependent genes, whereas the *E. coli* Δ*ahpCF* mutant is devoid of only the OxyR-dependent *ahpCF*.

The deletions causing the observed growth defects could be complemented by plasmids containing the corresponding genes, including physiologically relevant promoters restoring wild-type behavior (see Table S2 in the supplemental material). The restoration of the wild-type phenotype confirmed that the observed growth retardation of the knockout mutants in the presence of sucrose was not due to a secondary mutation.

High levels of medium osmolality did not result in the formation of free radicals. Aldsworth and Dodd proposed that free radicals are formed in response to heat, osmotic stress, or ethanol stress (1, 10). To elucidate the relation between free radical formation and the observed induction of OxyR-dependent genes, dihydrorhodamine 123-stained *E. coli* cells were exposed to H_2O_2, glucose, lactose, or sucrose (Fig. 9). Fluorescence, which is proportional to free radical formation, increased 2-fold after exposure to 600 μM H_2O_2 or 50 mM glucose ($P < 0.01$) compared to incubation in medium without fermentable substrates. Addition of 50 mM lactose increased the free radical production 1.7-fold. However, this increase was not statistically significant. Nonfermentable sucrose (400 mM) did not induce the formation of free radicals. Increases in medium osmolality mediated by the addition of 400 mM fermentable glucose or lactose did not further increase the radical formation observed at a 50 mM concentration of the same carbohydrate. These experiments show that the catabolism of carbohydrates is accompanied by the formation of free radicals. In contrast, high levels of medium osmolality caused by glucose, lactose, or sucrose did not have the same result. These results and those of the *in vitro* luciferase reporter gene assays and growth experiments show that osmotic pressure is the crucial factor that leads to the induction of the OxyR-dependent proteins AhpF and Dps.

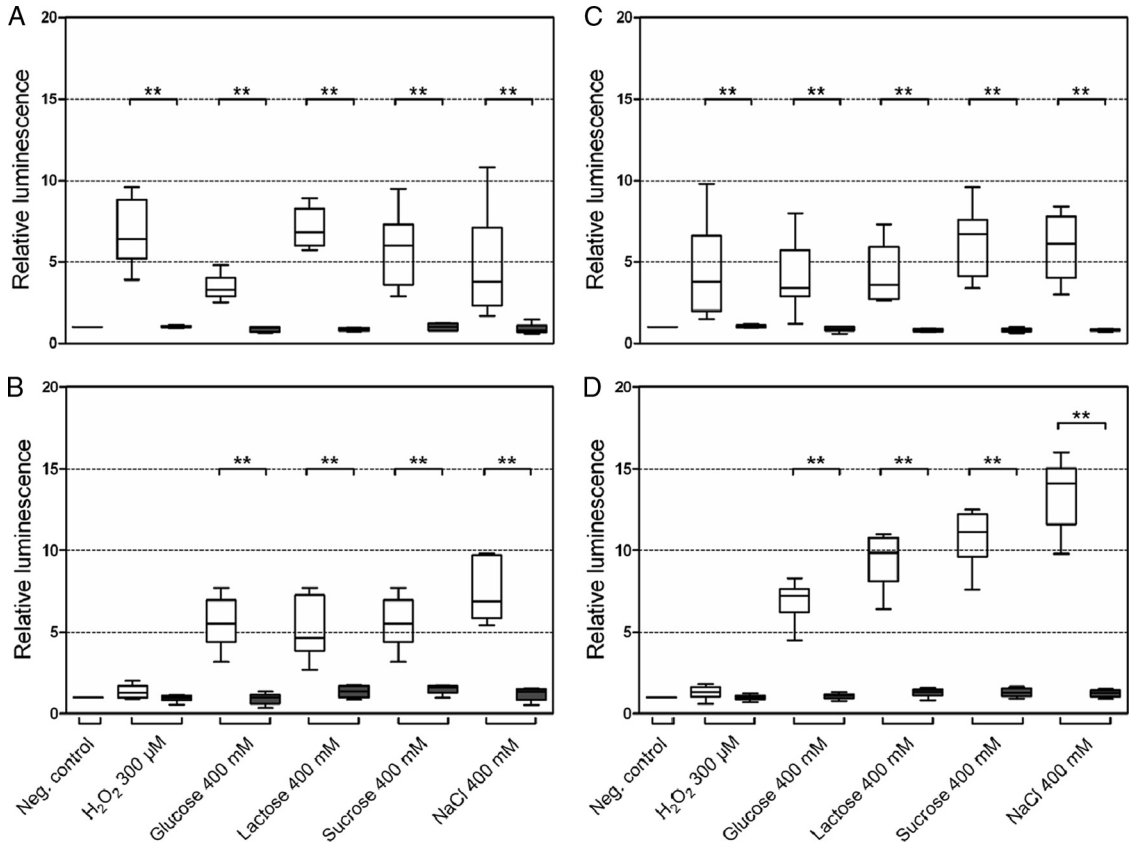

FIG 6 Comparison of levels of induction of the *ahpCF* and *dps* promoters in *E. coli* MG1655 and the *E. coli* Δ*oxyR* mutant by different osmolytes after 30 min of incubation under aerobic (A and C) and anaerobic (B and D) conditions. Relative luminescence data for *E. coli* MG1655 (white bars) and the *E. coli* Δ*oxyR* mutant (gray bars) carrying either p*ahpCFp*::*luxAB* (A and B) or p*dpsp*::*luxAB* (C and D) are shown. Units of luciferase activity were normalized based on values determined for clones grown on LB medium without the addition of osmolytes. Data are expressed as medians ($n = 6$). The Mann-Whitney test was applied. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

Comparison of the proteome of intestinal *E. coli* of mice fed the lactose diet with that of mice fed the starch diet reflected the *in situ* adaptation of this organism to lactose. Induction of Leloir pathway enzymes and of enzymes for amino acid and nucleotide biosynthesis demonstrates the validity of our experimental approach. While the Leloir pathway enzymes are required for the utilization of lactose, enzymes such as GdhA and CarA are involved in the biosynthesis of glutamate (31) or of arginine and pyrimidine nucleotides (34). Their upregulation indicates a shortage of intestinal amino acids and pyrimidine in the intestine. A shortage of nucleosides in the mouse intestine has previously been reported by Vogel-Scheel et al., who identified key enzymes of purine and pyrimidine biosynthesis (encoded by *purC* and *pyrBI*) as necessary for *E. coli* for successful colonization of the mouse intestine (37).

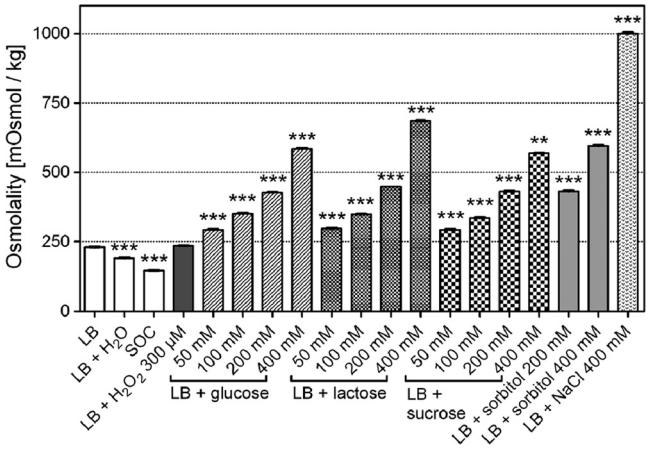

FIG 7 Osmolality of LB medium, SOC medium, and LB medium supplemented with water, NaCl, H_2O_2, or different types and concentrations of carbohydrates. Data are expressed as means ($n = 3$). One-way ANOVA and Dunnett's multiple-comparison test were used for calculations. **, $P < 0.01$; ***, $P < 0.001$.

The induction of enzymes involved in the degradation of peptides and amino acids in intestinal *E. coli* of mice fed the casein diet was hardly detectable because of fewer and smaller differences in the organism's proteome compared to the results seen with the lactose diet (and for the proteome of mice fed each of those diets in comparison to the proteome of mice fed the starch diet). This may be due to the fact that synthesis and degradation of amino acids involves numerous enzymes that undergo many changes that are subtler and less obvious than those observed in response to the lactose diet. Therefore, the lactose diet appears more suitable than the casein diet for investigating bacterial adaptation to dietary nutrients in the intestine.

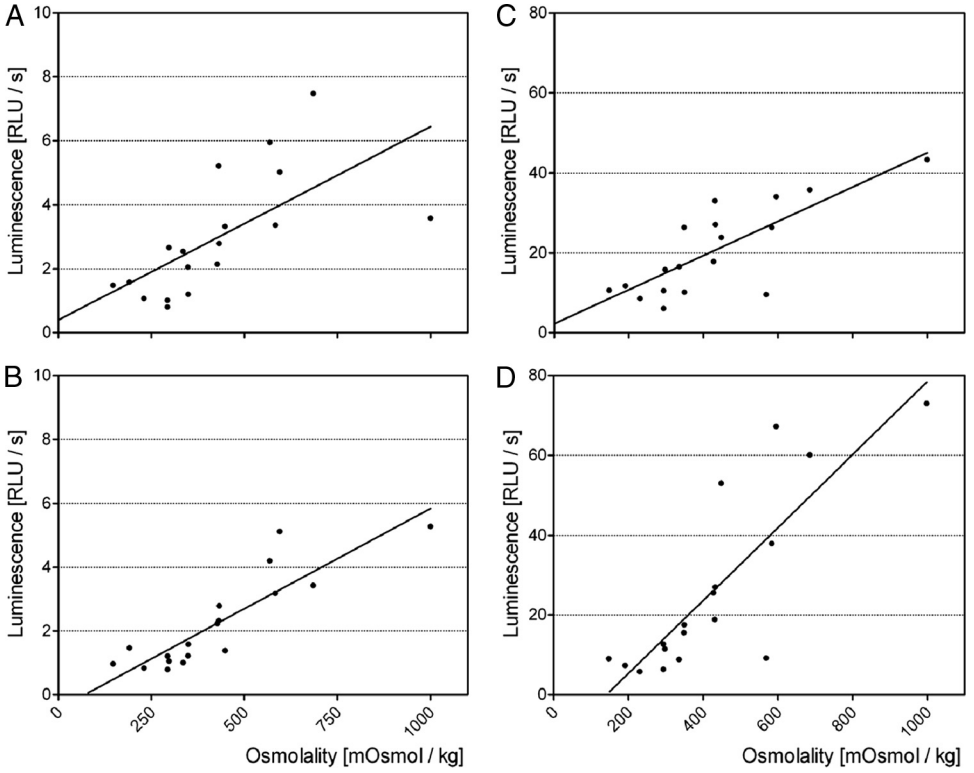

FIG 8 Correlation of *ahpCF* and *dps* promoter activities in *E. coli* MG1655 and medium osmolality for all investigated *in vitro* luciferase reporter gene assays. (A) *ahpCF*, aerobic growth conditions ($R^2 = 0.836$; $P \leq 0.001$). (B) *ahpCF*, anaerobic growth conditions ($R^2 = 0.878$; $P \leq 0.001$). (C) *dps*, aerobic growth conditions ($R^2 = 0.716$; $P = 0.01$). (D) *dps*, anaerobic growth conditions ($R^2 = 0.868$; $P \leq 0.001$). Data are expressed as medians for promoter activity and means for osmolality; statistical analysis was done with Spearman's rank correlation coefficient.

That proteins involved in the oxidative stress response of *E. coli* were upregulated in mice fed the lactose diet but mostly downregulated in mice fed the casein diet was an unexpected finding. The genes encoding the upregulated proteins such as Dps, AhpF, and Fur belong to the *oxyR* regulon. Other members of this regulon encoding such proteins as Sod, KatG, and TrxB were not identified. Since only approximately 60% of the total number of detected differentially expressed proteins were clearly identified by mass spectrometry, we cannot exclude the possibility that other members of the *oxyR* regulon were among the nonidentified spots.

Although the OxyR transcriptional regulator activates the expression of target genes under prooxidant conditions (35), this regulon includes genes helping in the protection of the bacterial cell not only against oxidative stress but also against heat shock, pH, and salt stress (8, 17). Since it is difficult to imagine that the lactose caused oxidative stress in the intestine, we tested the possibility that other forms of stress activated the *oxyR* regulon.

TABLE 5 Growth of *E. coli* MG1655, Δ*ahpCF*, and Δ*oxyR* strains under aerobic conditions[a]

Medium	Parameter	Value (minimum:maximum) for indicated *E. coli* strain		
		MG1655	Δ*ahpCF*	Δ*oxyR*
LB	OD_{600} after 24 h	6.9 (5.5:8.0)	6.3 (5.6:7.0)	6.6 (6.3:6.7)
	t_d (min^{-1})	33 (31:35)	32 (27:36)	39 (34:39)
LB plus 400 mM sucrose	OD_{600} after 24 h	4.5 (3.8:4.5)	2.6 (2.5:2.9)[b]	2.7 (2.5:2.9)[b]
	t_d (min^{-1})	36 (35:37)	36 (34:40)	47 (44:51)[b]
LB plus 700 mM sucrose	OD_{600} after 24 h	2.8 (2.5:3.1)	1.0 (1.0:1.2)[b]	1.3 (1.3:1:3)[b]
	t_d (min^{-1})	36 (33:37)	40 (30:44)	49 (44:56)[b]

[a] Data are expressed as medians and minimums versus maximums ($n = 4$). t_d, doubling time.

[b] Data represent comparisons of the results obtained with *E. coli* MG1655 versus mutant *E. coli* under conditions that included use of the same medium (Mann-Whitney test; $P \leq 0.05$).

To identify the nature of the stress response and to clarify the role of lactose as an inducer, we compared its effect on the expression of *ahpCF* and *dps* with that of various other carbohydrates by the use of *in vitro* luciferase reporter gene assays. Glucose, lactose, sucrose, and sorbitol activated the *ahpCF* and *dps* promoters under both aerobic and anaerobic conditions. Proteins had no effect on the transcription of these genes, suggesting that the osmotic pressure exerted by the carbohydrates caused this effect. Since the observed effects were not detected in the *oxyR* deletion mutants, other transcription factors such as sigma 24, 38, and 70, which can bind to the promoter regions of the analyzed genes, were not involved (4, 25, 30, 33).

The carbohydrate concentrations needed for the *in vitro* induction of *ahpCF* and *dps* were much higher than those observed in the intestines of mice fed the lactose diet. To estimate the maximal carbohydrate concentrations that are possibly reached in the mouse intestine, we assumed an uptake of approximately 3 g of diet and 6 ml of water per mouse and day. Based on this assumption, the concentration of lactose in the small intestine would be approximately 100 mM and that of sucrose around 200 mM. Compared with the carbohydrate concentrations needed for the *in vitro* induction of the *ahpCF* and *dps* promoters (200 mM to 400 mM), these theoretical concentrations would be sufficient to in-

TABLE 6 Growth of *E. coli* MG1655, Δ*ahpCF*, and Δ*oxyR* strains under anaerobic conditions[a]

Medium	Parameter	Value (minimum:maximum) for indicated *E. coli* strain		
		MG1655	Δ*ahpCF*	Δ*oxyR*
LB	OD_{600} after 24 h	0.85 (0.79:0.89)	0.82 (0.76:0.9)	0.78 (0.77:0.79)
	t_d (min^{-1})	41 (35:45)	41 (39:43)	37 (36:42)
LB plus 400 mM sucrose	OD_{600} after 24 h	0.43 (0.39:0.52)	0.31 (0.30:0.33)[b]	0.33 (0.30:0.36)[b]
	t_d (min^{-1})	50 (39:54)	54 (48:63)	60 (59:68)[b]
LB plus 700 mM sucrose	OD_{600} after 24 h	0.22 (0.19:0.27)	0.11 (0.11:0.11)[b]	0.11 (0.10:0.12)[b]
	t_d (min^{-1})	66 (49:84)	85 (61:98)	89 (85:93)[b]

[a] Data are expressed as medians and minimums versus maximums ($n = 4$). t_d, doubling time.
[b] Data represent comparisons of the results obtained with *E. coli* MG1655 versus mutant *E. coli* under conditions that included use of the same medium (Mann-Whitney test; $P \leq 0.05$).

duce the expression of *oxyR*-dependent genes. The fluctuating nutrient availability during the feeding and sleeping periods of the mice and the resulting differences in the absorption rate (26) might be the reason for the lower concentrations detected *in vivo*. We assume that a higher osmotic stress present in the upper part of the small intestine immediately after feeding induced the expression of Dps, AhpF, and Fur. Since changes in the proteome are expected to require some time to become effective, it is reasonable to assume that proteins upregulated in response to osmotic stress remained detectable after sucrose absorption by the host.

The observed induction of *ahpCF* and *dps* by carbohydrates is in agreement with results of Weber et al., who reported the induction of AhpC and Dps by 400 mM NaCl and of Dps by 700 mM sorbitol (38). Our results demonstrate that both monosaccharides and disaccharides induce OxyR-dependent genes. The digestibility of the supplemented carbohydrates had no influence on the induction of *ahpCF* and *dps*, because both fermentable and nonfermentable carbohydrates induced their transcription. Since the latter are not converted, they cannot cause oxidative stress.

Growth inhibition of *ahpCF* or *oxyR* deletion mutants by sucrose under aerobic and anaerobic conditions indicates a role of these genes in *E. coli*'s osmotic stress response. However, we expected a stronger growth inhibition of the *E. coli* Δ*oxyR* mutant than of the *E. coli* Δ*ahpCF* mutant at high sucrose concentrations, because the deletion of *oxyR* affects several genes, including *ahpCF*. Interestingly, maximal cell densities for the mutants were similar, whereas the growth rate of the *E. coli* Δ*oxyR* mutant was lower than that of the *E. coli* Δ*ahpCF* mutant (Tables 5 and 6). Our data suggest that expression of genes belonging to the *oxyR* regulon, in particular, *ahpCF*, enables *E. coli* to better cope with osmotic stress.

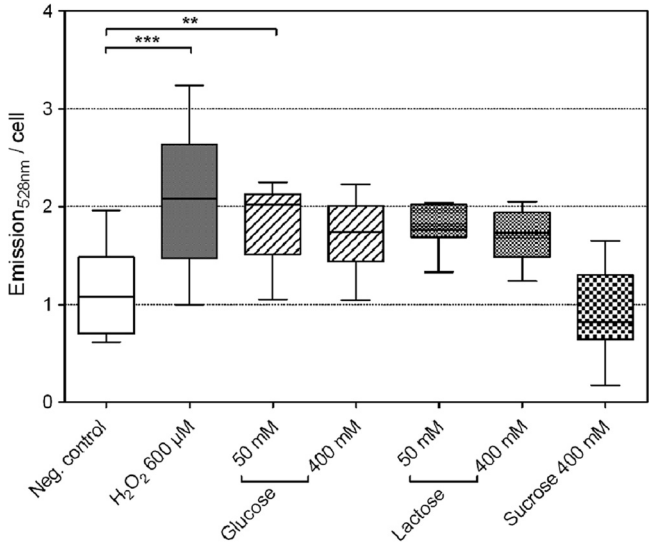

FIG 9 Generation of free radicals after exposure of *E. coli* MG1655 to H_2O_2, fermentable carbohydrates (glucose or lactose), or nonfermentable sucrose. Data are expressed as medians ($n = 17$ for negative control and H_2O_2, $n = 7$ for others). Kruskal-Wallis one-way analysis of variance and Dunn's multiple-comparison test were used for calculations. **, $P < 0.01$; ***, $P < 0.001$.

Under conditions of high osmolality, water leaves the cell, resulting in reduced cell turgor. To adapt to high extracellular solute concentrations, bacteria increase their intracellular solute pool by uptake or synthesis of organic osmolytes such as trehalose, polyols, or free amino acids (23). We hypothesize that compatible solutes in the mouse intestine are sufficiently scarce that *E. coli* has to synthesize them. The semisynthetic diets fed to the mice undergo almost complete absorption in the mouse intestine, as reflected by the low wet weights of the collected intestinal contents. The intestinal concentration of compatible solutes is therefore expected to be marginal. The observed induction of glutamate dehydrogenase *in vivo* seen with the lactose diet might be an adaptation to the osmotic stress situation, as glutamate serves to maintain the steady-state K^+ pool after an osmotic upshift (23). The intracellular synthesis of compatible solutes might not be sufficient or might take too long to alleviate osmotic stress in *E. coli*. Therefore, induction of the *oxyR* regulon could be a supportive measure for protecting *E. coli* against osmotic stress.

OxyR-regulated *ahpCF* codes for an alkyl hydroperoxide reductase, which converts alkyl hydroperoxides to their corresponding alcohols (7). We currently do not know how this enzyme might help bacterial cells in adapting to osmotic stress. OxyR-regulated Dps protects bacterial DNA from damage by oxidative stress as caused by H_2O_2 through direct binding and formation of a DNA-protein crystal (12, 27, 39). Interestingly, hyperosmotic stress imposed by NaCl causes DNA damage and results in DNA double-strand breaks in murine kidney cells (24). Such breaks of the DNA phosphodiester backbone are commonly induced by ionizing radiation or H_2O_2 (16). Therefore, we hypothesize that there is a link between osmotic stress and DNA damage also in bacteria, as is supported by our results that indicate DNA protection is an adaptive mechanism during osmotic stress.

One scenario explaining how such DNA damage is caused might be the formation of free radicals in response to osmotic stress as proposed by Aldsworth et al. (1, 10). The "suicide through

stress" theory proposes that bacteria produce a burst of intracellular free radicals such as H_2O_2 and O_2^- under conditions of heat, osmotic, or ethanol stress, leading to cell injury or death. These free radicals have so far been identified only in aerobic organisms with respiratory metabolism (*Salmonella enterica* serovars, *E. coli*, *Staphylococcus aureus*, *Mycobacterium smegmatis*) but not in a strictly fermentative organism (*Streptococcus mutans*), leading to the assumption that the proposed suicide response is linked to aerobic metabolism (10). In our experiments, induction of OxyR-dependent genes occurred under both aerobic and anaerobic conditions. Since high osmolality caused by carbohydrates did not affect formation of free radicals detected by dihydrorhodamine 123, other mechanisms of osmotic stress-dependent OxyR activation have to be envisaged.

First, the mechanism of OxyR activation might be similar to those seen under conditions of oxidative stress, in which H_2O_2 directly activates OxyR via the formation of an intramolecular disulfide bond between Cys199 and Cys208 (40). After hyperosmotic shock, the rapid cellular consequences such as water efflux across the cell membrane might result in closer contact of normally separated protein domains, which in turn might lead to the spontaneous formation of disulfide bridges. Such a mechanism has been proposed for protein damage after freezing injury (28). Second, it is possible that OxyR itself is a sensor not only for oxidative but also for osmotic stress or that an additional component exists that interacts with OxyR.

In conclusion, our report demonstrates the induction of some OxyR-dependent proteins in intestinal *E. coli* of mice fed a lactose-rich diet. Further *in vitro* experiments revealed that these proteins are responsible for the ability of *E. coli* to better cope with high-osmolality growth conditions. These results indicate an overlap of oxidative and osmotic stress responses in *E. coli* and the importance of these responses for the organism's adaptation to carbohydrate-rich host diets.

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