Metabolic Shift of *Escherichia coli* under Salt Stress in the Presence of Glycine Betaine

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An important area of food safety focuses on bacterial survival and growth in unfavorable environments. In order to understand how bacteria adapt to stresses other than nutrient limitation in batch cultures, we need to develop mechanistic models of intracellular regulation and metabolism under stress. We studied the growth of *Escherichia coli* in minimal medium with added salt and different osmoprotectants. To characterize the metabolic efficiency with a robust parameter, we identified the optical density (OD) values at the inflection points of measured “OD versus time” growth curves and described them as a function of glucose concentration. We found that the metabolic efficiency parameter did not necessarily follow the trend of decreasing specific growth rate as the salt concentration increased. In the absence of osmoprotectant, or in the presence of proline, the metabolic efficiency decreased with increasing NaCl concentration. However, in the presence of choline or glycine betaine, it increased between 2 and 4.5% NaCl before declining at 5% NaCl and above. Microarray analysis of the transcriptional network and proteomics analysis with glycine betaine in the medium indicated that between 4.5 and 5% NaCl, the metabolism switched from aerobic to fermentative pathways and that the response to osmotic stress is similar to that for oxidative stress. We conclude that, although the growth rate appeared to decrease smoothly with increasing NaCl, the metabolic strategy of cells changed abruptly at a threshold concentration of NaCl.

The growth efficiency of microorganisms is traditionally evaluated in two ways: by evolutionary principles which are based on fitness measurements, usually division rate, in a competitive environment, and by the demands of biotechnology, which is focused mostly on the growth yield of bioreactors, in terms of product or biomass obtained per unit of substrate. However, optimum fitness and growth yields are not always compatible (1). To optimize industrial applications, several modeling approaches have been proposed to facilitate the design of efficient strains. For instance, it is possible to predict biomass growth from substrate consumption in chemostats using flux balance analysis (FBA), a technique based on stoichiometric constraints applied to the metabolic network of a cell. For the model microorganism *Escherichia coli* in particular, it has been shown that the bacteria optimize their biomass production based upon the constraints on the fluxes of substrate consumption (2, 3). This is not valid for all bacteria, as, for example, *Lactobacillus plantarum* produces lactic acid at the expense of its optimal state (4). In batch culture, where nutrients are abundant, even in exponential phase, which is often assumed to be balanced growth, it has been shown that growth is not optimized in the same way as in chemostats (3, 5), so the physiological state of bacteria in batch cannot be extrapolated from experiments in chemostats.

In the food industry, predictions of the specific growth rates of food-borne pathogens are based on experiments carried out in batch. The most important environmental factors affecting the specific growth rate are temperature, pH, and water activity (6). A simple means to control the latter is by the addition of salt. Under osmotic stress, the metabolism of the bacterium is modified to accommodate changes in cytoplasmic water activity and it accumulates osmoprotectants (7). Some osmoprotectants, such as glycine betaine or proline, can be directly imported into the cell through the channels encoded by the *proP* gene and the *proU* operon (8). If there is no osmoprotectant in the medium, *Escherichia coli* converts glucose into trehalose by activation of the *ostA*

and *ostB* genes (9). Alternatively, the cell can metabolize some precursors, such as choline, for example, into the osmoprotectant glycine betaine. This two-step conversion is catalyzed by *betA* and *betB* and is regulated by a specific system, which is sensitive to oxygen, osmotic stress, and temperature (10, 11). With *E. coli*, we have shown *in silico* that the diversion of carbon source to import or produce osmoprotectants and modify the glutamate content of the cell could not alone explain the observed decrease of growth in the presence of NaCl if biomass production was optimized (12). This was especially evident in the case of the presence of glycine betaine, which is one of the most widespread osmoprotectants found in the environment and in food (13, 14). In this study, we propose to evaluate the effect of NaCl and the presence of different osmoprotectants on the cell physiology by optical density (OD) using a temperature-controlled automated plate reader, measuring OD in 200 wells (Bioscreen, Finland).

OD is commonly used to measure the kinetics of bacterial growth both in batch and in chemostat. In food safety studies, techniques to determine the growth rate have been developed using a Bioscreen, because it is less labor-intensive than traditional plate counts (15). One of the most accurate ways to determine the specific growth rate with a Bioscreen is to estimate it from the time to reach a set detection level, as a function of inoculation level (16–18). Krist et al. (19) measured the maximum OD in a limiting

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range of glucose concentrations to infer the energetic expenditure during different stresses. They concluded that, with osmotic stress, the energy expenditure was not correlated with the growth rate and that the addition of glycine betaine extended the range of stress that the bacteria could withstand.

Here, we propose to investigate both the kinetics and the intracellular changes of the model bacterium *E. coli*, in order to better understand its response to increasing NaCl at the systems level. First, we introduce a novel quantification of metabolic efficiency by describing how the inflection point of the “OD versus time” growth curves (primary modeling) depends on the glucose concentration (secondary modeling). A parameter of the secondary model is considered a measure of metabolic efficiency, and the effect of NaCl on this parameter is assessed. We compare the effect of different osmoprotectants (glycine betaine, choline, and proline) on the metabolic efficiency and the specific growth rate. In order to better understand the effect of NaCl on metabolism, we study the changes of gene expression as well as proteomic responses as a function of NaCl concentration in the presence of glycine betaine. Finally the implications of our findings are discussed in terms of modeling bacterial strategy as a tradeoff between energetic growth and stress management.

MATERIALS AND METHODS

Organism. *Escherichia coli* K-12, MG1655, was stored in 40% glycerol at -80°C .

Determination of viable count and dry weight. *E. coli* was inoculated into 1-liter lots of basic minimal medium (BMM) (20) with no added salt and incubated statically at 37°C . When growth was visible, a sample was taken for OD measurement in a spectrophotometer (OD_{600} , wavelength of 600 nm) and for viable count (CFU) on tryptone soy agar plates incubated 24 h at 37°C . At the same time, samples ($n = 2$; 250 ml) were transferred to centrifuge tubes and centrifuged at $14,300 \times g$ for 20 min. The supernatant was carefully removed and the pellet resuspended in 10 ml BMM with no glucose (wash BMM). The suspension was centrifuged again and the pellet resuspended in 2 ml wash BMM in a weighed 2-ml centrifuge tube. This was spun for 2 to 3 min at $14,460 \times g$ and the liquid carefully removed. Samples were dried at 42°C for 4 days and the tubes weighed. This was repeated for a range of OD measurements. According to the Beer-Lambert law, the OD is proportional to biomass (BM). We found that, for OD_s of >0.3 , the equation $\text{BM (mg/ml)} = -0.11 + 0.6 \times \text{OD}_s$ is a good description of this proportionality ($R^2 = 0.99$).

Optical density experiments. *E. coli* was recovered from frozen glycerol stock in BMM, incubated for 24 h at 37°C , and transferred three times, 100 μl to 10 ml, in low-glucose BMM (0.05%), each incubated 24 h at 37°C . The cells were washed by centrifugation, and the pellet was resuspended in wash BMM. This was repeated three times. The final pellet was resuspended in 10 ml wash BMM and 25 μl dispensed into wells in Bioscreen plates. For growth rate experiments, the washed cells were diluted in wash BMM (1/2, 1/10, 1/20). Viable counts were made of cell suspensions to determine the inoculum level in each well.

BMM was prepared with a range of glucose and salt concentrations and filter sterilized. Osmoprotectants (filter sterile stock solutions, 150 mM) were added (10 μl in 10 ml BMM to give 150 μM) as required. Aliquots (375 μl) were added to wells containing washed cells. Plates were incubated at 37°C , without shaking, for up to 5 days; OD readings were recorded at 10-min intervals (wavelength, 600 nm).

Comparison of Bioscreen and spectrophotometer OD measurements. To link the optical density measurements in the Bioscreen to the optical density measurements obtained in the spectrophotometer, a calibration curve was determined. *E. coli* was grown in BMM, and duplicate samples with or without osmoprotectant were taken for OD readings in the Bioscreen (400 μl per well) and in the spectrophotometer (2-ml cu-

FIG 1 “OD versus time” curve with 0.9 g/liter of glucose and 3% NaCl. No maximum OD can be defined as a parameter of the sigmoid curve, instead its inflection (break) point is considered, where the second derivative of the curve turns from positive to negative. It can be assumed there is a sudden change in the system at this point. On this plot, the time at the inflection is ca. 15 h, and the respective OD_b value is ca. 0.34.

vettes), using uninoculated BMM as a blank. The relationship between the OD values was linear, with $\text{OD}_s = 0.7695 \times \text{OD} + 0.0817$ ($R^2 = 0.99$), and did not depend on the nature of the osmoprotectant added to the medium.

Determination of growth rates and metabolic efficiency. The specific growth rate, μ , was determined by calculating the inverse of the slope when linear fitting was applied to detection time data (time to reach an OD of 0.12) against the natural logarithm of the respective inoculum levels in the wells of the Bioscreen (17).

In theory, the maximum biomass concentration obtained in wells with a limiting concentration of glucose increases linearly with glucose (19). However, as shown in Fig. 1, in many of our experiments, instead of reaching a maximum OD, a slow linear increase could be observed after the exponential phase. The vast majority of OD growth curves had a distinctive breakpoint, where the slope of the OD curve changed suddenly, most often indicating an inflection point. The locations of these breakpoints, OD_b , were calculated by a numerically smoothed approximation of the slope of the curve, utilizing the fact that the observation times were equidistant. For each time point, t , the slope was approximated by fitting a linear function to seven points located symmetrically around t . The breakpoint was defined as the point at which such adjacent numerical slopes changed by more than 85%.

It is reasonable to assume that this is a characteristic point in the metabolic process, even if we do not know exactly what happens at this time point. Therefore, the differential quotient $\Delta\text{OD}_b/\Delta\text{glucose}$ could be a possible measure of metabolic efficiency. However, after inspecting the data, it became evident that using the logarithm of the initial glucose made the relationship with OD_b linear, which makes statistics much more robust. Moreover, the residuals to the linear model became homogeneous after this transformation. These observations, and the transformation of the OD_b values to biomass, BM_c , using the calibration curves above, led to the definition of metabolic efficiency, $\Delta\text{BM}_c/\Delta\log(\text{glucose})$. In Fig. 4, the intercepts of the BM_c versus $\log(\text{glucose})$ curves are also shown, because the estimates (linear regression) for the slope and intercept are not independent.

Microarray measurements. *E. coli* was grown to exponential phase in BMM with a range of salt concentrations at 2, 3.5 (2 replicates), 4.5, 5, and 5.5% NaCl. Cells were inoculated at 10^4 CFU/ml, and cultures were incubated to reach 10^7 CFU/ml, corresponding to an OD_s of about 0.3 (cultures entered stationary phase at 10^8 CFU/ml) before harvesting. Total RNA was extracted, and cDNA was synthesized by reverse transcription as

described previously (21). Aliquots (2 μ g) of total RNA were labeled with Cy5-dUTP (GE Healthcare, United Kingdom) during reverse transcription to cDNA. Genomic DNA (gDNA) was purified from exponentially growing cells, digested with Sau3AI, and labeled with fluorescent nucleotides as previously described (21), except that Cy3-dUTP was substituted for Cy3-dCTP. A 0.5- μ g aliquot of Cy3-labeled *Escherichia coli* K-12 MG1655 DNA was added to the Cy5-labeled cDNA, the mix was hybridized on a microarray (*E. coli* gene expression microarray, G4813A-020097, 8 by 15 K; Agilent Technologies United Kingdom Ltd.) (21). Microarrays were washed and then scanned using an Axon 4000B laser scanner (Axon Instruments, California, USA). The data from detected features were initially processed using the GenePix Pro v.6.0 software supplied with the scanner, and the .gpr files were exported to Excel for further analysis.

Microarray data normalization. For each spot, the median of the spot intensity minus the background was considered. Because we wanted to compare different environmental conditions, the cDNA signal under each condition was normalized to that of gDNA with $y = \ln(\text{cDNA/gDNA})$. To ensure that the fluorescence measurements were independent of the overall signal intensity of the spot, the values of y were made independent of $(\ln \text{cDNA} + \ln \text{gDNA})/2$ with a linear shift (22). The y values were normalized to have 0 mean and a constant deviation.

Statistical analysis of the gene expression profiles. Monte-Carlo sampling was used to determine whether there exists a boundary value of the NaCl concentration that divides the expression data into two regimes (23). The methodology is summarized below and can be followed in Tables S2 and S3 of the supplemental material.

Let x be the NaCl concentration shifted to its smallest value, $x = \text{NaCl}\% - 2\%$, and let x_j be those concentrations at which expression data were generated. Consider the normalized expression values for each x_j , where $j = 1 \dots S$ and S is the number of experimental salt concentrations, with replication: $y_{ij} = y_i(x_j)$ ($j = 1 \dots S$; $i = 1 \dots N$, where N is the number of gene probes).

Our null hypothesis is that, for each probe, the expression value changes with the NaCl concentration in a monotonous manner. In other words, assuming simple linearity, the null model M_0 reads $y_i(x) = ax + b_i + \delta$ ($i = 1 \dots N$), where, due to the normalization, the δ random error is from the same distribution irrespective of the probe and the salt concentration.

We used F-tests, for each probe, to check whether a two-regime model, M_1 , describes the data significantly better (24). In M_1 , a gene-independent threshold NaCl value, s_i^* , divides the transcriptomic response into two separate linear models (see Fig. 5): $y_i(x) = c_i^{(1)}x + d_i^{(1)} + \varepsilon$ ($x < s_i^*$) and $y_i(x) = c_i^{(2)}x + d_i^{(2)} + \varepsilon$ ($x \geq s_i^*$) ($i = 1 \dots N$).

The questions are these: (i) which s_i^* experimental salt value has the highest probability to be a boundary if two regimes are assumed ($i = 1 \dots N$) and (ii) would it be significantly accurate to choose a common s^* for all probes, proving the two-regime assumption is universal?

For each probe, we calculated the $P_{ij}(F)$ ($j = 1 \dots S$) values quantifying the improvement when x_{ij} is chosen for the boundary in the M_1 model compared to M_0 . We selected the s_i^* value, the most likely boundary, for which P is the smallest for that gene probe.

Then we counted, for each experimental salt concentration, how many times, out of N , it had the smallest P value. We compared these frequencies with similar statistics derived from a Monte-Carlo sampling, where the y_{ij} expression data were generated randomly around the M_0 model. This means that we used the a_p , b_i coefficients and the standard errors of the individual regressions to generate test values for y_{ij} . In effect, this is a cross-validation test with data generated by the null model (25).

Transcriptional network reconstruction. A list of candidate genes most likely to participate in the change of regime was drawn by selecting those with P values of <0.3 , an arbitrary threshold value. The genes were linked to the transcription factors by which they are regulated (from the RegulonDB database [26], <http://regulondb.ccg.unam.mx/>). The threshold value was chosen to create the backbone of the regulatory network. As some transcription factors may be posttranscriptionally regulated, they

were retained if they were linked to at least 2 genes that were shown to change regime to ensure the coherence of the network even if they were not selected with the criteria given above.

Proteomics measurements. The samples were prepared in the same way as for the microarray measurement, with 3 biological replicates with NaCl concentrations of 2, 4.5, and 5.5%, which were centrifuged to obtain a dry pellet. The protein extracts were prepared and analyzed by two-dimensional (2D) gels using the method of Shaw et al. (27) with three gels run for each sample. The resulting 16-bit tiff images of the gels were analyzed using ProGenesis SameSpots (version 4.5; Non Linear Dynamics), and protein spots with altered levels of expression between the three conditions were excised from the gel using a ProPick excision robot (Genomic Solutions). The significantly differently expressed proteins were determined by analysis of variance (ANOVA) by comparing spots between any 2 NaCl concentrations with at least two technical replicates for all 3 biological replicates. The concentrations of NaCl at which the normalized volume was significantly different and associated P values are indicated in Tables 1 and 2. The proteins within the excised spots were digested using the method of Hockin et al. (28) and the liquid chromatography-tandem mass spectrometry (LC/MS-MS) analysis of the digests on an LTQ-Orbitrap (Thermo) using the method of Shaw et al. (27). The proteins were identified by searching the resulting RAW files converted to Mascot generic format using Proteome Discoverer (Thermo) against sequences in an *E. coli* K-12 database (downloaded from UniProt 19 July 2013) using an in-house version (v2.4.1) of the Mascot search tool (Matrix Science, Inc.).

Nucleotide sequence accession number. Both the raw and normalized data are deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE54512.

RESULTS

Growth rate depends on cytoplasmic osmoprotectant. The specific growth rates decreased with NaCl concentration and were dependent on the nature of the osmoprotectant (Fig. 2; see also Table S3 in the supplemental material). This is in agreement with previous studies with *E. coli* and *Salmonella* (20, 29). Growth rates were similar when there was either no osmoprotectant or when proline was added to the medium. They were also similar when either glycine betaine or choline was added. The natural logarithm of the suboptimal growth rate was fitted empirically by a linear function of the square of the added NaCl for no osmoprotectant or proline ($\ln \mu = -0.33 + 0.06 \cdot \text{NaCl}^2$; $R^2 = 0.98$) and of the fourth power of NaCl for glycine betaine or choline ($\ln \mu = -0.254 + 0.001 \cdot \text{NaCl}^4$; $R^2 = 0.97$). When no osmoprotectant is present in the medium, bacteria synthesize trehalose from glucose (9). If proline is present, it accumulates in the cell cytoplasm together with trehalose (7). The results presented here suggest that the growth rate is similar in the presence of proline or with no osmoprotectant in the medium, although the cell is able to grow at higher osmolarity in the presence of proline. These two conditions may be linked by the production of trehalose. Proline is an amino acid which is metabolized by *E. coli* in the absence of NaCl but is also accumulated in the cell cytoplasm in the case of osmotic stress (30).

If choline is present in the medium, it is taken up and converted to glycine betaine in the cytoplasm (10, 11), so the cytoplasmic osmoprotectant is the same as if glycine betaine is present in the medium. Cayley and Record (29) found that the growth rate with choline was slightly less than the growth rate with glycine betaine in medium containing 0.5 M NaCl, which corresponds to about 3% NaCl in our experiments. This result was confirmed by their measurement of internal osmoprotectant accumulated in

TABLE 1 Proteins whose expression changes abruptly at an NaCl concentration between 4.5% and 5%

Protein ^a	Description	Gene	H value	L value	Proteomics <i>P</i> value (ANOVA)	Fold change	Transcription <i>P</i> value (F-test) ^c
AdhE	Aldehyde-alcohol dehydrogenase	<i>b1241</i>	2	4.5	5.78E−03	2.3	0.083
AdhE	Aldehyde-alcohol dehydrogenase	<i>b1241</i>	5	4.5	4.37E−02	1.6	0.083
AmpA	Cytosol and leucine aminopeptidase	<i>b4260</i>	2	4.5	1.88E−02	1.7	0.71
CspA	Cold shock protein	<i>b3556</i>	2	4.5	1.56E−06	1.9	0.23
CysK	Cysteine synthase A	<i>b2414</i>	4.5	5	3.22E−02	1.1	0.18
DapB	4-Hydroxy-tetrahydrodipicolinate reductase	<i>b0031</i>	4.5	2	1.16E−07	1.7	0.095
GatZ	Subunit of the tagatose-1,6-bisphosphate aldolase 2	<i>b2095</i>	2	4.5	3.33E−03	1.8	0.62
GatZ	Component of the tagatose-1,6-bisphosphate aldolase 2	<i>b2095</i>	4.5	2	1.37E−06	1.8	0.62
GlnQ	Glutamine ABC transporter, ATP binding subunit	<i>b0809</i>	2	4.5	3.97E−04	1.9	0.35
GltD	Glutamate synthase, small subunit	<i>b3213</i>	4.5	5	2.82E−04	1.3	0.11
GrcA	Stress-induced alternate pyruvate formate-lyase subunit	<i>b2579</i>	2	4.5	9.40E−03	1.3	0.0060
H-NS	H-NS DNA-binding transcriptional dual regulator	<i>b1237</i>	2	4.5	2.10E−02	1.8	0.29
H-NS	H-NS DNA-binding transcriptional dual regulator	<i>b1237</i>	2	4.5	1.79E−03	1.2	0.29
IlvB	Component of acetohydroxybutanoate synthase/acetolactate synthase	<i>b3671</i>	4.5	2	2.39E−05	1.6	0.43
IlvI	Large subunit of the acetohydroxybutanoate synthase/acetolactate synthase	<i>b0077</i>	4.5	2	1.41E−04	1.3	0.95
KatE ^b	Catalase II	<i>b1732</i>	4.5	2	7.27E−10	2.5	0.84
KatE ^b	Catalase II	<i>b1732</i>	5	2	8.22E−09	2.4	0.84
NapA	Large subunit of periplasmic nitrate reductase	<i>b2206</i>	2	4.5	8.06E−04	2.9	0.83
NrdD	Ribonucleoside-triphosphate reductase, highly sensitive to O ₂	<i>b4238</i>	2	4.5	2.78E−02	4.5	0.30
OmpA	Outer membrane porin OmpA	<i>b0957</i>	4.5	2	1.78E−06	5	0.054
OmpA	Outer membrane porin OmpA	<i>b0957</i>	4.5	2	7.43E−07	3.9	0.054
OmpA	Outer membrane porin OmpA	<i>b0957</i>	4.5	2	3.64E−03	1.7	0.054
OmpT	Outer membrane protease VII	<i>b0565</i>	4.5	5	2.23E−04	1.8	0.53
OmpT	Outer membrane protease VII	<i>b0565</i>	5	2	2.58E−07	1.7	0.53
OppA	ATP-dependent oligopeptide transporter	<i>b1243</i>	2	4.5	5.28E−08	1.6	0.099
PflB	Pyruvate formate-lyase, signature of fermentation	<i>b0903</i>	5	4.5	2.76E−02	1.6	0.061
Pgm	Phosphoglucosyltransferase	<i>b0688</i>	4.5	2	5.86E−04	1.4	0.54
Ppx	Exopolyphosphatase	<i>b2502</i>	2	4.5	3.75E−02	1.5	0.89
ProA	Glutamate-5-semialdehyde dehydrogenase	<i>b0243</i>	2	4.5	3.12E−02	1.2	0.47
ProX ^b	Glycine betaine/proline ABC transporter	<i>b2679</i>	4.5	5	3.08E−02	1.1	0.64
RlmH	Methyltransferase	<i>b0636</i>	4.5	2	2.06E−03	4.1	0.49
RpsD	Component of the 30S subunit of the ribosome	<i>b3296</i>	2	4.5	2.32E−02	1.3	0.30
RpsF	Component of the 30S subunit of the ribosome	<i>b4200</i>	2	4.5	1.81E−02	1.6	0.094
SuhB	Inositol monophosphatase	<i>b2533</i>	2	4.5	2.25E−03	1.4	0.83
TrpB	Tryptophan synthase, β subunit dimer	<i>b1261</i>	4.5	2	4.69E−05	1.4	0.13
TrpE	Anthranilate synthase component I	<i>b1264</i>	4.5	2	6.38E−03	1.5	0.53
TrpG	Anthranilate synthase component II	<i>b1263</i>	4.5	2	4.06E−03	1.3	0.18
UvrA	Subunit of the UvrABC nucleotide excision repair, part of the SOS response	<i>b4058</i>	4.5	2	4.16E−03	1.7	0.65
YdcP	Peptidase	<i>b1435</i>	2	4.5	2.78E−04	2.4	0.18
YeaG	Protein kinase	<i>b1783</i>	4.5	2	9.13E−10	1.7	0.15
YgaU ^b	Conserved hypothetical protein	<i>b2665</i>	4.5	2	4.09E−12	3.4	0.156
YnfF	Putative selenate reductase	<i>b1588</i>	2	4.5	1.79E−03	2.9	0.43

^a For these proteins, expression was found to be either at its highest (H) or lowest (L) level at 4.5% NaCl, as determined by ANOVA.

^b Indicates proteins that were identified by Weber et al. (58).

^c The *P* values in the last column indicate the significance of the switch hypothesis, using the F-test with stagewise linear models for the NaCl dependence of the individual protein expression levels (or their square), as described in the microarray data analysis section. The lower the *P* value (the lowest of the two *P* values is indicated), the more likely that the switch hypothesis, considering only that protein, is justified.

the cell. However, at this salt concentration with these osmoprotectants in the medium, the stress is relatively mild (the growth rate is more than 90% of its optimum according to their measurements) and was based on one NaCl concentration only, so this difference may not be significant. Another difference to our experiments is that they used a buffer, MOPS (morpholinepropane-sulfonic acid), in their minimal medium, which has been shown to interfere with the accumulation of osmoprotectants, although it is not an osmoprotectant itself (31).

An abrupt change in metabolic efficiency occurs between 4.5 and 5% NaCl in the presence of glycine betaine or choline. The OD₆₀₀ values showed a linear trend with the logarithm of glucose for concentrations greater than 0.5 g/liter (the OD detection level is not reached at lower glucose concentrations; see Fig. 3). We used the slope of this linear relationship to characterize the cellular metabolic efficiency at different NaCl concentrations. The OD₆₀₀ was converted into biomass, BM_c, using the linear relationship described in Materials and Methods. Figure 4 shows the effect of

TABLE 2 Proteins that change monotonously with NaCl concentration^a

Protein	Description	Gene	H value	L value	Proteomics P value (ANOVA)	Fold change	Transcription P value (F-test)
ArtJ	Arginine ABC transporter	<i>b0860</i>	2	5	2.98E-06	1.4	0.71
Bcp	Thiol peroxidase induced by oxidative stress	<i>b2480</i>	2	5	4.85E-02	1.3	0.94
CspE	Cold shock protein	<i>b0623</i>	2	5	3.46E-05	1.5	0.97
GdhA	Glutamate dehydrogenase	<i>b1761</i>	2	5	3.71E-06	3.7	0.08
DppA	Dipeptide ABC transporter	<i>b3544</i>	2	5	2.98E-04	1.3	0.63
FabA ^b	Dehydratase/isomerase which is essential for fatty acid synthesis	<i>b0954</i>	2	5	3.55E-07	1.5	0.50
GlnH	Glutamine ABC transporter	<i>b0811</i>	2	5	5.15E-07	2.3	0.35
IlvE	Branched-chain amino acid aminotransferase	<i>b3770</i>	2	5	2.65E-08	1.6	0.53
Adk	Adenylate kinase	<i>b0474</i>	2	5	4.54E-07	1.4	0.87
ThrB	Homoserine kinase	<i>b0003</i>	2	5	3.74E-05	1.3	0.95
LeuB	3-Isopropylmalate dehydrogenase	<i>b0073</i>	2	5	2.91E-05	1.5	0.96
LivJ	Branched-chain amino acid ABC transporter	<i>b3460</i>	2	5	9.96E-09	2.7	0.080
MetE	Cobalamin-independent homocysteine transmethylase	<i>b3829</i>	2	5	1.43E-03	2.2	0.55
MetE	Cobalamin-independent homocysteine transmethylase	<i>b3829</i>	2	5	4.58E-03	2.1	0.55
MetE	Cobalamin-independent homocysteine transmethylase	<i>b3829</i>	2	5	7.28E-04	1.4	0.55
MetF ^b	5,10-Methylenetetrahydrofolate reductase	<i>b3941</i>	2	5	9.57E-08	1.4	0.54
NuoCD	NADH:ubiquinone oxidoreductase, chain CD	<i>b2286</i>	2	5	1.44E-07	2	0.87
MalP	Maltodextrin phosphorylase	<i>b3417</i>	2	5	4.62E-02	1.9	0.012
MalP	Maltodextrin phosphorylase	<i>b3417</i>	2	5	2.42E-05	1.7	0.012
Rho	Transcription termination factor Rho	<i>b3783</i>	2	5	5.43E-03	2.1	0.65
RfbB	dTDP-glucose 4,6-dehydratase	<i>b2041</i>	2	5	2.21E-05	1.3	0.53
RpsH	30S ribosomal subunit protein S8	<i>b3306</i>	2	5	3.49E-03	1.5	0.49
LysS	Lysyl-tRNA synthetase	<i>b2890</i>	2	5	5.67E-03	1.4	0.91
TopA	DNA topoisomerase I	<i>b1274</i>	2	5	1.88E-05	3.2	0.80
AceA	Isocitrate lyase (glyoxylate shunt)	<i>b4015</i>	5	2	1.06E-09	2.1	0.62
AckA	Acetate kinase	<i>b2296</i>	5	2	1.30E-02	1.2	0.58
AcnA ^b	Aconitate hydratase 1, resistant to oxidation	<i>b1276</i>	5	2	1.66E-04	1.5	0.80
CbpA	Cochaperone with DnaK	<i>b1000</i>	5	2	1.51E-04	2.8	0.47
ElaB	Conserved protein	<i>b2266</i>	5	2	9.59E-07	2.9	0.16
GalK	Galactokinase	<i>b0757</i>	5	2	8.71E-07	2.3	0.96
GalE	UDP-glucose 4-epimerase	<i>b0759</i>	5	2	7.70E-04	1.5	0.99
GlgX	Glycogen phosphorylase-limit dextrin α -1,6-glucohydrolase	<i>b3431</i>	5	2	3.69E-05	1.8	0.34
Gmd	GDP-mannose 4,6-dehydratase	<i>b2053</i>	5	2	1.58E-11	11	0.86
Ivy	Inhibitor of vertebrate C-type lysozyme	<i>b0220</i>	5	2	3.07E-12	4.9	0.29
AceB	Malate synthase A (glyoxylate shunt)	<i>b4014</i>	5	2	5.71E-11	2.6	0.57
AceB	Malate synthase A (glyoxylate shunt)	<i>b4014</i>	5	2	1.34E-08	2.5	0.57
MprA	MprA DNA-binding transcriptional repressor	<i>b2684</i>	5	2	1.11E-07	1.9	0.83
NagB	Glucosamine-6-phosphate deaminase	<i>b0678</i>	5	2	5.86E-09	2	0.79
OsmC ^b	Osmotically inducible peroxidase	<i>b1482</i>	5	2	4.44E-10	2.6	0.11
OsmY ^b	Hyperosmotically inducible periplasmic protein	<i>b4376</i>	5	2	3.98E-12	4.3	0.56
OtsA	Trehalose-6-phosphate synthase	<i>b1896</i>	5	2	3.76E-07	2	0.64
OtsB ^b	Trehalose-6-phosphate phosphatase	<i>b1897</i>	5	2	7.75E-10	2.5	0.11
PanC	Pantothenate synthetase	<i>b0133</i>	5	2	1.42E-10	1.5	0.63
PckA	Phosphoenolpyruvate carboxykinase	<i>b3403</i>	5	2	4.67E-07	1.7	0.90
PyrC	Dihydroorotase	<i>b1062</i>	5	2	3.23E-08	1.3	0.94
RplN	50S ribosomal subunit protein L14	<i>b3310</i>	5	2	5.23E-03	1.6	0.81
RspA	Mannonate/altronate dehydratase	<i>b1581</i>	5	2	1.82E-07	2.5	0.13
TalA ^b	Transaldolase (pentose phosphate pathway)	<i>b2464</i>	5	2	1.73E-09	2.4	0.47
ThiM	Hydroxyethylthiazole kinase	<i>b2104</i>	5	2	6.93E-07	1.8	0.22
TktB ^b	Transketolase II (pentose phosphate pathway)	<i>b2465</i>	5	2	1.95E-07	2.1	0.79
TolB	Periplasmic protein member of the Tol-Pal system	<i>b0740</i>	5	2	4.61E-07	1.8	0.65
Ydcf	Conserved protein	<i>b1414</i>	5	2	9.00E-06	1.9	0.033
Ygfb	Folate-binding protein	<i>b2898</i>	5	2	1.22E-02	1.4	0.84
YghA	Predicted glutathionylspermidine synthase	<i>b3003</i>	5	2	2.67E-07	3.6	0.13
YhbO ^b	Protein involved in stress response	<i>b3153</i>	5	2	6.17E-13	2.5	0.44
YqhD	NADP-dependent aldehyde reductase	<i>b3011</i>	5	2	1.64E-05	1.6	0.44

^a Columns represent the same as in Table 1 with the exception of the P value for transcription, which indicates the significance of a linear dependence of gene expression on NaCl concentration.

^b Indicates proteins that were identified by Weber et al. (58).

FIG 2 Specific growth rate decreases with increasing NaCl concentration and depends on the intracellular osmoprotectant. The osmoprotectants added to the medium were as follows: ■, no osmoprotectant; □, proline; ▲, glycine betaine; △, choline. The logarithm of the specific growth rate with no osmoprotectant and proline in the medium was fitted by a linear function of the square of the NaCl concentration, while that of the specific growth rate with glycine betaine and choline in the medium was fitted by a linear function of the fourth power of the NaCl concentration.

different osmoprotectants on the slope and intercept of the “ BM_c versus $\log(\text{glucose})$ ” curves as a function of salt concentration and osmoprotectant in the medium.

With no osmoprotectant or with proline in the medium, the metabolic efficiency decreased smoothly with increasing salt concentration. However, with choline or glycine betaine in the medium, the trend was not monotonous with salt (Fig. 4). In both cases, it slightly decreased for NaCl below 2% and then increased when the salt was between 2 and 4.5%. Between 4.5 and 5% NaCl the trend was reversed. Finally, for concentrations of NaCl higher than 6%, the metabolic efficiency was significantly lower than that calculated for NaCl less than 6%, in agreement with the observation in Fig. 3 and with the findings of Krist et al. (19), although those authors used a different definition of metabolic efficiency. Indeed, they found that the ΔOD decreased sharply at water activities around 0.96, which corresponds to an NaCl concentration close to 6%. Glycine betaine is the most efficient osmoprotectant with the highest metabolic efficiency maintained for NaCl up to

FIG 3 For a given NaCl concentration, OD_{600} measurements depend on the logarithm of glucose concentration in a linear manner. We used these linear relationships to compare the metabolic efficiency in different conditions. △, NaCl concentration ranging from 0% to 5.5%; +, NaCl > 6% in minimal medium in the presence of glycine betaine; continuous lines represent the regression. We observed a significant decrease in metabolic efficiency when NaCl was > 6%.

FIG 4 The effect of NaCl on metabolic efficiency is not always correlated to the growth rate. Metabolic efficiency [represented by the slope and intercept of BM_c versus $\log(\text{glucose})$] as a function of the salt concentration and the osmoprotectant in the medium: (A) no osmoprotectant; (B) proline; (C) choline; (D) glycine betaine. The left-hand axis corresponds to the slope and its standard error (□); the right-hand axis is for the intercept (■). A dash represents a linear trend estimation based on less than 10 data points.

6%. Although the addition of choline also maintained the metabolic efficiency up to 6% NaCl and the growth rates were similar to those obtained with glycine betaine, its effect on the value of the metabolic efficiency is more similar to that of proline. For proline, the decrease in metabolic efficiency was so sharp that we could not measure it; at 5.7% NaCl, the OD did not reach the detection level of the Bioscreen. These results are consistent with those of Nagata et al. (32) who showed that at 1 M NaCl, in the presence of glycine betaine, cultures reached higher optical densities faster than in the presence of proline. With choline or glycine betaine in the medium, no correlation could be seen between the specific growth rate and the metabolic efficiency in low-stress conditions.

The transcriptional network revealed by microarray data suggests a fundamental change of metabolic regime in the presence of glycine betaine between 4.5 and 5% NaCl. To better understand how glycine betaine, the most efficient osmoprotectant, may help *E. coli* to grow under osmotic stress, transcriptional gene expression in medium containing glycine betaine during exponential growth, before OD_{600} was reached, was measured with microarrays as a function of NaCl concentration. A change of trend of the logarithm of the cDNA/gDNA ratio was observed between 4.5 and 5% NaCl, as shown in Fig. 5 for a sample of genes. These genes are known to be involved in the response to osmotic stress. The pattern of gene expression for the *osmC* gene, which is responsive to osmotic stress and encodes a peroxiredoxin (33), shows that the best model is a change of gene expression at NaCl between 4.5 and 5% NaCl ($P = 0.28$). Our data also indicate a shift in porin gene transcription rather than a continuous trend with osmotic stress: *ompF* is downregulated up to 4.5% NaCl and then upregulated at concentrations higher than 4.5% ($P = 0.03$ for a linear model as a function of the square of NaCl concentration). The consensus for porin balance between OmpC (the porin with the

FIG 5 Examples of significant changes in gene expression pattern when NaCl increased from 4.5% to 5%. (A) Relative expression of the osmotic stress gene *osmC* as a function of the shifted NaCl concentration; (B) relative expression of the *ompF* gene as a function of the square root of the shifted NaCl concentration. The lines represent linear regressions with a 2-regime model with a switch between 4.5 and 5% NaCl.

smaller diameter) and OmpF (the porin with the larger diameter), both being regulated by OmpR in the case of osmotic stress, is that the higher the osmolarity, the less OmpF porins there are. However, this is based on experiments with sucrose rather than NaCl and not in the presence of glycine betaine, which may interfere with porin stoichiometry (34, 35) together with other factors, such as glucose availability (31). In addition, small RNAs are involved posttranscriptionally with the regulation of these porins under osmotic stress, so their gene expression would not exactly correlate with levels of mature protein (36).

To show that this change of regime is unlikely to happen by chance, we calculated the $p_{i,j}$ values for each probe ($i = 1 \dots N$; $n = 3,997$) and x_j salt concentration. For each x_j , we estimated how many probes had the smallest P value out of N measurements. The highest frequency was obtained for 4.5% NaCl with 1,369 probes and the second highest at 2% NaCl with 1,075 probes (see Tables S1 and S2 in the supplemental material). To test whether the dominance of NaCl at 4.5% is significant, the same statistic was calculated from a data set of identical size, generated by Monte-Carlo random sampling where the underlying assumptions followed our null hypothesis (i.e., the $y_{i,j}$ values depended linearly on NaCl, but this trend was perturbed by a random noise from the same distribution for each probe). We found that out of 100 simulations, the smallest P values were always at 2 or 5% NaCl, at the two ends of the interval, and never within the interval. Therefore, we can conclude at a $P \leq 0.01$ significance level that it is very unlikely that the above-demonstrated dominance of the NaCl 4.5% boundary salt value could happen by chance.

To test the choice of linear model to represent the original hypothesis, we repeated the calculations with $x = (\text{NaCl}\% - 2\%)^2$. This rescaling transformation could be justified by the observation that the relative inhibitory effect of salt increased with

salt concentration. The result was the same: the change of response to osmotic stress at NaCl at $>4.5\%$ is statistically significant.

Among the genes showing a change of regime, there were not only those known to be associated with osmotic stress but also metabolic genes, in particular, genes linked to anaerobiosis and those linked to stresses other than osmotic stress. In order to better interpret the change of regime, we reconstructed the transcriptional network with those genes which showed a change of regime and their associated transcription factors according to the criteria described in Materials and Methods. Using these criteria, 1,124 of the 3,997 gene probes considered in the microarray analysis were found to change regime between 4.5 and 5% NaCl (Fig. 6; see Table S4 in the supplemental material).

The gene which had the highest degree (the node with the highest number of links, see Fig. 6) in the network was that encoding the catabolic receptor protein, Crp. It has been shown that cyclic AMP (cAMP) acts as an osmoregulatory protein through its receptor Crp (37). We showed here that it is central to osmotic stress regulation during exponential growth. Crp is directly linked to genes implicated in osmotic stress, like those encoding the porin (*ompF*) (Fig. 5) (38), glutamate biosynthesis (*gltB*) (39), or the periplasmic protein (*osmY*) (40).

DNA architecture has also been shown to play an important role in osmoregulation (41, 42). Two other genes with high degrees are *ihfA* and *ihfB*, coding for the integration host factor protein involved in chromosome structure (43). The relative expression of *ftrR*, the primary transcriptional regulator that mediates the transition from aerobic to anaerobic growth through the regulation of hundreds of genes (44), increased abruptly when the NaCl concentration increased from 4.5 to 5% NaCl, and this has the second highest degree. This suggests that the cells shift from an aerobic metabolism to an anaerobic state. The results confirm previously observed correlations between osmotic stress, DNA supercoiling, and anaerobiosis (45–47). Although the catabolic repressor activator, *fruR* or *cra*, was not itself selected as a gene showing a change of regime using the selection criteria chosen here, 28 out of the 63 genes it is linked to (26) were (Fig. 6). In addition to participating in regulation of the use of catabolic pathways, *cra* has been linked to the expression of the osmotically induced operon *betAB*, the gene products of which convert choline into glycine betaine (48). All together, the microarray data suggest a fundamental change of metabolic regime when NaCl concentration was increased from 4.5 to 5%.

The response to osmotic stress is coupled to other stresses. During osmotic stress, the cell envelope is affected and, in particular, fluxes of water and osmoprotectants are modified (38) (see, for instance, the *ompF* gene encoding porin expression in Fig. 5). Expression of the *rcs* phosphorelay which plays a role in osmotic adaptation (49) also changed. Another aspect linked to envelope stress response is protein folding, as illustrated by expression of *rpoH*, encoding the envelope stress sigma factor (which is not represented in the regulatory network but which was selected as a gene showing a change of regime, $P = 0.12$) and the *rseA* gene ($P = 0.0013$) (50). The gene expression of *lexA*, the regulator induced by DNA damage or SOS response but which also modulates antibiotic resistance, also increased in the second regime ($P = 0.12$) (51).

In addition to genes linked with envelope stress, the other response that was significantly represented in the regulatory network is that to oxidative stress and iron homeostasis. We noticed that the import of iron increases significantly at 4.5% NaCl, as

FIG 6 Transcriptional network affected by the change of regime. The nodes represent either genes or transcription factors, the edges interactions between them, based on RegulonDB. Red/blue nodes, up/downregulated when stress reaches a critical level; yellow nodes, transcription factors linked to genes that were significantly up- or downregulated but themselves not significantly affected.

suggested by the expression of the enterochelin esterase encoded by *fes* or the *ent* genes which encode proteins used in the synthesis of siderophores. However, this inferred iron import then drops at 5% NaCl. To check that our medium was not limiting in iron, we measured the growth rates with other forms and concentrations of iron in the medium, but this did not change the growth rate (results not shown). The expression of iron uptake genes correlated with the expression of those regulating oxidative stress, such as *oxyR* ($P = 0.062$) (52). All together, these results suggest that the regime switch is correlated to an internal oxidative stress. The response to iron availability has been linked to the response to osmotic stress in other bacteria (53–55).

A proteomics analysis confirmed a switch to fermentative pathways and a metabolic response similar to that to oxidative stress. To confirm the metabolic switch detected by kinetics and transcriptional analysis, a proteomic analysis was carried out at 2, 4.5, and 5% NaCl in the presence of glycine betaine in the medium. Of the proteins corresponding to the spots which changed significantly with NaCl concentration, two types of profiles could be detected: those which were maximum or minimum at 4.5% NaCl (Table 1) or those which were monotonously decreasing or increasing with NaCl (Table 2). The former pattern confirmed the switch detected by transcriptional analysis, and 16 out of the 35 proteins identified by proteomics belonged to the list of genes

detected with the microarray data with the criteria used in this study. Notably, the *P* values for the metabolic proteins pyruvate formate lyase (PflB), aldehyde alcohol dehydrogenase (AdhE), and ribonucleoside-triphosphate reductase (NrdD) were at their lowest at 4.5% NaCl (namely, 2.76×10^{-2} , 5.48×10^{-3} , and 2.78×10^{-2} , respectively), confirming that between 4.5% NaCl and 5% NaCl, the bacteria switch from aerobic to fermentative pathways consistent with their own gene expression and the gene expression pattern of the regulator *fnR* (56, 57). Concomitant with aerobic respiration at 4.5% NaCl is the catalase II, KatE ($P = 7.27 \times 10^{-10}$), which helps to alleviate oxidative stress and which was also found by Weber et al. (58) to change with osmotic stress in both aerobic and anaerobic states but less so in the latter. The histone-like nucleoid structuring protein (HNS) DNA binding regulator also confirmed that DNA architecture is associated with osmotic stress ($P = 2.10 \times 10^{-2}$). The data also corroborated an increase of trehalose synthesis, with OstA and OstB, the two proteins converting glucose into trehalose, increasing by more than 2-fold between 2 and 5% NaCl (the *P* values were 3.76×10^{-7} and 7.75×10^{-10} , respectively) in agreement with the gene expression for *otsB* ($P = 0.11$). The osmotically inducible protein OsmC and the periplasmic protein OsmY also increased significantly in agreement with Weber et al. (58) ($P = 4.44 \times 10^{-10}$ and 3.98×10^{-12} , respectively; see Table 2). The gene expression of the latter did not change ($P = 0.56$), perhaps because its expression is induced only transiently (40) and hence would not be detectable in exponential-phase cultures.

The two pentose-phosphate pathway proteins transaldolase A (TalA) and transketolase B (TktB) increased significantly ($P = 1.73 \times 10^{-9}$ and 1.95×10^{-7} , respectively) with increasing NaCl in agreement with Weber et al. (58). In addition, we found that the expression of both proteins of the glyoxylate shunt, i.e., isocitrate lyase (AceA) and malate synthase (AceB), as well as the phosphoenolpyruvate carboxykinase (PckA), also increased (the *P* values were 1.66×10^{-4} , 5.71×10^{-11} , and 4.67×10^{-7} , respectively). Taken together, this suggests that the metabolic response to osmotic stress induced by NaCl is similar to that for oxidative stress induced by paraquat measured by flux distributions (59).

Proteins that decreased with NaCl concentration include the homocysteine transmethylase (MetE) and the 5,10-methylenetetrahydrofolate reductase MetF, involved in the biosynthesis of methionine (the *P* values were 7.28×10^{-4} for MetE and 9.57×10^{-8} for MetF). The glutamine synthetase, GlnA, was not picked as decreasing, as in Weber et al. (58), but the glutamate dehydrogenase, GdhA, was found to decrease by 3.7-fold and the glutamine ABC transporter (GlnH) by 2.3-fold. The amount of GltD, the glutamate synthase, was also found to be altered by NaCl concentrations in agreement with the microarray analysis ($P = 2.82 \times 10^{-4}$; Table 1), confirming that glutamate and glutamine play a role in adaptation to osmotic stress (29).

To summarize, the metabolic response to osmotic stress is consistent with that for oxidative stress, but the significant difference is that in the former, the cells switch to fermentative pathways at a threshold NaCl concentration.

DISCUSSION

We have shown by three independent methods that the apparent smooth decrease in specific growth rate with increasing NaCl concentration conceals a dramatic change of metabolic regime mediated by the addition of glycine betaine.

Methodology. Krist et al. (19) proposed the characterization of cell energy expenditure as a function of stress by measuring the maximum OD in wells of a Bioscreen where the maximum OD was limited by the glucose concentration. In theory, the differential OD obtained at different glucose concentrations is proportional to the differential glucose concentration and defines a straight line up to a saturation level. Although we indicated that the measurements would not be accurate using this method because of the shape of the OD measurement curves, the yield may be estimated by considering the maximum ODs. As an order of magnitude, we find an increase of about 0.2 in the Bioscreen reading for a differential glucose concentration of 0.5 g/liter, which according to our calibration curves corresponds to about 0.1 g/liter dry weight biomass/0.5 g/liter glucose. This is more than that found by Andersen and von Meyenburg (60), but the experimental conditions were different (larger batch, different strain, etc.).

With the method used here, the location of the OD_b breakpoint was estimated by numerical derivation. In effect, this is equivalent to the estimation of an inflection point by numerical approximation. Although such numerical derivation is an unstable approximation, we found that the trend of the OD_b points, as a function of the logarithm of glucose, was consistently linear. In only a few circumstances was this method not applicable; these were at high glucose and high NaCl concentrations with proline or no osmoprotectant.

The modeling of the “BM_c versus log(glucose)” curve is empirical, and the slope before the breakpoint depends on both NaCl and glucose concentration. This may explain why the metabolic efficiency defined in this way is proportional to the logarithm of glucose rather than exhibiting a biphasic relationship to glucose, which would be the case if the maximum OD was considered (19). Krist et al. (19) did not have a sufficient number of measurements at higher NaCl concentrations to be able to observe the change of trend we detected here.

The measurement of the metabolic efficiency with a Bioscreen, as described here, required the generation of lot of data. Direct measurement of oxygen consumption is not feasible in the Bioscreen, and oxygen concentration may be limiting. To check this, growth experiments were carried out in sealed bottles, thus restricting the oxygen availability, and the oxygen in the headspace was monitored. The oxygen level remained high until the end of the exponential phase, and no difference in the growth rate was observed whether or not the tubes were shaken (results not shown).

When analyzing the microarray data, we chose to normalize the cDNA to genomic DNA rather than a cDNA-cDNA cohybridization. Provided the signal data are properly corrected for low and high intensity reference signals, it was possible to carry out a uniform comparison across experiments (61). Concerning the statistical analysis of gene expression, since there were only six salt conditions, one cannot expect to individually identify genes that deviate from the null hypothesis (which was that expression increases or decreases monotonously with NaCl concentration). However, by cross-validation with Monte-Carlo sampling of the null hypothesis applied to about 4,000 probes, it was possible to show a switch in gene expression pattern.

In this study, more proteins were detected as significantly changing than in Weber et al. (58), but the ratios tended to be lower. One of the reasons is that the experimental conditions were very different: Weber et al. measured the time-dependent effect of

the addition of 2.3% NaCl and sorbitol to cells grown under non-osmotic stress. In our case, the cells were grown under osmotic pressure throughout. The likelihood of variability is much greater in our design, as it limits possible variation occurring during growth. We are also examining changes from cells already under a level of osmotic stress at 2% NaCl going up to 5% NaCl (compared to the Weber et al. maximum stress level of 2.3% NaCl) in the presence of glycine betaine. This would reduce the ratios, as the cells are already under stress.

Although the gene-to-protein agreement between transcriptomic and proteomics data is poor, as is usually found when measuring stress response (62, 63), the trend indicating a change of regime at a threshold NaCl concentration was confirmed by both methods of analysis. The effect of NaCl on intracellular components of the cell is still to be elucidated, but our study indicated that metabolism is similar to that seen in response to oxidative stress.

Osmotic and oxidative stress. The coupling of the response to osmotic stress to that for other stresses has been reported in different studies (41–43, 46, 47, 50, 64), but we illustrate them here in a more comprehensive manner. Our study also indicated that there is a metabolic shift at a threshold NaCl concentration and that the response to stress in batch is not continuous, despite the fact that the growth rate decreases continuously with increasing NaCl. It further suggested that this shift may be linked to intracellular iron homeostasis and that internal oxidative stress is correlated to the metabolic switch. Oxidative stress has been shown to be linked to a change of metabolic state such as entering stationary phase or the viable but nonculturable state (VBNC) but is also linked to starvation (65–67). However, in our case, the bacteria are not deprived of carbon, as glucose is still plentiful in the exponential phase. Nor is iron limiting, as adding iron did not change the growth rate. There was no indication from the transcriptional data that ppGpp was involved in the change of regime, as *relA* and *spoT* were not affected.

Modeling a switch of regime. To the best of our knowledge, this is the first time that the existence of two distinct metabolic regimes, responding to a continuous increase in salt, is shown. In the first regime, the cell's priority seems to be rapid growth (aerobic regime); in the second regime, the metabolism seems to be geared to managing stress. The switch from one regime to another is not detectable when observing only the growth rate, even though this parameter is often used as a measurement of fitness, as this varies continuously with NaCl. The specific growth rate is routinely determined in predictive microbiology as the most important physiological parameter, but, as shown here, its trend does not necessarily reflect the true changes in the metabolic mechanism.

One way of modeling bacterial strategy is to use objective functions, that is, functions that are optimized given a set of constraints. Objective functions are useful in biology because they may be related to evolution (e.g., optimization of the growth rate), and optimization problems tend to be solvable. To model growth in a chemostat, it has been proposed that the objective function is the optimization of biomass production with constraints on the nutrients by FBA (2). In batch, with models based on stoichiometric coefficients, with excess of carbon, it has been proposed that it is ATP production that is maximized (3). To explain the use of overflow pathways when carbon and oxygen sources are abundant in a chemostat, rather than by applying additional constraints on

nutrient uptake, it has been proposed that the growth rate may be limited by molecular crowding. This was modeled by adding constraints of the volume occupancy of the enzymes to stoichiometric models (68, 69). With this approach, the metabolic fluxes are further constrained by a coefficient proportional to the inverse of the enzymatic catalytic rates, k_{cat} , the turnover number. In the case of osmotic stress, cytoplasmic crowding has been shown to increase (29), but the effect on diffusion of macromolecules, for instance, is disputed (70, 71). Additional experimental evidence is needed to check whether cytoplasmic crowding is an intrinsic constraint or not. Alternatively, metabolic switches, such as the one we observed here, have been proposed to be the result of a tradeoff between using kinetically and metabolically efficient pathways (1, 72). In this approach, both the kinetic and metabolic efficiency are taken into account, and the metabolic switch is explained by feedback loops, while the growth rate is optimized. The same principle could be envisaged in the case of osmotic stress, the switch from aerobic to fermentative pathways being the result of the response of the cells to oxidative stress (1).

Conclusions. To conclude, the specific growth rate seems to be linked to one or both of the following two parameters: which particular osmoprotectant is used internally by the cell and/or whether or not the cell produces trehalose. The efficiency of osmoprotectants may be evaluated by looking at different aspects: the growth rate at a given NaCl concentration, the maximum osmotic stress at which the metabolic efficiency is maintained, or the metabolic efficiency itself. The efficiencies of these osmoprotectants may be ranked, in decreasing order: glycine betaine, choline, proline, trehalose. Compatible solutes are thought to relieve osmotic stress by maintaining preferential exclusion of the proteins that would otherwise be affected by ions such as potassium (14). It has been shown *in vitro* that glycine betaine relieves osmotic stress better than trehalose and that the cytoplasmic water activity is higher with glycine betaine *in vivo* (29). However, the diversion of glucose to accumulate these osmoprotectants is unlikely to be sufficient to fully explain the decrease of growth rate (12), suggesting that other metabolic, regulatory changes or physical constraints are limiting to cell growth.

The existence of a sudden metabolic switch in the case of stress, rather than a progressive switch depending on nutrient availability, has far-reaching implications for understanding bacterial strategy and modeling growth under unfavorable environments. In the case of salt stress, the metabolic switch seems to happen at a threshold NaCl concentration, a phenomenon masked by the fact that growth rate decreases smoothly with increasing NaCl. Further experiments, for instance using a sugar as a source of osmotic stress, would show how general this response is, but metabolic efficiency results suggest it does not happen with proline or without osmoprotectant in the presence of NaCl. This switch is correlated to oxidative stress. Although more data are needed to fully characterize the phenomenon, the switch seems to separate one regime where the growth rate is optimized from another one where stress management is prioritized over the growth rate.

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