Physiological and Proteomic Adaptation of “*Aromatoleum aromaticum*” EbN1 to Low Growth Rates in Benzoate-Limited, Anoxic Chemostats

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“*Aromatoleum aromaticum*” EbN1 was cultivated at different growth rates in benzoate-limited chemostats under nitrate-reducing conditions. Physiological characteristics, proteome dynamics, phospholipid-linked fatty acid (PLFA) composition, and poly(3-hydroxybutyrate) (PHB) content were analyzed in steady-state cells at low (μ_{low}) (0.036 h^{-1}), medium (μ_{med}) (0.108 h^{-1}), and high (μ_{high}) (0.180 h^{-1}) growth rates. A positive correlation to growth rate was observed for cellular parameters (cell size, and DNA and protein contents). The free energy consumed for biomass formation steadily increased with growth rate. In contrast, the energy demand for maintenance increased only from μ_{low} to μ_{med} and then remained constant until μ_{high} . The most comprehensive proteomic changes were observed at μ_{low} compared to μ_{high} . Uniformly decreased abundances of protein components of the anaerobic benzoyl coenzyme A (benzoyl-CoA) pathway, central carbon metabolism, and information processing agree with a general deceleration of benzoate metabolism and cellular processes in response to slow growth. In contrast, increased abundances were observed at μ_{low} for diverse catabolic proteins and components of uptake systems in the absence of the respective substrate (aromatic or aliphatic compounds) and for proteins involved in stress responses. This potential catabolic versatility and stress defense during slow growth may be interpreted as preparation for future needs.

Most terrestrial and aquatic habitats are characterized by dynamic fluctuations in the composition and concentration of nutrients, supporting heterotrophic growth of bacteria in a feast-and-famine manner. In most cases, poor carbon and nutrient availabilities restrict or even arrest microbial growth (see, e.g., references 19, 27, and 33). Thus, the capacity of bacteria to cope with nutrient limitation is a key determinant for their survival and success in the environment. Substrate limitation and its long-known intimate coupling to bacterial growth rates are best studied by continuous cultivation in chemostats (for an overview, see, e.g., references 20 and 27). Constrained by a defined and constant supply of the limiting nutrient at a specific rate (dilution rate [D]), the bacterial population in a chemostat approaches a steady state characterized by a specific growth rate (μ) and stable growth parameters (39, 45). Combining these quantities with chemostat models can describe the dynamics of bacterial populations at many levels (see, e.g., reference 25).

Steady-state responses are best understood in *Escherichia coli* and other enterobacteria. Early physiological studies revealed a tight coupling of growth rate with the size and molecular composition of cells (7, 42, 61). Subsequently, growth rate-dependent global alterations in gene expression (21, 30) and proteome signatures (49, 76) were observed. An interesting outcome from these global surveys of slowly growing, carbon-limited cells was the apparently preemptive adaptation to future needs for stress resistance and multiple substrate utilization (for an overview, see reference 19).

The denitrifying strain “*Aromatoleum aromaticum*” EbN1 is a versatile aquatic betaproteobacterium that anaerobically degrades 24 different monoaromatic compounds, including petroleum hy-

drocarbons (57), phenolic solvents (79), and 3-phenylpropanoids (69). With few exceptions, these aromatic substrates are channeled via specific reaction sequences into the central anaerobic benzoyl coenzyme A (benzoyl-CoA) pathway (22), placing benzoate in a central position within the complex catabolic network. Aromatic compounds are abundant constituents of terrestrial organic biopolymers (e.g., lignin and proteins), the mineralization of which leads to a continuous release of aromatic building blocks into predominantly anoxic environments. The availability of the complete genome sequence (55) and comprehensive proteomic data on substrate-dependent regulation of the catabolic network (see, e.g., reference 79) qualify strain EbN1 as a promising model system for investigating the influence of growth rates on the anaerobic metabolism of aromatic compounds.

In this study, benzoate-limited, anoxic (nitrate-amended) chemostats were established to study the physiological and proteomic adaptation of strain EbN1 to different steady-state growth rates.

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MATERIALS AND METHODS

Batch cultivation. The denitrifying bacterium *A. aromaticum* EbN1 was cultivated in a phosphate-buffered mineral medium (modified from that described in reference 71) with 7 mM nitrate as an electron acceptor at 28°C. The basal medium had the following composition (values in g liter⁻¹): KH₂PO₄, 1.1; K₂HPO₄, 5.6; NH₄Cl, 0.8; Na₂SO₄, 0.23; and NaCl, 1.0. The medium was composed as described previously (57); additionally, 1 ml of a solution containing MgCl₂ (324 g liter⁻¹) and CaCl₂ (29.4 g liter⁻¹) was added per liter of medium. Anoxic batch medium was prepared as described previously in butyl rubber-sealed 20-ml glass tubes (containing up to 15 ml anoxic medium) or 250-ml flat glass bottles (containing 200 ml anoxic medium) under an N₂-CO₂ (9:1 [vol/vol]) atmosphere (57). Soluble substrates, including benzoate (4 mM) and acetate (8 mM), were added from sterile aqueous stock solutions. Filter-sterilized hydrocarbons and other poorly soluble organic substrates were diluted in 2,2,4,4,6,8,8-heptamethylnonane (HMN) as an inert carrier phase. These cultures were incubated horizontally on a rotary shaker, and contact between the carrier phase and the butyl rubber stoppers was generally avoided (57). All chemicals used were of analytical grade. Optical density (OD) was measured at 660 nm (UV-mini 1202; Shimadzu GmbH, Duisburg, Germany).

Continuous cultivation. Strain EbN1 was cultivated in a 1-liter double-walled Duran glass bioreactor (diameter, 13.5 cm, and height, 25 cm; Ochs GmbH, Bovenden, Germany). The cultivation vessel was kept anoxic by continuous flushing of the headspace with sterile N₂ gas at a flow rate of 20 to 25 ml min⁻¹. Tubing and O-ring seals were made of Viton (Ochs GmbH). Liquid loss due to evaporation (especially at low *D*) was prevented by moisturizing the N₂ gas with sterile anoxic water. An in-house-developed, custom-made control unit was used to monitor and adjust the cultivation settings. Dissolved oxygen (i.e., for verification of anoxia) was monitored with an autoclaved 220-mm long partial O₂ pressure (pO₂) electrode (InPro6800; Mettler-Toledo GmbH, Giessen, Germany). Using the pO₂ electrode also as thermometer allowed controlling the temperature of the cultivation vessel (28 ± 0.1°C) by adjusting the temperature of the heating water circuit. The pH was monitored with an autoclaved 225-mm-long pH electrode (405-DPAS-SC-K8S; Mettler-Toledo GmbH) and maintained constant at pH 7.35 ± 0.1 using autoclaved 1 M HCl. Due to the online pH control, the chemostat medium contained 4 mM total phosphate, compared to 40 mM total phosphate in the otherwise equally composed batch mineral medium. An S1610 syringe dosing system (Sykam GmbH, Fürstfeldbruck, Germany) equipped with two 1-ml gas-tight syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) delivered sterile, anoxic medium via fluorethylenepropylene tubings (Chromatographie Handel Müller, Fridolfing, Germany) from the medium reservoir to the bioreactor through a sterile, replaceable in-line nitrocellulose membrane filter (0.2 μm) (Schleicher & Schuell, Dassel, Germany). The feed medium contained 3.9 ± 0.1 mM benzoate or 7.6 ± 0.6 mM acetate as the limiting substrate and 15.4 ± 0.8 mM nitrate as an electron acceptor. The culture was vigorously mixed at 250 rpm using an 8-cm-long Teflon-coated magnetic stirrer bar. The culture volume was kept constant with an overflow pipe and varied only slightly between independent chemostat runs (1,006 ± 7 ml). The amount of electrons provided by the substrate (benzoate or acetate) matched the amount of electrons accepted by nitrate/nitrite fairly well, with a deviation of 14% ± 6% for benzoate and 5% ± 13% for acetate.

(i) Preparation of chemostat inoculum. To ensure maximal similarity of the inocula used for the 20 individual chemostat runs performed for this study, the same procedure for inoculum preparation was applied. Actively growing anaerobic benzoate- or acetate-adapted cells (at least 5 passages with the respective substrate) were diluted 1:2 in a glycerol-containing solution (20 mM MgCl₂ in 50% [vol/vol] glycerol), immediately frozen in liquid nitrogen, and stored at -80°C. Cryocultures were revived by serial dilution in benzoate- or acetate-containing anoxic medium. Initial batch cultures in the bioreactor were inoculated with 5% (vol/vol) of

a freshly revived culture. The continuous supply of medium was started at or shortly before entry of cells into stationary growth phase.

(ii) Determination of dilution rate ranges in benzoate- or acetate-limited chemostats. The growth behavior of strain EbN1 in carbon-limited chemostats was monitored during stepwise increases from low (*D*_{low}) to critical (*D*_{crit}) dilution rates (0.036 to 0.205 h⁻¹ with benzoate or 0.06 to 0.25 h⁻¹ with acetate). In all cases, *D* was increased following ~8 complete exchanges of the culture volume.

(iii) Adaptation to a fixed growth rate and harvesting of cells. To obtain sufficient cell material for molecular analyses [proteome, phospholipid (PL)-linked fatty acids (PLFAs), and poly(3-hydroxybutyrate) (PHB)], strain EbN1 was cultivated independently at a fixed *D* (giving a fixed μ) of 0.036 h⁻¹ (μ_{low}), 0.108 h⁻¹ (μ_{med}), or 0.180 h⁻¹ (μ_{high}). For each of these three growth rates, four independent chemostat cultures (biological replicates) were harvested at steady state after 12 ± 0.9 complete exchanges of the culture volume. Likewise, four independent benzoate-grown batch cultures (cultivated in the bioreactor) were harvested at the half-maximal optical density (OD = 0.3). Cells were harvested as described previously (8), and cell pellets were immediately frozen in liquid nitrogen and stored at -80°C.

Sampling for chemical, spectrometric, microscopic, and gravimetric analyses. To withdraw samples during continuous cultivation, the bioreactor was connected to a sterile 250-ml Duran bottle equipped with a lower glass port (Ochs GmbH). The lower glass port was coupled via Viton tubing to exchangeable, sterile plastic syringes (B. Braun, Melsungen, Germany). Prior to sampling, the tubing between the bioreactor and the sampling bottle was flushed with culture liquid, which was discarded. Sample volumes were <3% of the total culture volume to minimize disturbance of continuous cultures. Samples were processed as follows. (i) For high-pressure liquid chromatography (HPLC) analysis, cells were immediately removed by filtration (0.2 μm) (Schleicher & Schuell) and the supernatant was stored in aliquots at -20°C. (ii) For determination of total cell numbers, cells were immediately diluted in Tris-HCl buffer (100 mM Tris-HCl, 5 mM MgCl₂, pH 7.5) and counted at least in duplicates (standard deviations of <5%) using a Neubauer hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). (iii) For whole-cell hybridization and flow cytometry, cells were fixed in 4% (vol/vol) formaldehyde-containing phosphate-buffered saline (PBS) (145 mM NaCl, 8 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, pH 7.4) for 1 h at 20°C. Cells were washed with PBS and PBS-96% ethanol (1:1) and stored at -20°C. (iv) The dry mass of cells in steady state was determined after washing with Tris-HCl buffer (100 mM Tris-HCl, 5 mM MgCl₂, pH 7.5) and drying at 85°C to a constant weight (the weight of the Tris-HCl buffer was subtracted).

HPLC analysis. Benzoate was determined with an HPLC system (Sykam GmbH) previously described for the determination of nitrate and nitrite (57) at a retention time of 3.2 min and a detection limit of 20 μM benzoate. The eluent was composed of 20 mM NaCl in 45% (vol/vol) ethanol. Concentrations of low-molecular-weight organic acids were determined with an HPLC system (Sykam GmbH) equipped with an anion neutral precolumn (4 by 20 mm; Sykam GmbH) and an Aminex HPX-87H separation column (300 by 7.8 mm; Bio-Rad, Munich, Germany) that was temperature controlled to 60°C. With 5 mM H₂SO₄ in HPLC-grade water as the eluent and a flow rate of 0.6 ml min⁻¹, organic acids were detected using a refractive index (RI) or UV detector (210 nm) at retention times (detection limit) of 10.6 min (100 μM) for succinate (RI) and 13.8 min (25 μM) for acetate (UV).

Modeling. A cell population model was fitted to experimentally determined steady-state cell numbers and residual substrate concentrations. The applied model described by Droop (15, 16) was adopted to include the relevant solute uptake systems; i.e., acetate uptake is predicted to proceed via an unassigned permease, while two uptake systems (the symporter BenK and the ABC transporter EbA5303-09) are predicted for benzoate. Acetate uptake is described by a Monod kinetic (40) as follows

$$\rho^{\text{acetate}}(N) = \frac{\rho_{\text{max}}^{\text{perm}} N}{\rho_{\text{s}}^{\text{perm}} + N} \quad (1)$$

with acetate concentration N , maximum growth rate $\rho_{\text{max}}^{\text{perm}}$, and half-saturation constant $\rho_{\text{s}}^{\text{perm}}$. For benzoate, the combination of two uptake systems was modeled as

$$\rho^{\text{benzoate}}(N) = \frac{\rho_{\text{max}}^{\text{symp}} N}{\rho_{\text{s}}^{\text{symp}} + N} + \frac{\rho_{\text{max}}^{\text{ABC}} N}{\rho_{\text{s}}^{\text{ABC}} + N} \quad (2)$$

with benzoate concentration N , maximum growth rates $\rho_{\text{max}}^{\text{symp}}$ and $\rho_{\text{max}}^{\text{ABC}}$ (specific to the symporter and ABC transporter, respectively), and half-saturation constants $\rho_{\text{s}}^{\text{symp}}$ and $\rho_{\text{s}}^{\text{ABC}}$ for the respective uptake systems.

The Droop equations (equations 3 to 5) were applied to describe the dynamics of the bacterial population in the chemostat: the extracellular substrate concentration N , population density P , and cell quota Q (nutrient content per cell) vary according to

$$\dot{N} = D(N_{\text{in}} - N) - \alpha \rho(N)P \quad (3)$$

$$\dot{P} = [\mu(Q) - D]P \quad (4)$$

$$\dot{Q} = \rho(N) - \mu(Q)Q \quad (5)$$

with dilution rate D , input substrate concentration N_{in} in the feed medium ($N_{\text{in}} = 7.6$ mM for acetate and $N_{\text{in}} = 3.9$ mM for benzoate), conversion coefficient α , and quota-dependent growth $\mu(Q) = \mu_Q[1 - (K_Q/Q)]$, where μ_Q denotes the maximum growth rate and K_Q is the minimal amount of nutrient per cell. Nutrient uptake is described by $\rho(N) = \rho^{\text{acetate}}(N)$ for acetate and $\rho(N) = \rho^{\text{benzoate}}(N)$ for benzoate. For parameter estimation, relative quadratic deviations of measured cell numbers and residual substrate concentrations at steady state and corresponding equilibrium values of model simulations were minimized using the metaheuristics differential evolution (63) and adaptive simulated annealing (31) implemented by Oldenhuis and Vandekerckhove (48). The model parameters obtained are provided in Table S1 in the supplemental material.

Thermodynamic calculations. The total free energy ($\Delta G'_{\text{total}}$) transformed during substrate catabolism, as well as the free energy consumption rates for biomass formation ($\Delta G'_{\text{biomass}}$) and growth-dependent maintenance [$m(\mu)$], were calculated at physiological conditions from experimentally determined benzoate, acetate, nitrate, and nitrite concentrations (Fig. 1A; see Fig. S1 in the supplemental material). Free energy values under standard conditions ($\Delta G'^0$) were calculated from published ΔG_f^0 values (65, 77) for benzoate and acetate degradation coupled to nitrate reduction or denitrification (equations 6 to 9).

HCO_3^- concentrations (4 to 18 mM) were estimated from the amount of substrate that was dissimilated. The N_2 pressure was constant at 10^5 Pa. Free energy consumption for biomass formation ($\Delta G'_{\text{biomass}}$) was calculated from experimentally determined dry weight using the empirical relation that the formation of 1 g (dry weight) of biomass requires ~ 100 mmol ATP (65). The estimated free energy to generate 1 mol ATP is 100 kJ. The maintenance energy consumption rate m_e corresponds to the free energy consumed by a nongrowing population (i.e., $\mu = 0.0 \text{ h}^{-1}$), e.g., to sustain membrane integrity and proton gradients (26, 66). Under anoxic conditions, (facultative) anaerobic bacteria, regardless of species, consume $4.58 \text{ kJ (g dry weight)}^{-1} \text{ day}^{-1}$ for such maintenance-related functions (66). The growth-dependent maintenance energy consumption

rate $m(\mu)$ in actively growing bacterial cultures was calculated as follows: $m(\mu) = \Delta G'_{\text{total}} - \Delta G'_{\text{biomass}} - m_e$ (26).

Flow cytometry. The relative amounts of DNA and protein per cell were determined using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an air-cooled argon laser at 488 nm. Formaldehyde-fixed cells of strain EbN1 were diluted 1:150 in PBS and stained on ice for 20 min with SYBR green I (1:10,000 dilution of the commercial stock; Molecular Probes, Eugene, OR). Alternatively, fixed cells were incubated for 3 h with Flamingo (1:10 dilution of the commercial stock; Bio-Rad) in the presence of 0.03% (wt/vol) SDS. Green fluorescence (band-pass filter, 530 nm) was measured with logarithmic signal amplification. All measurements were standardized to the fluorescence of yellow-green fluorescent latex beads (0.99- μm diameter; Molecular Probes) added to each sample (84). A total of 70,000 events were recorded per measurement; each sample was measured in triplicates. Data acquisition and analysis were performed with CellQuest Pro software (Becton Dickinson).

TEM. Cells from steady-state cultures were stained with 2% (wt/vol) uranyl acetate for 5 min on Formvar/carbon-coated copper grids (3.05 mm, 400 mesh; Plano GmbH, Wetzlar, Germany). The grids were then gently washed three times in 50- μl droplets of distilled water. The EM 902A electron microscope (Zeiss, Jena, Germany) was operated at 80 kV, and digital images were recorded using a 1k Proscan high-speed slow-scan charge-coupled device (SSCCD) camera (Proscan Elektronische Systeme GmbH, Lagerlechfeld, Germany) controlled by iTEM software (Olympus Soft Imaging System GmbH, Münster, Germany). Cell sizes were determined manually for an average of 220 cells per sample from transmission electron microscopy (TEM) images using measureIT software (Olympus Soft Imaging System GmbH). The biovolume was calculated for cylindrical cells with hemispherical ends from measured maximal cell lengths and cell widths (64). Dividing cells were visually distinguished by the appearance of cell constriction.

Control of culture purity in chemostats. Anaerobic growth tests, light microscopy, and whole-cell hybridization were used to verify that continuous cultures were free of contamination. Anaerobic growth tests were performed in glass tubes containing 15 ml anoxic medium. Based on the known anaerobic substrate range of strain EbN1 (R. Rabus et al., unpublished data), the following growth-supporting (+) or non-growth-supporting (−) substrates were used (concentrations are given as percentages [vol/vol in HMN] unless stated otherwise): toluene (2; +), ethylbenzene (2; +), *p*-ethylphenol (0.25; +), *n*-propylbenzene (2; −), *p*-cymene (5; −), *m*-xylene (2; −), *p*-ethyltoluene (2; −), *n*-hexane (5; −), propiophenone (1; −), α -terpinene (1; −), α -phellandrene (1; −), and glucose (5 mM; −). Tubes were inoculated with cells retrieved directly from the chemostats via the sampling port (inoculum size, 5% [vol/vol]). Controls lacked either the organic substrate or the inoculum. Growth tests were monitored for 4 weeks. In all cases, growth was observed only in the presence of the three growth-supporting substrates mentioned above.

Whole-cell hybridization was performed as described previously (38) at 46°C for 3 h in hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.01% (wt/vol) SDS, and 30% (vol/vol) formamide. The probe concentration was $5 \text{ ng } \mu\text{l}^{-1}$. Hybridization was followed by a stringent washing step at 48°C for 25 min in prewarmed washing buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.01% (wt/vol) SDS. Subsequently, slides were rinsed with distilled water and air dried. Cells were stained with $1 \mu\text{g ml}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) for 3 min and rinsed again with distilled water and 96% ethanol. Air-dried slides were embedded in Citifluor (Citifluor Ltd., London, United Kingdom) and examined with an Axiophot epifluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). The ARB software package (36) was used to design a new oligonucleotide probe termed EbN825, which targets the 16S rRNA of strain EbN1 at *Escherichia coli* positions 825 to 842 (5' $\rightarrow$ 3' probe sequence, ACCGCACCGAACAAGT AG). The online resource probeCheck (35) was used to assess probe spec-

FIG 1 Growth of *A. aromaticum* EbN1 in anoxic, benzoate-limited chemostats with nitrate as an electron acceptor during stepwise increase of the dilution rate. (A) Cell numbers and residual concentrations of nitrate, nitrite, and benzoate. The detection limit for benzoate was $20 \mu\text{M}$. (B) Measured (Y) and maximal (Y_G) growth yields, as well as average DNA and protein contents per cell in arbitrary units (AU). (C) Cell length, cell width, and calculated biovolume. TEM images of an average steady-state cell and the percentages of dividing cells at the indicated dilution rates are also shown.

ificity, which was evaluated experimentally by whole-cell hybridization at optimized stringency with close relatives of strain EbN1 (*Aromatoleum* strains pCyN1, HxN1, and PbN1). The eubacterial probe EUB338 (2) was used as positive hybridization control. Probe NON338 (74) served as negative control for nonspecific binding. Cy3-labeled oligonucleotide probes were purchased from Biomers (Ulm, Germany).

Analysis of soluble protein fraction by 2D DIGE and MALDI-TOF MS. Preparation of cell-free protein extracts and two-dimensional difference gel electrophoresis (2D DIGE) were carried out as recently reported (23, 56). Cells were disrupted with the PlusOne sample grinding kit (GE Healthcare, Munich, Germany), and the protein concentration was determined as described by Bradford (6). Isoelectric focusing (IEF) was per-

formed using the IPGphor system (GE Healthcare) and commercial 24-cm immobilized pH gradient (IPG) strips with a nonlinear (NL) pH gradient of 3 to 11 (GE Healthcare). The EttanDalt II system (GE Healthcare) was used for protein separation according to molecular mass in 12.5% acrylamide gels. Pre-electrophoretic labeling with different fluorescent dyes allows coseparation of three protein samples (50 μ g each) in a single gel, representing the reference state, the test state, and the (pooled) internal standard. Protein extracts from steady-state cells at μ_{high} served as the reference state, and each of the four biological replicates was separately labeled with Cy5. Individual test states (4 biological replicates each) were prepared from protein extracts of steady-state cells at μ_{low} or μ_{med} or from protein extracts of batch-grown cells at $\mu_{\text{max (batch)}}$, all of which were separately labeled with Cy3. The internal standard was composed of equal amounts of protein extracts of the reference and all test states and was labeled with Cy2.

2D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (GE Healthcare). Cropped images were analyzed with the DeCyder software (version 5.0; GE Healthcare). To achieve statistical confidence, four gels (i.e., one for each biological replicate) were included in the analysis for each test state, yielding a total of 12 gels (representing 36 gel images). Parameters for codetection of spots were as described recently (79). The master gel contained 1,341 detected spots. Statistical analysis of the total variation of 2D electrophoresis (2DE)-resolved spots among the four biological samples of the reference and each test state was based on 622 protein spots that were matched in all gels. The determined total variation of biological replicates (coefficient of variation [CV], <18%) justified an average ratio (n -fold change in protein abundance) of ≤ -1.5 or ≥ 1.5 as threshold for significant abundance changes, as already reported recently for strain EbN1 (81). Differentially regulated spots had to fulfill the following criteria: analysis of variance (ANOVA) P value of <0.05 , t test value of $<10^{-4}$, and matched in at least 75% of all gels.

Protein spots with changed abundances were manually excised from preparative 2DE gels (300- μ g protein load) stained with colloidal Coomassie brilliant blue (cCBB) as described previously (12). Tryptic digestion of excised 2DE-separated proteins was performed as previously described (32). Peptide masses were determined with matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) by Toplab GmbH (Martinsried, Germany). Peptide mass fingerprints were mapped to the *in silico* digests of the predicted proteins by using the MS-Digest program (9). Database searching of the monoisotopic masses was performed with Mascot using the previously published list of annotated genes in the genome sequence of strain EbN1 (55).

Shotgun proteomics by liquid chromatography-electrospray ionization tandem MS (LC-ESI-MS/MS). For shotgun proteomics, cell pellets (110 to 160 mg [wet weight]) representing μ_{low} and μ_{high} were resuspended in 200 μ l lysis buffer (30 mM Tris-HCl [pH 8.5], 2 M thiourea, 7 M urea). Cell disruption was performed with the PlusOne sample grinding kit (GE Healthcare). Subsequently, cell debris was removed by centrifugation (20 min, 15,000 $\times$ g, 17°C). Disulfide bonds were reduced with 45 mM dithiothreitol at 55°C for 30 min, and alkylation of free cysteines was achieved with 100 mM iodoacetamide (15 min at room temperature). The protein solution was diluted with HPLC-grade water to obtain a concentration of 2 M urea prior to digestion with 0.5 μ g trypsin Gold (Promega, Mannheim, Germany) at 37°C overnight. To avoid clogging, the solution was ultracentrifuged (1 h, 75,000 $\times$ g, 17°C).

Separation of peptides (200 ng total protein) was performed with an UltiMate3000 nanoLC system (Dionex GmbH, Munich, Germany). Peptides were loaded on a trap column with 0.05% trifluoroacetic acid (TFA) as the solvent at a flow rate of 6 μ l min⁻¹ (PepMap nanoTrap [C₁₈, 100 Å, 3- μ m bead size, 75- μ m inner diameter {i.d.}, 2-cm length]; Dionex GmbH). Separation of peptides was achieved with an analytical column (PepMapRSLC [C₁₈, 100 Å, 2- μ m bead size, 75- μ m i.d., 15-cm length]; Dionex GmbH) and a 215-min gradient at a flow rate of 0.3 μ l min⁻¹ (solvents with 0.05% TFA): 3 to 5% acetonitrile (ACN) in 6 min, 5 to 22%

ACN in 106 min, 22 to 26% ACN in 24 min, 26 to 32% ACN in 44 min, 32 to 76% ACN in 35 min, and 80% ACN for 30 min.

Mass spectrometric analysis of the nanoLC effluent was performed with an online-coupled ion trap mass spectrometer (amaZon ETD; Bruker Daltonik GmbH, Bremen, Germany). For electrospray ionization, a distal coated fused SilicaTip (NewObjective, Woburn, MA) with an inner diameter of 10 μ m was used at a voltage of 1,600 V. The MS method consisted of a full MS scan (mass range, 300 to 1,500 m/z) with subsequent MS/MS of the 10 most intense MS peaks (successive exclusion after 1 spectrum for 0.2 min).

Protein identification was performed with ProteinScape (version 2.1; Bruker Daltonik GmbH) on a Mascot server searching against a genomic database of strain EbN1, translated into amino acid sequences, including a target-decoy strategy. The following settings were applied: taxonomy, all entries; enzyme, trypsin; may missed cleavage, 1; fixed modification, carbamidomethyl (C); variable modification, oxidation (M); peptide mass tolerance, 0.4 Da; fragment mass tolerance, 0.4 Da; mass values, monoisotopic; instrument type, ESI-TRAP; and inclusion of peptide decoy. A false discovery rate (FDR) of <1% was applied as threshold of significance. In total, 325 different proteins were identified, with identification scores ranging from 29 to 2,894 (median of 148) and sequence coverage from 2 to 99% (median of 25%).

Analysis of membrane protein-enriched fraction by SDS-PAGE and LC-ESI-MS/MS. Differences in the membrane protein compositions of cells grown at μ_{low} and μ_{high} were analyzed. Following mechanical breakage of cells (~120 mg [wet weight]), membrane preparations were obtained by ultracentrifugation and treatment with ice-cold carbonate, essentially as described elsewhere (44). Proteins were solubilized from the prepared membranes by treatment with hot (95°C) SDS (1% [wt/vol]). Protein concentrations were determined with the detergent-compatible RC DC protein assay (Bio-Rad). Protein separation (20 to 40 μ g per lane) was achieved by conventional SDS-PAGE (12.5% acrylamide, 16- by 20-cm gels), and proteins were stained with cCBB.

For both states (μ_{low} and μ_{high}), one complete protein lane was cut into 33 gel slices. These were then further cut into smaller pieces (about 1 mm) and washed twice with 50 mM NH₄HCO₃ and 50 mM NH₄HCO₃:ACN (1:1 [vol/vol]) prior to reduction, alkylation, and tryptic digestion with 5 ng μ l⁻¹ trypsin solution (Promega) in 25 mM NH₄HCO₃. Following incubation overnight at 37°C, peptides were extracted once with 60% ACN and 1% TFA and once with 90% ACN and 1% TFA. The supernatants were pooled prior to analysis.

Separation of peptides was performed with an UltiMate3000 nanoLC system (Dionex GmbH) (see above) and a 95-min linear gradient at a flow rate of 0.3 μ l min⁻¹ (solvents with 0.05% TFA): 3 to 5% ACN in 6 min, 5 to 40% ACN in 89 min, 40 to 76% ACN in 2 min, and 80% ACN for 15 min. Mass spectrometric analysis with an ion trap mass spectrometer (amaZon ETD) and subsequent protein identification were performed as described above. In total, 1,041 different proteins were identified, with identification scores ranging from 28 to 3,309 (median of 112) and sequence coverage from 1 to 94% (median of 15%). Putative transmembrane domains were detected using the TMHMM 2.0 prediction server (34). Approximately 25% of the identified proteins contain between 1 and 15 transmembrane helices.

Analysis of PHB. Poly(3-hydroxybutyrate) (PHB) was analyzed in cell pellets (80 to 120 mg [wet weight]) as described previously (68). The total amount of PHB was analyzed in relation to methyl esters obtained from purchased sodium salt of 3-hydroxybutyric acid. Three biological replicates were analyzed per growth condition.

Analytical procedure for investigation of membrane PLs. Cell pellets (three biological replicates per growth condition, 80 to 130 mg [wet weight]) were extracted for inventory of their phospholipids (PLs) (cytoplasmic and outer membrane) using a method modified from that described by Bligh and Dyer (5). The organic extracts were separated into fractions of different polarity using a pure silica column and a Florisil column in sequence (for details, see reference 82). Intact PLs were ana-

lyzed by HPLC (SCL-10a VP; Shimadzu GmbH) coupled with a Finnigan MAT TSQ 7000 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray interface as described by Zink and Mangelsdorf (82). The head group inventory was quantified using a procedure reported by Mangelsdorf et al. (37). The fatty acyl side chain inventory was analyzed by gas chromatography (GC)-MS after *trans*-esterification of the phospholipid fraction to obtain the methylated formerly phospholipid-linked fatty acids using the method described by Mueller et al. (41). The position of the double bond(s) in unsaturated phospholipid-linked fatty acids (PLFAs) was determined as described by Pepe et al. (50).

RESULTS

Growth in benzoate-limited chemostats. The anaerobic growth behavior of benzoate-limited cells of strain EbN1 under nitrate-reducing conditions was characterized from low (D_{low}) (0.036 h^{-1}) to critical (D_{crit}) ($>0.20 \text{ h}^{-1}$) dilution rates (Fig. 1). At D_{low} , cell numbers were maximal, and they then gradually decreased with increasing D until cells were washed out exponentially at D_{crit} (Fig. 1A). At a D of $<0.18 \text{ h}^{-1}$, residual benzoate concentrations were $<20 \mu\text{M}$. Low levels of excreted succinate ($<0.25 \text{ mM}$) were detected across the complete D range (data not shown). At D_{low} , similar residual concentrations of nitrate ($<1 \text{ mM}$) and nitrite ($<0.5 \text{ mM}$) were observed, whereas at D_{high} (0.18 to 0.20 h^{-1}), low nitrate ($<100 \mu\text{M}$) and high nitrite (3 to 8 mM) concentrations were detected (Fig. 1A). At D_{crit} , residual concentrations of nitrate and benzoate increased exponentially, as expected. At D_{high} , the DNA and protein contents of cells were more than 2- and 7-fold, respectively, higher than at D_{low} (Fig. 1B). The average biovolume of cells almost doubled from D_{low} ($1.4 \pm 0.5 \mu\text{m}^3$) to D_{high} ($2.6 \pm 0.7 \mu\text{m}^3$), which was paralleled by an increase in the percentage of dividing cells from 20 to 50% (Fig. 1C). During washout (nonlimited conditions), the biovolume ($5.5 \pm 1.8 \mu\text{m}^3$) and DNA content per cell further increased (Fig. 1B and C).

Comparison of benzoate- with acetate-limited growth in chemostats. The growth behavior of strain EbN1 in acetate-limited chemostats was analyzed from D_{low} to D_{crit} (0.06 to 0.25 h^{-1}) (Fig. 2; see Fig. S1 in the supplemental material). Growth functions and population models were fitted to experimental data by nonlinear optimization (Fig. 2). Irrespective of the limiting carbon source (benzoate or acetate), only a population model including intracellular nutrient contents (cell quota) resulted in a satisfactory match of simulation results and experimental data. Profiles of cell numbers were essentially congruent in acetate- and benzoate-limited chemostats (Fig. 2A). Acetate consumption followed Monod kinetics, with a graphically determined half-saturation constant (K_S) of 0.44 mM acetate (Fig. 2B). Residual benzoate concentrations in the nondetectable ($<20 \mu\text{M}$) range prevailed from D_{low} until close to D_{high} . At D_{high} , benzoate concentrations reaching the millimolar range followed a steep saturation kinetic, with a $K_{S(\text{benzoate})}$ of 0.24 mM (Fig. 2B). For fitting the model to the experimental data, residual substrate concentrations (where available) and determined cell numbers were taken into account. Since cell numbers were quantified from D_{low} to D_{high} , they could partly compensate for benzoate concentrations that were nondetectable. Corresponding fitting attempts suggested the involvement of two benzoate uptake systems, which were modeled by superimposing two Monod functions (equation 2). The resulting parameters predict the two assumed transporters to exhibit significant differences in benzoate affinity (see Table S1 in the supplemental material).

FIG 2 Experimental data from benzoate- or acetate-limited anoxic chemostat cultures of *A. aromaticum* EbN1 and fitted models. Cell numbers (A) and residual concentrations of benzoate or acetate (B) as a function of growth rate are shown. Graphically determined K_S values: $K_{S(\text{acetate})}$, 0.44 mM ; $K_{S(\text{benzoate})}$, 0.24 mM .

Similar nitrate/nitrite concentration profiles were observed with acetate (see Fig. S1A in the supplemental material) and benzoate (Fig. 1A) at D_{low} . At $>D_{\text{low}}$, however, nitrite concentrations increased to markedly higher levels with acetate (up to 11 mM) than with benzoate (up to 8 mM). This difference is paralleled by a μ -dependent increase in calculated nitrite consumption rates with benzoate (~ 4 -fold), contrasted by unchanged rates with acetate ($\sim 0.5 \text{ mM nitrite h}^{-1}$). Conversely, the μ -dependent increase in calculated nitrate consumption rates (4 - to 6 -fold) was in the same range with benzoate and acetate.

Free energy consumption rates for biomass formation ($\Delta G'_{\text{biomass}}$) and growth-dependent maintenance [$m(\mu)$] were calculated. During benzoate-limited growth of strain EbN1, energy consumption for biomass formation increased steadily from 8 to $48 \text{ kJ (g dry weight)}^{-1} \text{ day}^{-1}$ with increasing D (Fig. 3A). At D values of $\leq 0.108 \text{ h}^{-1}$, energy consumption for maintenance-related cellular functions increased linearly from 22 to $71 \text{ kJ (g dry weight)}^{-1} \text{ day}^{-1}$. At higher D values, these expenditures were constant at $73 \pm 9 \text{ kJ (g dry weight)}^{-1} \text{ day}^{-1}$ (Fig. 3A and B). Overall, similar absolute values were obtained for acetate-limited growth. However, at D values of $>0.20 \text{ h}^{-1}$ (which equals D_{crit} during benzoate-limited growth), $m(\mu)$ further increased with growth rate by about 2 - to 3 -fold (Fig. 3B). Benzoate- and acetate-limited cells consumed between 60 and 78% of the available free energy for maintenance [as the sum of m_e at $\mu = 0.0 \text{ h}^{-1}$ and growth-

FIG 3 Free energy consumption rates in carbon-limited anoxic chemostat cultures of *A. aromaticum* EbN1. (A) Free energy from benzoate catabolism ($\Delta G'_{total}$) is consumed for biomass formation ($\Delta G'_{biomass}$) and maintenance [m_e and $m(\mu)$]. m_e corresponds to the maintenance energy consumption of nongrowing populations (i.e., $\mu = 0.0 \text{ h}^{-1}$). Actively growing populations have higher maintenance energy demands, which is described by the growth-dependent maintenance energy consumption rate $m(\mu)$. (B) Comparison of $m(\mu)$ during benzoate- and acetate-limited growth.

dependent $m(\mu)$]. Benzoate-limited cells utilized $\sim 75\%$ of the available energy for maintenance at D values of $\leq 0.108 \text{ h}^{-1}$, whereas with increasing D , this share gradually decreased to 60% at 0.20 h^{-1} . Notably, the opposite trend was observed with acetate-limited cells. At low growth rates, a higher deviation between Y (measured growth yield) and Y_G (maximal attainable growth yield corrected for maintenance) (51, 72) was observed in benzoate-limited cells (Fig. 1B).

Experimental design for studying the molecular adaptation to μ_{low} during benzoate-limited anaerobic growth. To study growth rate-dependent effects on the proteome (Fig. 4 and 5) and membrane phospholipid and poly(3-hydroxybutyrate) (PHB) content (Fig. 5), biomass of strain EbN1 was generated in a series of chemostat cultures individually adapted to μ_{low} (0.036 h^{-1}), μ_{med} (0.108 h^{-1}), or μ_{high} (0.180 h^{-1}). The replicate cultures displayed similar steady-state parameters (Table 1), which also agreed with those of the initial chemostat culture used for detailed physiological characterization (Fig. 1).

Proteome analysis with 2D DIGE employed steady-state cells at μ_{low} or μ_{med} and batch-grown cultures at $\mu_{max} \text{ (batch)}$ (0.196 h^{-1}) as test states, while steady-state cells at μ_{high} served as the reference

state. The largest number of proteins with changed abundance (151; $\sim 18\%$ of the 2DE-resolvable proteome) was observed at μ_{low} (Table 2 and Fig. 4A; see Table S2 in the supplemental material). To cover proteins not captured by 2D DIGE, whole-cell extracts (shotgun) and SDS-PAGE-resolved, membrane protein-enriched fractions were analyzed by LC-ESI-MS/MS (Fig. 4BC; see Table S2 in the supplemental material).

Anaerobic metabolism of benzoate at μ_{low} . Anaerobic benzoate degradation proceeds via the canonical benzoyl-CoA pathway, yielding three molecules of acetyl-CoA and one molecule of CO_2 (22). Cells of strain EbN1 growing at μ_{low} contained uniformly decreased amounts of the protein components of this pathway, ranging from -1.5 -fold for benzoate-CoA ligase (BclA) to -3.0 -fold for BcrB, the β subunit of benzoyl-CoA reductase (Table 2; Fig. 5). The associated transcriptional repressor BzdR was identified in cells at μ_{low} and μ_{high} (see Table S2 in the supplemental material). Moreover, most enzymes of acetyl-CoA conversion via the tricarboxylic acid (TCA) cycle (e.g., AcnB and KorCAB), the glyoxylate shunt (e.g., AceA), and anaplerotic reactions (e.g., MaeB1) displayed decreased abundances at μ_{low} . In contrast, NAD^+ -dependent isocitrate dehydrogenase (Icd) and two subunits of 2-oxoglutarate dehydrogenase (SucB and Lpd) increased in abundance.

For energy conservation, anaerobic benzoate oxidation to CO_2 is coupled to denitrification. The 5-fold-lower nitrate consumption rate at μ_{low} (Table 1) is paralleled by 3.0- and 2.5-fold-decreased abundances of the α and β subunits of dissimilatory nitrate reductase (NarGHI) (Table 2). Other denitrification enzymes (including chaperones), two nitrate transporters (NarK1 and -2), and three predicted regulators (NarX, Dnr, and NosZ) were identified in the membrane protein fraction (Fig. 4B and 5; see Table S2 in the supplemental material).

Acetyl-CoA from benzoate oxidation can be diverted from entry into the TCA cycle toward PHB synthesis. The highest quantities of PHB were observed at μ_{high} and $\mu_{max} \text{ (batch)}$ ($\sim 30 \text{ fg PHB cell}^{-1}$), while at μ_{low} and μ_{med} , cells contained 3- and 10-fold less PHB, respectively (Fig. 5). This reduced PHB level correlated most markedly with the decreased abundance of phasin EbA1323 (Table 2; Fig. 5). Among the various phasins of strain EbN1, EbA1323 displays the highest sequence identity (51%) to PhaP1 of *Ralstonia eutropha* H16, synthesizing only such amounts of this major phasin that can be bound to the PHB granules (43, 78). Opposite abundance profiles were observed for two putative acetoacetyl-CoA reductases (PhbB and Adh) of strain EbN1 at μ_{low} . Other detected proteins of PHB metabolism comprised three additional phasins, four paralogueous PHB synthases (PhbC), a PHB depolymerase (PhbZ), and PhbR, a transcriptional repressor of PHB synthesis (Table 2 and Fig. 4B and 5; see Table S2 in the supplemental material).

Formation of diverse catabolic proteins in the absence of their respective substrates. In contrast to the reduced levels of proteins of the anaerobic benzoyl-CoA pathway at μ_{low} , several proteins of other anaerobic or aerobic degradation pathways were newly formed in most cases, despite absence of the respective substrate (Table 2 and Fig. 5; see Table S2 in the supplemental material). Notably, the largest changes in abundance (up to 85.8-fold) were observed for all protein components of the aerobic degradation pathway of *m*-hydroxybenzoate and gentisate (79). Several catabolic proteins detected at μ_{low} were previously linked to anaerobic degradation pathways of aromatic alcohols (AdhB) and aldehydes (AldB), *m*-hydroxybenzoate (HbrB) (79), 3-phenylpropanoids (EbA5320) (69), ethylbenzene (Ped) (54), and *p*-eth-

FIG 4 Protein inventory of *A. aromaticum* EbN1 at different growth rates. (A) 2D DIGE gel (pH 3 to 11 NL; left to right) comparing the protein profiles of cells at μ_{low} to those at μ_{high} . For predicted functions of annotated proteins, see Table 2. (B) Selected proteins (see Fig. 5) identified from SDS-PAGE-resolved, membrane protein-enriched fractions at μ_{low} or μ_{high} . The predicted functions of selected and additional proteins are given in Table S2 in the supplemental material. (C) Total proteomic coverage at $\mu_{low/med/high}$ and $\mu_{max(batch)}$ and assignment of identified proteins to different cellular functions. The share of “changed” proteins includes proteins with quantified abundance changes (2D DIGE) as well as proteins that were detected by LC-ESI-MS/MS exclusively at μ_{low} or μ_{high} or that displayed large differences (>10 -fold) in identification scores (Mascot).

ylphenol (PchF and ChnA) (80). Moreover, proteins involved in catabolism of the aliphatic substrates propionate (PrpE), pyruvate (AceEF), acetate (AcsA), and formate (FdH) showed increased abundances. While all protein constituents of the aerobic *m*-hy-

droxybenzoate/gentisate pathway displayed increased abundances, for all other aerobic or anaerobic pathways only single to few proteins were detected. In batch cultures adapted to various aromatic substrates, the formation of protein constituents of the

FIG 5 Integrated scheme of proteomic and cellular responses of *A. aromanticum* EBNI to μ_{low} . Proteins with abundance differences determined by 2D DIGE (Fig. 4A) are color coded, and their predicted functions are provided in Table 2. Proteins detected by LC-ESI-MS/MS (Fig. 4B) are marked in black, and their predicted functions are provided in Table S2 in the supplemental material. Proteins not captured by proteomics are marked in gray. Of the 159 proteins displayed in this scheme, 143 were identified by MS. Growth rate-dependent profiles of PTHB, intact phospholipids (PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol), and phospholipid-derived fatty acids are displayed as bar charts.

TABLE 1 Steady-state physiological characteristics of *A. aromaticum* EbN1 in benzoate-limited chemostats harvested for molecular analyses

Physiological characteristic	Value ^a at growth rate (steady state):		
	μ_{low}	μ_{med}	μ_{high}
Doubling time (h)	19.4 ± 0.2	6.4 ± 0.1	3.9 ± 0.0
Cell no. (10 ⁷ ml ⁻¹)	100.0 ± 7	97.0 ± 12	55.0 ± 8
Biovolume (μm ³)	1.1 ± 0.2	1.4 ± 0.3	1.9 ± 0.5
DNA cell ⁻¹ (AU)	1.2 ± 0.1	1.3 ± 0.0	1.6 ± 0.1
Protein cell ⁻¹ (AU)	2.2 ± 0.8	2.5 ± 0.2	5.7 ± 0.9
Dividing cells (% of total cells)	16.8 ± 2.6	26.8 ± 7.1	44.4 ± 3.1
Y [g dry wt (mol benzoate) ⁻¹]	26.9 ± 3.4	39.4 ± 4.7	50.9 ± 8.6
ΔG ^b biomass [kJ (g dry wt) ⁻¹ day ⁻¹]	8.9 ± 1.9	26.0 ± 2.0	43.0 ± 2.0
m(μ) [kJ (g dry wt) ⁻¹ day ⁻¹]	46.9 ± 14.9	90.0 ± 15.3	94.3 ± 21.6
Dissimilation-to-assimilation ratio	4.3 ± 0.6	3.0 ± 0.3	2.2 ± 0.4
Nitrate consumption rate (mmol liter ⁻¹ h ⁻¹)	0.5 ± 0.02	1.7 ± 0.05	2.6 ± 0.05
Nitrite consumption rate (mmol liter ⁻¹ h ⁻¹)	0.5 ± 0.02	1.5 ± 0.07	2.2 ± 0.06

^a Average values and standard deviations calculated from four biological replicates per growth rate.

m-hydroxybenzoate/gentisate pathway also displayed lowest substrate specificity (79).

Formation of high-affinity solute uptake transporters at μ_{low} . Several proteins involved in high-affinity solute uptake displayed maximal abundance at μ_{low} , declining considerably with increasing growth rate (Table 2 and Fig. 5; see Table S2 in the supplemental material).

(i) **Benzoate uptake.** The active uptake of benzoate is predicted to be facilitated by an ABC transporter (EbA5303-09) and by the benzoate:H⁺ symporter BenK (55). In contrast to the reduced abundance of protein components of the anaerobic benzoyl-CoA pathway at μ_{low} , the predicted periplasmic benzoate-binding protein (EbA5303) and an ATP-binding subunit (EbA5307) of the ABC transporter increased in abundance. The presence of the other components of the ABC transporter and of BenK in the membrane protein enriched fraction of cells at μ_{low} and μ_{high} was confirmed (Fig. 4B).

(ii) **Uptake of other substrates.** Several periplasmic ABC-type amino acid-binding proteins (LivK, GltI, and EbA3556) displayed up to 12.4-fold-increased abundances at μ_{low} . Their corresponding permeases were detected primarily at μ_{low} . In addition, strongly increased abundances (up to 16.9-fold) were observed for various TRAP-type periplasmic solute-binding proteins, including two putative aromatic compound-binding proteins (EbA1033 and EbA4994) as well as C₄-dicarboxylate-binding DctP (EbA4158) (67).

Modification of cellular membranes at μ_{low} . Phosphatidylethanolamines (PEs) and phosphatidylglycerols (PGs) constitute the major phospholipids (PLs) of strain EbN1. Phosphatidylcholines (PCs) accounted for 7.3% of phospholipids at μ_{low} , decreasing to 1.5% at μ_{med} and vanishing at μ_{high} and μ_{max} (batch) (Fig. 5). PG levels remained constant, while loss of PC was paralleled by an increase in PE. Thus, one may speculate that strain EbN1 synthesizes the choline moiety by methylation of a PE precursor. A putative PE *N*-methyltransferase (PemT) catalyzing this reaction is present in the genome of strain EbN1, whereas the enzyme of an alternative pathway (PC synthase; Pcs) (62) could not be assigned. In addition to these changes in head groups at μ_{low} , phospholipid

side chains contained C_{17:1} fatty acids (up to 1.5%) and larger amounts of C₁₆ than of C₁₈ fatty acids (Fig. 5). This resulted from a decrease in C_{18:1}ω7, while levels of C_{16:1}ω7 increased. C₁₆ (saturated and monounsaturated) fatty acids constitute ≥90% of the total PL-linked fatty acids (PLFAs) in strain EbN1 (see also reference 83). Differences in the overall degree of PLFA unsaturation were not observed.

Nucleotide synthesis and DNA replication at μ_{low} . The lower DNA content per cell at μ_{low} than at μ_{high} (Table 1) was paralleled by a decreased abundance of proteins involved in DNA synthesis (Table 2 and Fig. 5; see Table S2 in the supplemental material). Deoxynucleoside triphosphate (dNTP)-generating ribonucleotide reductase (NrdA) (−2.7-fold) plays a key role in the regulation of DNA replication rates (29). Nucleoside diphosphate kinase (Ndk) (−1.7-fold) uses ATP to produce other NTPs, and guanine monophosphate synthetase (GuaA) (−2.3-fold) is involved in *de novo* purine biosynthesis. The largest decrease in abundance was observed for the α-subunit of DNA gyrase (GyrA) (−17.2-fold).

Transcription and protein synthesis at μ_{low} . The transcription factor inventory changed at μ_{low} compared to μ_{high} (see Table S2 in the supplemental material). σ⁵⁴ and several transcriptional regulators (e.g., Crp and GntR- or LysR-type regulators) were detected only at μ_{low} , while σ⁷⁰ was only observed at μ_{high} .

Proteins related to translation uniformly displayed lowest abundances at μ_{low} (Table 2; also see Table S2 in the supplemental material), e.g., the 50S (RplI) and 30S (RpsAB) ribosomal proteins, translation initiation factor 2 (InfB), translation elongation factor Ts (Tsf), and several aminoacyl-tRNA synthetases. This indicates a diminished *de novo* protein synthesis, which would agree with the reduced protein content per cell at μ_{low} (Table 1). Likewise, major cellular chaperones (e.g., Tig, GroEL, and DnaK) displayed up to 2.0-fold-decreased abundance at μ_{low} . Considering the unchanged abundance of these chaperones (~1.1-fold) in strain EbN1 under diverse substrate conditions previously studied (79), the μ_{low} -specific abundance decrease might be linked to diminished protein synthesis.

Formation of stress-related proteins at μ_{low} . Several proteins involved in reactivation and repair of denatured proteins displayed most pronounced abundance changes at μ_{low} (Table 2 and Fig. 5; see Table S2 in the supplemental material). The abundances of peptidyl-prolyl *cis/trans* isomerase (PpiD) and two chaperones (ClpB and EbA4332) increased, while that of protease ClpA decreased.

Increased abundances of superoxide dismutase (SodB), two peroxiredoxins (Tpx and AhpC), glutathione S-transferase (Gst), and DNA-binding ferritin-like protein (Dps) might prevent proteins, DNA, and fatty acids from undergoing modification by reactive oxidizing agents at μ_{low} . Remarkably, several plasmid-encoded proteins involved in tellurite resistance (TerDEZ) exhibited abundance profiles differing from those of the aforementioned stress-related proteins; i.e., they had equally high abundances (up to 38.6-fold) at μ_{low} , μ_{med} , and μ_{max} (batch) compared to μ_{high} (see Table S2 in the supplemental material). A notable observation was the identification of several other plasmid 1- or plasmid 2-encoded proteins exclusively at μ_{low} (see Table S2 in the supplemental material). However, most of these proteins cannot unambiguously be assigned to distinct physiological functions.

Carbon-restricted (μ_{high}) versus unrestricted [μ_{max} (batch)] growth. During restricted growth, μ is limited by the rate of substrate supply. Conversely, during unrestricted (batch) growth, the type of substrate instead of its concentration defines μ (if non-

TABLE 2 Changes in abundances of selected proteins at different growth rates^a

Category and protein ^b	Predicted function ^b	Fold change in abundance compared to that at μ_{high} at ^c :		
		μ_{low}	μ_{med}	μ_{max} (batch)
Catabolism of aromatic compounds				
Benzoate (anaerobic)				
BcrC (EbA5282)	Benzoyl-CoA reductase, γ subunit	−1.6	−1.0	−1.3
BcrB (EbA5284)	Benzoyl-CoA reductase, β subunit	−3.0	−1.5	1.1
BcrD (EbA5286)	Benzoyl-CoA reductase, δ subunit	−2.0	−1.2	−1.2
BcrA (EbA5287)	Benzoyl-CoA reductase, α subunit	−2.0	−1.2	−1.2
BzdV (EbA5294)	NADPH:ferredoxin oxidoreductase	−2.7	−2.1	−1.1
Oah (EbA5298)	6-Oxo-cyclohex-1-ene-carbonyl-CoA hydrolase	−2.0	−1.3	1.0
BclA (EbA5301)	Benzoate-CoA ligase	−1.5	1.1	−1.4
GcdH (EbA2993)	Glutaryl-CoA dehydrogenase	−1.7	−1.4	1.7
Aromatic alcohols or aldehydes (anaerobic)				
AdhB (EbA4623)	Alcohol dehydrogenase II	11.3	1.7	1.1
AldB (EbA4625)	Putative aldehyde dehydrogenase	6.3	1.3	1.0
EbA5005	Aldehyde:ferredoxin oxidoreductase, tungsten containing	2.7	1.3	1.1
Benzoate (aerobic)				
BoxC (EbA2763)	Enoyl-CoA-hydratase/isomerase	−1.0	1.1	2.2
BoxB (EbA2765)	Benzoyl-CoA oxygenase, B component	1.6	1.4	3.5
EbA2768	β -Ketoadipyl-CoA thiolase	2.9	2.1	1.3
<i>m</i> -Hydroxybenzoate/gentisate (aerobic)				
NagI (EbA1373)	Gentisate 1,2-dioxygenase	6.4	−1.1	−1.0
NagK (EbA1377)	Fumarylpyruvate hydrolase	85.8	1.7	3.1
Hbh (EbA1378)	Hydroxybenzoate hydroxylase	16.3	−1.1	1.4
NagL (EbA1379)	Maleylpyruvate isomerase	9.2	1.2	1.3
Phenylacetate (aerobic)				
PaaK (EbA3545)	Phenylacetate-CoA ligase	2.6	1.6	−1.1
Catabolism of aliphatic compounds				
AcsA (EbA172)	Acetate-CoA ligase	2.9	1.4	−1.0
AcsA (EbA1206)	Putative acetate-CoA ligase (AMP dependent)	2.4	1.6	1.0
PrpE (EbA7220)	Propionate-CoA ligase	4.2	1.9	−1.2
FdhA (EbA2936)	Formate dehydrogenase, α subunit	7.3	3.6	−1.0
TCA cycle and central metabolism				
AceE (EbA133)	Pyruvate dehydrogenase, E1 component	3.1	1.6	1.1
AceF (EbA135)	Pyruvate dehydrogenase, E2 component	2.4	1.2	2.4
FumA (EbA173)	Fumarate hydratase	−1.5	−1.3	−1.2
GlcB (EbA819)	Malate synthase G	−1.8	−1.5	1.0
Icd (EbA829)	Isocitrate dehydrogenase, NAD ⁺ dependent	6.2	3.6	2.3
PpcK (EbA4040)	Phosphoenolpyruvate carboxykinase	−1.7	−1.3	1.2
MaeB1 (EbA4392)	Malic enzyme, NADP ⁺ dependent	−2.5	−1.8	1.3
AceA (EbA4473)	Isocitrate lyase	−2.0	−1.5	1.7
KorC (EbA4587)	2-Oxoglutarate:NADP ⁺ oxidoreductase, γ subunit	−2.9	−2.0	−1.2
KorA (EbA4588)	2-Oxoglutarate:NADP ⁺ oxidoreductase, α subunit	−2.7	−1.8	−1.2
KorB (EbA4589)	2-Oxoglutarate:NADP ⁺ oxidoreductase, β subunit	−3.2	−1.7	−1.2
AcnB (EbA5265)	Aconitase	−2.2	−1.8	1.1
Pps (EbA5830)	Phosphoenolpyruvate synthetase	−3.0	−2.0	−1.5
Lpd (EbA6683)	2-Oxoglutarate dehydrogenase, E3 component	1.6	1.2	1.2
SucB (EbA6684)	2-Oxoglutarate dehydrogenase, E2 component	1.8	1.4	−1.1
Polyhydroxybutyrate metabolism				
PhbB (EbA4729)	Acetoacetyl-CoA reductase	5.3	2.4	1.0
Adh (EbA4731)	Acetoacetyl-CoA reductase (59% sequence identity to PhbB)	−2.4	−1.8	−1.1
EbA1323	Predicted PHB granule-associated phasin (51% sequence identity to PhaP1 of <i>R. eutropha</i> H16)	−6.7	−14.8	1.6
PhbP (EbA6668)	PHB granule-associated phasin (27% sequence identity to PhaP1 of <i>R. eutropha</i> H16)	2.3	1.5	−1.4

(Continued on following page)

TABLE 2 (Continued)

Category and protein ^b	Predicted function ^b	Fold change in abundance compared to that at μ_{high} at ^c :		
		μ_{low}	μ_{med}	μ_{max} (batch)
Solute uptake, nutrient scavenging				
TRAP transporter components				
EbA1033	Periplasmic solute-binding protein	3.3	2.5	−1.3
DctP (EbA4158)	Periplasmic C ₄ -dicarboxylate-binding protein	9.2	2.9	−1.1
EbA4994	Periplasmic solute-binding protein	16.9	3.1	1.6
ABC transporter components				
LivK (EbA3428)	Periplasmic branched-chain amino acid-binding protein	8.9	4.6	1.2
EbA3556	Periplasmic amino acid-binding protein	12.4	6.6	−1.2
EbA3560	ATP-binding subunit of amino acid ABC transporter	1.8	1.5	−1.1
GltI (EbA4561)	Periplasmic glutamate-binding protein	5.6	2.5	1.3
EbA5303	Periplasmic benzoate-binding protein	6.4	3.2	1.1
EbA5307	ATP-binding subunit of predicted benzoate ABC transporter	2.1	1.5	−1.2
Iron uptake				
EbA1861	Uncharacterized protein probably involved in high-affinity Fe ²⁺ transport	−14.5	−2.4	5.0
EbA4918	ABC-type periplasmic iron-binding protein	−3.0	−1.7	3.3
EbA3939	Siderophore receptor, TonB dependent	−5.3	−6.6	9.1
EbA5333	Siderophore receptor, TonB dependent	−2.3	−2.2	6.0
Energy conservation and denitrification				
AtpD (EbA3007)	F ₁ -ATP synthase, β subunit	−1.8	−1.2	−1.4
NarH (EbA6285)	Nitrate reductase, β subunit	−3.0	−2.3	−1.7
NarG (EbA6286)	Nitrate reductase, α subunit	−2.5	−2.1	−1.3
Nucleotide synthesis and DNA replication				
NrdA (EbA4850)	Ribonucleotide reductase, coenzyme B ₁₂ dependent	−2.7	−1.7	1.1
GyrA (EbA909)	DNA gyrase, α subunit	−17.2	−5.3	−2.7
Protein synthesis				
AspS (EbA4339)	Aspartyl-tRNA synthetase	−3.7	−1.7	−1.6
RpsA (EbA903)	30S ribosomal protein S1	−1.9	−1.3	−1.1
InfB (EbA5841)	Translation initiation factor IF2	−3.0	−2.5	1.3
Tsf (EbA5987)	Translation elongation factor Ts	−2.0	−1.5	−1.2
Tig (EbA4969)	Trigger factor (ribosome-associated chaperone)	−1.7	−1.2	1.1
Stress response				
PpiD (EbA6665)	PpiC-type peptidyl-prolyl <i>cis-trans</i> isomerase	5.7	2.5	1.0
Tpx (EbA2918)	Thiol peroxidase	4.8	2.3	1.2
Gst (EbA5076)	Glutathione S-transferase	7.2	2.7	1.6

^a See Fig. 4 and 5; for a complete list, see Table S2 in the supplemental material.

^b As described previously (55).

^c 2D DIGE-determined fold changes in protein abundance compared to that in benzoate-limited cells at μ_{high} . If proteins were identified from more than one protein spot, the fold change of the major protein spot (i.e., largest spot volume) is given. Proteins with abundance changes above the threshold of significance of $>|1.5|$ are marked in bold.

toxic). Benzoate-restricted and -unrestricted cells of strain EbN1 at μ_{high} or μ_{max} (batch) exhibited highly similar protein profiles, PHB contents, and phospholipid compositions (Table 2 and Fig. 5; see Table S2 in the supplemental material). Among the observations specific for μ_{max} (batch) was the upregulation of iron uptake siderophore receptors and periplasmic iron-binding proteins (Table 2). Moreover, biovolume ($5.3 \pm 0.7 \mu\text{m}^3$) and DNA (2.7 ± 0.3 arbitrary units [AU]) and protein (9.0 ± 2.5 AU) contents increased about 2-fold at μ_{max} (batch) compared to μ_{high} (Table 1).

DISCUSSION

Correlation of cellular parameters with growth rate. In their seminal study on tryptophan-limited growth of *Salmonella enterica* serovar Typhimurium try A-8, Schaechter et al. (61) ob-

served (exponential) increases in RNA, DNA, and cell size across a μ range of 0.5 to 2.0 h^{−1}. During balanced growth of *E. coli* B/r in batch cultures with μ ranging from 0.7 to 2.4 h^{−1}, correlations of cellular parameters and μ did not always follow simple exponential relations (11). The present study is distinct in that a betaproteobacterial strain and anoxic conditions were used and markedly lower growth rates (0.04 to 0.24 h^{−1}) were observed. In benzoate- or acetate-limited cultures of strain EbN1 (Fig. 1; see Fig. S1 in the supplemental material) the μ -dependent changes of cellular parameters are smaller than one order of magnitude. Thus, as previously reported (61), also here this positive correlation cannot unambiguously be described as exponential. The necessity to include cell quota in the model agrees with the complex relationship between cellular parameters and μ .

With benzoate at μ_{high} , doubling of the DNA content per cell, of cell length (equivalent to cell mass) (13), and of the number of dividing cells (Table 1) agreed with the general observation that bacteria maintain a constant DNA-to-cell mass ratio (1). Notably, the protein content increased much more strongly than the DNA content with benzoate compared to growth with acetate. The share of enzyme constituents of the anaerobic benzoyl-CoA pathway increased from a cumulative 10% of the 2DE-resolvable proteome (including 7% for Bcr only) at μ_{low} to 20% (including 15% for Bcr only) at μ_{high} .

Growth rate-dependent modulation of benzoate uptake?

Chromosomal colocalization of genes for the predicted benzoate-specific ABC transporter (EbA5303-09) and benzoate:H⁺ symporter (BenK; EbA5311) downstream of the gene cluster for the anaerobic benzoyl-CoA pathway suggested the possible involvement of both transport systems in benzoate uptake (55). Agreeing with the predicted role of the ABC transporter, its periplasmic solute-binding protein (EbA5303) displays high sequence identities (54%) to RPA0668 of *Rhodopseudomonas palustris* CGA009, which has recently been demonstrated to bind benzoate and closely related monoaromatic acids (24). Residual benzoate concentrations remained constant in the <20 μM range from D_{low} until close to D_{high} . Only at D_{high} (0.18 to 0.20 h⁻¹), benzoate concentrations steadily increased, approaching the millimolar range (Fig. 2B). One may speculate that this biphasic benzoate profile reflects a solute concentration-dependent deployment of the two predicted uptake systems. Thus, at < D_{high} the high-affinity ABC transporter may dominate benzoate uptake. Only when the ABC transporter becomes saturated, BenK, which is predicted to have a lower affinity (see Table S1 in the supplemental material), may also contribute to benzoate uptake. Both benzoate uptake systems were detected at μ_{low} and μ_{high} by qualitative LC-ESI-MS/MS analysis. Quantitative 2D DIGE analysis captured only the periplasmic solute-binding protein (EbA5303) and the cytoplasmic ATP-binding protein (EbA5307) of the ABC transporter. Remarkably, both displayed increased abundances at μ_{low} , agreeing with the assumed predominant role of the ABC transporter at μ_{low} .

Under acetate-limited conditions, a monophasic saturation kinetic and low substrate affinity (K_s of 440 μM) were observed (Fig. 2B). Comparison of acetate- with benzoate-limited growth revealed higher substrate affinity and Y for benzoate. This may reflect the specialization of strain EbN1 in uptake and catabolism of benzoate and other monoaromatic carboxylates. This may further be supported by the markedly lower $K_{s(\text{acetate})}$ observed with other facultative anaerobes, e.g., 28 and 63 μM for *Paracoccus pantotrophus* and *Pseudomonas denitrificans*, respectively (60).

Benzoate and central carbon metabolism tuned to slow growth. At μ_{low} , uniformly decreased abundances of proteins involved in anaerobic metabolism of benzoate (e.g., BclA, BcrCBDA, and KorCAB) (Table 2; Fig. 5) indicate μ -dependent modulation of gene expression in strain EbN1. In the closely related *Azoarcus* sp. strain CIB, expression of the benzoate catabolic genes depends on liberation of the transcriptional repressor BzdR from the operators in the presence of benzoyl-CoA (3). The high sequence identity (76%) of BzdR, colocalization of *bzdR* with benzoate catabolic genes, and presence of similar operator sites in strains CIB and EbN1 may suggest that BzdR serves similar functions in both strains. In this scenario, a correspondingly lower pool size of benzoyl-CoA (cumulatively determined by benzoate

uptake, CoA activation, and dearomatization) would have to be expected at μ_{low} compared to $\geq \mu_{\text{med}}$.

Benzoate-derived acetyl-CoA is oxidized via the TCA cycle or used for anabolism via the glyoxylate shunt (18). The enzymatic ring reduction of benzoyl-CoA is linked to the TCA cycle by an inducible 2-oxoglutarate:NADP⁺ oxidoreductase (KorCAB) and the NADPH:ferredoxin oxidoreductase (BzdV) in *Azoarcus evansii* (18) and *Azoarcus* sp. strain CIB (3). The coinciding decreased abundances of KorCAB, BzdV, and BcrCBDA at μ_{low} support the coupling of benzoyl-CoA reduction to the TCA cycle also in strain EbN1. In addition, a reduced flux of acetyl-CoA through the TCA cycle and glyoxylate shunt at μ_{low} is indicated by uniformly decreased abundances of aconitase (AcnB), isocitrate lyase (AceA), and malate synthase G (GlcB) (Table 2, Fig. 5). The profiles of these three and other enzymes of central metabolism (e.g., Icd and MaeB1) agree with reduced anabolism at μ_{low} , which is in accord with a decreased benzoate conversion into biomass reflected also in the high share of free energy for maintenance and reduced Y (Table 1).

Growth rate-dependent PHB content. The μ -dependent PHB profile showed a modest content at μ_{low} , followed by a minimum at μ_{med} and a maximum at μ_{high} (Fig. 5). It has long been known that polyhydroxyalkanoates (PHAs) accumulate in the presence of surplus carbon source when bacterial growth is limited by another essential nutrient. Generally, PHA synthesis requires high intracellular ratios of NAD(P)H to NAD(P)⁺ and of acetyl-CoA to free CoA (59). However, other factors, which have not been studied in much detail yet, also have an impact on PHA synthesis (52, 53). *Pseudomonas oleovorans* has been demonstrated to accumulate PHA also under carbon-limited conditions (17, 58). In the present study, residual NH₄⁺ concentrations (provided as a nitrogen source) were >5 mM across the investigated μ range (data not shown). The strongly decreased abundance of iron uptake proteins at μ_{low} (Table 2; Fig. 5) indicated that iron, like ammonia, might not be limiting. Thus, a so-far undetected secondary nutrient limitation or other factors may cause the modest PHB formation by strain EbN1 at μ_{low} . In contrast, at μ_{high} and μ_{max} (batch), a limiting condition could result from insufficiently high rates of uptake of nutrients (e.g., iron) (Table 2) or from an imbalance between redox equivalent formation (benzoate degradation) and regeneration (denitrification). PHB was recently speculated to provide a sink for reducing equivalents in strain EbN1 when the aromatic substrates at elevated (toxic) concentrations impaired denitrification (68).

Preparedness for utilization of alternative substrates. A remarkable response of strain EbN1 to benzoate-limited growth at μ_{low} is the formation of a large number of proteins involved in anaerobic or aerobic degradation of various aromatic carboxylates, phenolics, alkylbenzenes, and aliphatic carboxylates, despite their absence from the medium. In addition, a variety of solute uptake systems for organic substrates (e.g., periplasmic solute-binding proteins as well as permeases for C₄-dicarboxylates and amino acids) showed increased abundances or seemed to be newly formed at μ_{low} . This contrasts with previous studies with batch cultures of strain EbN1 showing the substrate-dependent induction of the protein constituents of the catabolic pathways concerned (see, e.g., references 79 and 80) and the repression of DctP (C₄-dicarboxylate-binding protein of TRAP transporter) in the presence of elevated benzoate concentrations (67).

Considering the absence of the inducing substrates, formation

of these catabolic enzymes could theoretically result from broad-scale derepression (i.e., due to inactivity of pathway-specific or global repressors or by action of global activators) or from passive induction overarching gene expression of all pathways concerned. In enterobacteria, gene expression can be subject to passive regulation (46). At μ_{low} , *E. coli* and related bacteria reduce the extent of negative DNA supercoiling (GyrAB mediated) of certain promoter regions (e.g., stable RNAs), resulting in downregulation of transcription (70). As a consequence, the portion of available (nonstable RNA-transcribing) RNA polymerase increases, which in conjunction with alternative σ factors could lead to effector-independent passive induction (4, 10). The present observations at μ_{low} that GyrA represents the most strongly downregulated protein and that several transcriptional regulators were formed (e.g., Crp and MarR-, LysR-, and GntR-type regulators) could agree with such a scenario also occurring in strain EbN1. Moreover, the μ_{low} -specific detection of σ^{54} agrees with formation of several proteins involved in the anaerobic degradation of *p*-ethylphenol, the transcriptional control of which was previously suggested to be σ^{54} dependent (80).

Derepression and/or passive induction may represent a prospective survival strategy under conditions of maximal substrate limitation. Even in the absence of substrates, the organism, despite its energy-limited state, invests in the deployment of the respective uptake systems and degradation pathways. Cells then are ready to utilize the substrates without delay upon their becoming available. Under environmental conditions, organic substrates usually occur at low concentrations and in complex mixtures (19). In its anoxic/oxic habitat, strain EbN1 may encounter plant exudates and degradation products from plant polymers as predominant substrates. This is well reflected in the range of uptake systems and degradation pathways observed to be present at μ_{low} . Thus, preparedness for a “multivorous” lifestyle (for an overview, see references 19 and 28) may also be characteristic for strain EbN1 as a facultative anaerobic nutritional specialist. It is unclear at present whether the observed catabolic preparedness represents a stochastic or deterministic process. That is, have the catabolic regulatory circuits in strain EbN1 evolved such that preemptive opening of selected degradation routes enables a fast response to future occurrence of the corresponding substrate/redox conditions (“memory of the future” [F. Neidhardt, presented at the 108th General Meeting of the American Society for Microbiology, 2008])?

Adjustment of overall cell physiology to low growth rates. In benzoate-limited cells, the most profound changes in PL composition were observed at μ_{low} (Fig. 5), and this may reflect responses to decreased cell size as well as low energy charge. In contrast to PE, the appearing PC does not hydrogen bond (14) and therefore could contribute to increased membrane fluidity in combination with the shift to shorter fatty acid chain lengths. This could influence the functionality of membrane proteins in general and solute uptake (14, 47) under the conditions of reduced cell size at μ_{low} . Moreover, an increased content of C_{16} fatty acids could be of economic benefit, since one round of fatty acid biosynthesis (compared to C_{18}) could be saved. The observed μ_{low} -specific changes in intact PL were distinct from those recently described for strain EbN1 as a response to solvent stress (83).

At μ_{low} , proteins involved in repair of denatured proteins (e.g., ClpB and PpiD) displayed their largest increases in abundance (Table 2; also see Table S2 in the supplemental material). These

proteins are generally known to improve survival during stress or when *de novo* protein synthesis is restricted at low growth rates (73). Other μ_{low} -specific upregulated proteins involved in oxidative and stress responses may contribute to an enhanced more general stress resistance, as known from slowly growing or stationary-phase *E. coli* (for an overview, see reference 75), and may prepare cells of strain EbN1 for future stress conditions.

A general observation with strain EbN1 growing at μ_{low} was the decreased abundance of proteins involved in the different levels of information processing (Table 2). This correlation is also underpinned by the μ -dependent antiparallel behavior of σ^{54} and σ^{70} (see Table S2 in the supplemental material). While σ^{54} (a transcriptional regulator of stress-related genes, among others) was detected only at μ_{low} , the opposite was observed for σ^{70} (which is involved in transcriptional control of housekeeping genes, among others).

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