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Carbon and energy fluxes during haloadaptation of *Halomonas* sp. EF11 growing on phenol

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Abstract The haloalkaliphile *Halomonas* sp. EF11 can grow on phenol as sole source for carbon and energy, while maintaining an osmotic equilibrium predominantly by adjusting levels of a certain compatible solute. To determine the energy costs of haloadaptation and the fate of substrate-carbon, the strain was grown continuously in an isothermal compensation calorimeter, keeping all conditions constant except salinity. As salinity increased, slight linear reductions in exothermic heat flow and biomass formation occurred, and 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (ectoine) synthesis increased linearly. However, beyond a certain salinity threshold the stationary phenol concentration increased exponentially, while heat flow fell sharply, indicating intoxication or wash-out. The clear transition point between the phases, where ectoine formation peaked, suggests that calorimetric measurements could be used to control the conversion of growth-inhibiting substrates (like phenol) into ectoine and to optimize the process. Enthalpy balance and chemical determinations revealed acetate and formate were formed as side products when the C/N ratio in the feed was low, while 2-muconic acid semialdehyde and formate were produced when the ratio was high. These findings indicate that phenol assimilation occurs via the *meta* pathway. However, enzyme assays implied that assimilation occurs via the *ortho* and *meta* pathways at a low C/N ratio and exclusively via the *meta* pathway at a high C/N ratio.

Keywords Calorimetry · Degradation · Ectoine · *Halomonas* · Metabolomics · Phenol

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Introduction

Halophilic or halotolerant bacteria have the potential to mineralize or transform a wide range of organic pollutants in the presence of halostress that is fluctuating in amplitude and frequency (Oren et al. 1992; Woolard and Irvine 1994; Hinteregger and Streichsbier 1997; Fu and Oriel 1999; Kargi and Dinçer 2000). However, this potential is seldom optimized in technical systems. The velocity and efficiency of conversion are usually limited by both the halostress and the pollutants, which may even, depending on their concentration and nature, inactivate or poison the catalyst. A wide range of different microbial responses have been described that help counteract physiological dryness (Galinski and Trüper 1994; Galinski 1995; Kempf and Bremer 1998; Oren 2002) and denaturation by harmful agents (Russel 1989; Weber and de Bont 1996). These responses include, *inter alia*, changes in the cytoplasmic membrane, adjustment of transport activities, and synthesis of compatible solutes, chaperones, and other proteins. These alterations in the composition of cells and exchanged matter are accompanied by changes in heat flux and have to comply with the first law of thermodynamics. Thus, comparison of the measured heat flux with the enthalpy balance may reveal previously undetected, unexpected events, providing a powerful means for extending our physiological knowledge. Admittedly, not every change is energetically important enough to be measurable by monitoring the heat flow rate. However, the main distribution of substrate-carbon and substrate-enthalpy may be revealed in this way (Maskow and Babel 2002). A further advantage of calorimetric measurements is the fact that variations in heat flow rate instantaneously reflect metabolic changes, which may be exploited in biotechnological processes.

This possibility arises because halophilic or halotolerant bacteria growing and multiplying on harmful chemicals and counteracting osmotic imbalances by synthesizing small organic compounds called compatible

solutes (Brown 1990; Kempf and Bremer 1998) combine detoxification with the formation of potentially valuable compounds. Compatible solutes have attracted commercial interest due to their ability to stabilize biocatalysts—enzymes or whole cells—against low water activity and inactivation by heating, freezing, desiccation, or even chemical agents (Sauer and Galinski 1998; Knapp et al. 1999; Lamosa et al. 2000; Margesin and Schinner 2001). However, in practice, combining the degradation of potentially toxic agents with the synthesis of compatible solutes requires a fine balance to be maintained between formation of the desired product and intoxication of the microbial catalyst. Therefore, a key question addressed in this study was whether it is possible to isolate any bacterial strain that can grow and multiply on toxic substances while predominantly counteracting osmotic imbalances by synthesizing a certain compatible solute. If so, the next goal was to determine its distribution of substrate-enthalpy and substrate-carbon during the haloadaptation. The technologically inspired ambition was to find an indicator for the putative boundary between the maximum formation rate of the main compatible solute and the inhibitory level of the toxic substrate. The final intention was to compare the efficiency of conversion of the toxic substrate into the desired product with the theoretically calculated maximum yield.

Materials and methods

Isolation and identification of strain EF11

Strain *Halomonas* sp. EF11 was isolated from a pooled sample of water and sediment samples collected from an alkaline pond in the surroundings of the soda lake Szegedi Fehértó in southern Hungary. Chemical parameters of the sampling site have been described elsewhere (Kleinsteuber et al. 2001). Enrichment cultures for phenol-degrading bacteria were prepared at 30 °C in 500 ml shaking flasks containing 200 ml alkaline mineral medium (AMM, pH 10) with an initial phenol concentration of 2 mM. The AMM, prepared according to Maltseva et al. 1996, was composed of (in mg/l): KCl (3,000), NH_4NO_3 (500), $(NH_4)_2SO_4$ (300), $MgSO_4 \times 7\ H_2O$ (400), NaH_2PO_4 (300), biotin (0.02), folic acid (0.02), pyridoxine-HCl (0.1), thiamine-HCl×2 H_2O (0.05), riboflavin (0.05), nicotinic acid (0.05), D-Ca-pantothenate (0.05), vitamin B_{12} (0.001), *p*-aminobenzoic acid (0.05), lipoic acid (0.05), and 1 ml of a trace elements stock solution (Imhoff and Trüper 1977), buffered by adding 20 g/l Na_2CO_3 and 20 g/l $NaHCO_3$. Enrichment cultures contained 10% (w/v) NaCl. Phenol consumption was measured by HPLC as described in Analyses. After the phenol had been replenished several times, enrichment cultures were plated onto AMM agar containing 2 mM phenol and 5% (w/v) NaCl. Pure cultures were isolated by several passages on alkaline R2A agar containing 5% (w/v) NaCl, buffered by adding 20 g/l Na_2CO_3 and 20 g/l $NaHCO_3$, and afterwards examined for growth on phenol in AMM.

The Gram behavior of strain EF11 was determined by the KOH test (Ryu 1938). For identification based on 16S rDNA sequence analysis, genomic DNA was isolated from a liquid culture grown in alkaline R2A medium using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). The 16S rDNA was then amplified by PCR using a Taq PCR Master Mix (Qiagen, Hilden, Germany) and the primers 27F and 1525R (Lane 1991). PCR was performed in a PTC200 thermocycler (MJ Research, US) with the following temperature protocol: initial denaturation for 5 min at 95 °C, 30 cycles at 95 °C for 30 s, 58 °C for 45 s, 72 °C for 75 s, followed by a final extension step at 72 °C for 5 min. After checking that the expected PCR products had been generated by electrophoresis on a 0.8% agarose gel, they were purified using a NucleoSpin Extract 2 in 1 kit (Macherey-Nagel) and their concentration was determined spectrophotometrically at 260 nm. Cycle sequencing was performed using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Weiterstadt, Germany) and the sequencing primers 27F, 357F, 519R, 530F, 926F, 1100R, 1114F, and 1525R (Lane 1991). Electrophoresis and data collection were carried out on an ABI PRISM 310 Genetic Analyzer. Data were analyzed by ABI PRISM DNA Sequencing Analysis software and partial 16S rDNA sequences were assembled by ABI PRISM Autoassembler software. The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST; version 2.0; Altschul et al. 1990) was used to search for similar sequences in the nucleotide sequence databases, and the Sequence Match tool (version 2.7) was used to search for similar sequences recorded by the Ribosomal Database Project II (http://www.cme.msu.edu/RDP; Maidak et al. 2001). Phylogenetic analysis was performed using the ARB package (http://arb-home.de). The 16S rRNA sequence of strain EF11 was aligned to the ARB database by the ARB editor, with manual correction. The aligned sequence was added to the ARB tree using the quick add parsimony tool. The determined 16S rDNA sequence will appear in the GenBank, EMBL, and DDBJ nucleotide sequence databases under the accession number AY332559.

The salt dependency of growth was determined in R2A medium containing various concentrations of NaCl in the range 0–25% (w/v), corresponding to 0.02–4.3 M total sodium ion concentration. Specific growth rates under various salt concentrations were analyzed during exponential growth in batch cultures by linear regression of the logarithm of $OD_{700}$with time.

Cultivation

Precultures of *Halomonas* sp. EF11 for chemostat cultivation were prepared as described for the enrichment cultures, with repeated replenishment of phenol (1 mM start concentration) to reach a biomass concentration of about 0.3 g/l. Precultures contained 3% (w/v) NaCl. The NaCl and phenol contents of the media for the chemostat experiments are given in the descriptions of the respective experiments. Strain EF11 was grown continuously on phenol as sole source of carbon and energy in an isothermal heat flux calorimeter with power compensation (Berghof, Enningen, Germany), described in detail elsewhere (Meier-Schneiders et al. 1995). The pH was kept constant at 8.5 by titration with 1 M NaOH. The heat of neutralization was calculated from the loss of mass of the alkali using tabulated values (von Stockar et al. 1993). The growth temperature was 30 °C. The temperature of all feeds was adjusted to the reactor temperature before they entered the vessel. The calorimeter was aerated with 0.1 $m^3\ h^{-1}$ air (related to 101.325 kPa and 273.15 K), saturated with water at the reactor temperature. If the oxygen saturation fell below 20% then a certain amount of air was substituted by pure oxygen. No effects of this substitution on heat flow rate were observed. The culture was agitated using a Ruston turbine at a rate of 400 rpm. Torque measurements were used for detecting baseline shifts of heat flow rate due to changes in viscosity (Menoud et al. 1995).

Analyses

The culture broth was rapidly separated from the biomass by direct ultrafiltration of the efflux from the calorimeter (Müller and Babel 1995). The filtrate was analyzed for salt, phenol, total organic carbon (TOC), and side product contents. Salt concentrations were quantified by titrating the chloride ions with silver nitrate, using potassium chromate as an indicator (Rump and Krist 1992). Phenol concentrations were estimated by HPLC on a Nucleosil 100 PR 18 column (250×3 mm i.d.; Knauer-Säulentechnik, Berlin, Germany) with 70% acetonitrile in water

containing 0.1% acetic acid anhydride as eluant. The TOC measurements were performed with a Total Carbon Analyzer 550 (Shimadzu Europe, Duisburg, Germany). Samples were passed through an OnGuard-Ag cartridge (Dionex, Sunnyvale, CA, USA) to remove the chloride ions before analyzing for charged side products. These side products were estimated by ion chromatography on an IonPac AS11 column (250×4 mm i.d.; Dionex). 2-Hydroxymuconic acid semialdehyde was quantified by HPLC on a Nucleogel ION 300 OA column (300×7.8 mm i.d.; Macherey-Nagel) at 70 °C with 0.01 N sulfuric acid as eluant. The system was equipped with a UV/VIS photodiode array detector, and the maximum absorbency was found to be at 318 nm in the sulfuric acid. The required standard was synthesized from 1.9 mg/l catechol using catechol 2,3-dioxygenase in phosphate buffer (0.05 M, pH = 7.4) from *Pseudomonas putida* MT2. Enzymes for the subsequent reactions were deactivated by heating the enzyme extract for 10 min at 50 °C. The completeness of the conversion was confirmed photometrically at 375 nm. The standard was immediately cooled down to 5 °C and applied.

Separate samples were taken for determinations of cell dry mass, compatible solute content, and enzymatic activities. Cell dry mass was determined in quadruplicate after oven-drying at 105 °C. Determination of compatible solutes followed a method described by Maskow and Babel (2001), and was occasionally validated with anion exchange chromatography and pulsed amperometric detection (Riis et al. 2003). Harvested cells were washed twice with phosphate buffer (0.1 M, pH = 7.5) and disrupted by three passages through a French pressure cell (Aminco, Silver Spring, USA) at 124.1 MPa and 4 °C. Particle-free supernatants were obtained by centrifugation at 15,000 *g* for 20 min at 4 °C (centrifuge model 5403; Eppendorf, Germany) and used for the determination of enzyme activities. Catechol 1,2-dioxygenase activity (the key enzyme of the *ortho* pathway) was determined by monitoring the formation of *cis*,*cis*-muconic acid from catechol at 260 nm, and catechol 2,3-dioxygenase activity (the key enzyme of the *meta* pathway) was measured by following the formation of 2-hydroxymuconic acid semialdehyde from catechol at 375 nm according to Nakazawa and Nakazawa (1970). Catechol 2,3-dioxygenase was inactivated in the first assay by H_2O_2 as described by Pieper et al. (1988). Protein was assayed according to Bradford (1976).

The composition of the dried air entering and leaving the calorimeter was determined by passage through infrared carbon dioxide and electrochemical oxygen analyzers (Ultramat 23; Siemens, Karlsruhe, Germany).

Results and discussion

Identification of strain EF11

After growth on alkaline R2A agar, strain EF11 forms round, convex colonies, 1–2 mm in diameter, slightly glossy, and white to cream-colored. The strain is Gram-negative. The cells are strongly motile, straight rods.

The nucleotide sequence of the nearly complete 16S rRNA gene (1,484 bp) was determined and compared to previously recorded sequences. According to the BLAST search, the 16S rRNA sequence of strain EF11 shows 98% similarity to the 16S rRNA sequence of *Halomonas* sp. EF43 (accession number AF280796), which was isolated from the same sample (Kleinsteuber et al. 2001). Comparison with data collected by the Ribosomal Database Project revealed that strain EF11 clusters within the *Halomonas desiderata* subgroup, and its closest relative is *Halomonas* sp. ML-185 (accession number AF140007), a soda lake isolate from Mono Lake, California, with a similarity score of 0.941. The 16S rRNA sequence of strain EF11 contains all the 15 signature features characteristic of the family *Halomonadaceae* and the four additional signature features characteristic of the genus *Halomonas* (Dobson and Franzmann 1996). Based on these findings strain EF11 was assigned to the genus *Halomonas*.

As shown in Fig. 1, this conclusion was supported by phylogenetic analysis revealing that strain EF11 clusters within a *Halomonas* group that is dominated by various soda lake isolates such as *Halomonas* sp. EF43 (Kleinsteuber et al. 2001), the Mono Lake isolate *Halomonas* sp. ML-185, and isolates from the soda lakes situated in the Great Rift Valley in East Africa (Duckworth et al. 1996).

Strain EF11 is able to grow over a wide range of salt concentrations: growth was observed in R2A medium containing 1–15% (w/v) NaCl. No growth was observed above 20% NaCl. Sodium ions are required for growth. The optimal sodium ion concentration for growth was 0.2–0.6 M.

Mass and energy balances

Generally, steady states, i.e., chemostat states, are reached if the growth rate is governed by the concentration of a single nutrient that is present at a constant, growth-limiting concentration, and all the key metabolic

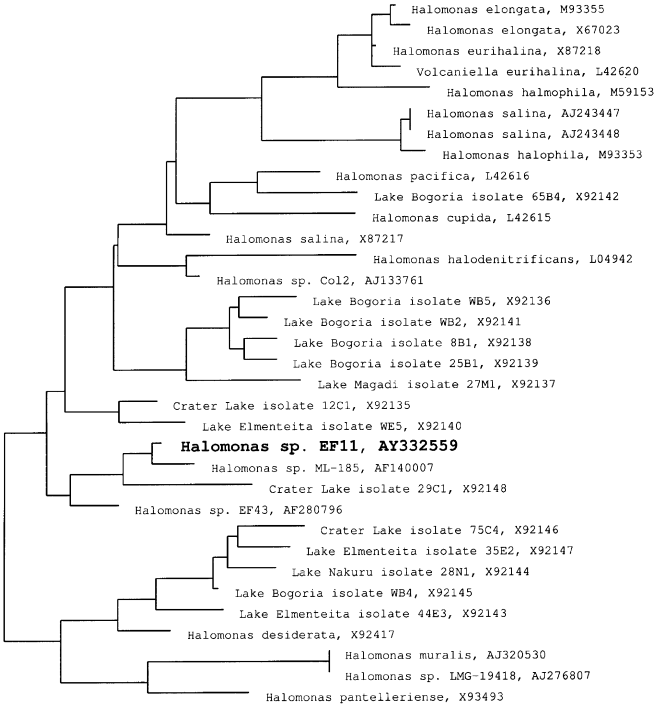

Fig. 1 Phylogenetic tree showing the relationship between *Halomonas* sp. EF11 (indicated in *bold*) and its closest relatives. The 16S rRNA sequence of strain EF11 was added to the existing ARB tree using the quick add parsimony tool. A subtree of the *Halomonas* clade is shown

and physiological rates are constant. The specific growth rate is fixed by the dilution rate. The efficiency of carbon conversion of most organic substrates ($C_{S1}H_{S2}O_{S3}$; Babel et al. 1993) is energetically limited and all other stoichiometric coefficients depend on it. Therefore, the carbon metabolism-determined yield coefficient $Y_{X/S}^{CMD}$ cannot be reached (Babel and Müller 1985; Müller et al. 1985). The energy, E_A, required for biomass ($C_{X1}H_{X2}O_{X3}N_{X4}$) synthesis (Eq. 1) must be supplied by the energy, E_D, gained from the biological combustion (Eq. 2) of the carbon-substrate offered. Note, the energy considered here includes both ATP units and reduction equivalents, which may be transformed into ATP units by electron transport phosphorylation.

$$\begin{aligned} &C_{S1}H_{S2}O_{S3} + a1NH_3 + a2O_2 + E_A \\ &\quad \rightarrow Y_{X/S}^{CMD}\, C_{X1}H_{X2}O_{X3}N_{X4} + a3\, CO_2 + a4\, H_2O \\ &\qquad a1 = Y_{X/S}^{CMD} X4 \\ &\qquad a2 = \frac{Y_{X/S}^{CMD}(3\,X4 - 4\,X1 - X2) + 4\,S1 + S2 - 2\,S3}{4} \\ &\qquad a3 = S1 - X1\, Y_{X/S}^{CMD} \\ &\qquad a3 = \frac{Y_{X/S}^{CMD}(3\,X4 - X2) + S2}{2} \end{aligned} \quad (1)$$

$$\begin{aligned} &C_{S1}H_{S2}O_{S3} + d1\, O_2 \quad \rightarrow \quad d2\, CO_2 + d3\, H_2O + E_D \\ &\qquad d1 = \frac{2\,S3 - 4\,S1 - S2}{4} \qquad d2 = S1 \\ &\qquad d3 = \frac{S2}{4} \end{aligned} \quad (2)$$

In the case of phenol, two main assimilation pathways, initiated by cleavage of the ring structure in the intradiol (*ortho*) or extradiol (*meta*) position of the catechol intermediate, are known which are not energetically equivalent (Müller and Babel 1994; Maskow and Babel 1998). The coefficients are $Y_{X/S}^{CMD} = 1.12$ mol-X mol-S^{-1}, $E_A = (9.90 - 0.44$ P/O) ATP, $E_D = (-1 + 10$ P/O) ATP for *ortho* assimilation and $Y_{X/S}^{CMD} = 0.75$ mol-X mol-S^{-1}, $E_A = (11.3 - 3.62$ P/O) ATP, $E_D = (-2 + 10$ P/O) ATP for *meta* assimilation (Babel 1994). This implies that carbon conversion efficiencies (CCEs) are 46.7% for the *ortho* pathway and 40.8% for the *meta* pathway, assuming a P/O quotient of 2.

If we increase the sodium chloride concentration, while keeping the level of all other growth conditions constant, it will influence the growth rate when it exceeds a specific level. Microorganisms counteract osmotic imbalances predominantly by synthesizing or taking up compatible solutes ($C_{CS1}H_{CS2}O_{CS3}N_{CS4}$) depends strongly on the sodium chloride concentration, at least within a certain range. For uptake, the growth yield coefficient is influenced by the energy required for uptake, E_{CS}, whereas for synthesis it is affected by both the carbon metabolism determined yield coefficient of compatible solutes $Y_{CS/S}^{CMD}$ and the energy required for their synthesis, E_{CS}, according to Eq. 3.

$$\begin{aligned} &C_{S1}H_{S2}O_{S3} + p1\, NH_3 + p2O_2 + E_{CS} \\ &\quad \rightarrow Y_{CS/S}^{CMD}\, C_{CS1}H_{CS2}O_{CS3}N_{CS4} + p3\, CO_2 + p4\, H_2O \\ &\qquad p1 = Y_{CS/S}^{CMD} CS4 \\ &\qquad p2 = \frac{Y_{CS/S}^{CMD}(3\,CS4 - 4\,CS1 - CS2) + 4\,S1 + S2 - 2\,S3}{4} \\ &\qquad p3 = S1 - CS1\, Y_{CS/S}^{CMD} \\ &\qquad p4 = \frac{Y_{CS/S}^{CMD}(3\,CS4 - CS2) + S2}{2} \end{aligned} \quad (3)$$

If the biosynthesis pathways are known then the values of $Y_{CS/S}^{CMD}$ and E_{CS} may be estimated (Oren 1999; Maskow and Babel 2001) using the Y_{ATP}-concept (Stouthamer 1973). In the case of the conversion of phenol into ectoine as the main osmotic counterweight, three main pathways can be distinguished:

1. *Ortho*

$$C_6H_6O + 2\,NH_3 + 3\,NAD(P)H + ATP \rightarrow C_6H_{10}O_2N_2 + FADH_2 \quad (4)$$

2. *Meta* via pyruvate carboxylation

$$C_6H_6O + 2\,NH_3 + 2\,NAD(P)H + 3\,ATP \rightarrow C_6H_{10}O_2N_2 \quad (5)$$

3. *Meta* via the glyoxylate shunt

$$C_6H_6O + 1.33\,NH_3 + 2.66\,ATP \rightarrow 0.66\,C_6H_{10}O_2N_2 + 1.33\,NAD(P)H + 0.66\,FADH_2 + 2\,CO_2 \quad (6)$$

The coefficients for these three equations are: (1) $Y_{CS/S}^{CMD} = 1$ mol-X mol-S^{-1}, $E_{CS} = (2 + 2$ P/O) ATP, (2) $Y_{CS/S}^{CMD} = 1$ mol-X mol-S^{-1}, $E_S = (3 + 2$ P/O) ATP, and (3) $Y_{CS/S}^{CMD} = 0.66$ mol-X mol-S^{-1}, $E_{CS} = (3.32 - 2$ P/O) ATP. The energy requirement for ectoine synthesis, without consideration of biomass formation, reduces the carbon-metabolism determined CCEs of ectoine formation from 100% to 76% in (1) and to 72% in (2). In (3) the ectoine formation is energy gaining, so there is no reduction in CCE in this case. If ectoine acts as the main compatible solute and a maximum content of 1.4 mmol/g dry weight is assumed which may be considered a good estimation in the family *Halomonadaceae* (Wohlfarth et al. 1990; Sauer and Galinski 1998; Maskow and Babel 2001) then the CCE of ectoine-free biomass will be reduced by both $Y_{CS/S}^{CMD}$ and E_{CS} in the case of *ortho* assimilation from 46.7% to 40.0%, and in the case of *meta* assimilation from 40.8% to 35.4% with carboxylation or to 35.0%

without carboxylation. Other known microbial responses, for example, energy-driven transport or changes in the cytoplasmic membrane may be considered in a similar way.

Above a certain salinity, the ability of the cells to withstand its adverse effects via responses such as synthesizing compatible solutes, is exhausted. If these compounds are commercially interesting, then the point at which this occurs could be considered the economic optimum, because the formation rate of compatible solutes is then at a maximum. If the salinity increases further, growth will be limited by physiological desiccation, causing the uptake rate of the carbon-substrate to diminish and the levels of the substrate to rise. The microbes will then be harmed and, consequently, wash out.

Because of such considerations, a special feeding system, such as the one used in this study, is required to gradually change the salinity while keeping all other growth conditions constant. The details of the feeding system and a mathematical model describing the salinity gradient are given by Maskow and Babel (2002).

The microbial adaptation process can be characterized using elemental balances (see the equation for carbon, for example, in Eq. 7) and the balance of enthalpy (Eq. 8) and of substrates considered to be relevant (Eq. 9) for the calorimeter. Equations 7 and 8 apply solely to linear evolution of the rates,

$$\frac{d\,\dot{n}_{CO_2}}{V_C\,dt} = -\sum_{i=1}^{n} \dot{r}_i\,C_i \tag{7}$$

$$\frac{d\,P}{V_C\,dt} = -\sum_{i=1}^{n} \dot{r}_i\,\Delta_C H_i \tag{8}$$

where $\dot{r}_i$, C_i, and $\Delta_C H_i$ are the change of a rate [mM/(h)^2], the molar fraction of carbon, and the combustion enthalpy (KJ/mol) of the considered substance, i, respectively. Note, consumption rates have a negative effect whereas formation rates have positive inputs. Biomass may be considered in a similar way to a substance, using its elementary composition ($C_4H_8O_2N_1$) and assessing its enthalpy content by applying the Thornton rule (1917) and using the more recently formulated enthalpy per electron-moles parameter presented by Cordier et al. (1987). The equations can be simply extended to describe more complex changes in the rates by considering higher order derivatives of the rate functions. Further equations can be generated by taking the curvature and skew of carbon dioxide release, or those of the heat flow rate, into consideration. Note, that the carbon balance is difficult to exploit because at pH 8.5 a substantial fraction of the carbon dioxide evolved is fixed in the culture broth as HCO_3^- and CO_3^{2-}. The connection between the measured time course of any concentration, Φ_i(M), and the evolution of rates of formation $r^{Gen} = r^{Gen}(t)$ or consumption rates $r^{Con} = r^{Con}(t)$ is given by the associated balance for the calorimeter (Eq. 9).

$$\frac{d\,\Phi}{dt} = r^{Gen} - r^{Con} + D\,(\Phi_0 - \Phi) \tag{9}$$

The analytical integration of the differential Eq. 9, giving Eq. 10, succeeds when any rate is expressed in terms of a polynomial of degree n (Eq. 11).

$$\Phi = \Phi^0 e^{-Dt} - \left(\tfrac{\Delta a_0}{D} + \Phi_0\right)\left(e^{-Dt} - 1\right) + \sum_{J=1}^{n} \tfrac{\Delta a_J}{D}\left((-1)^{(J+1)}\tfrac{J!}{D^J}e^{-Dt} + \sum_{I=1}^{J+1}(-1)^{(I+1)}\tfrac{J!\;\,t^{(J+1-I)}}{(J+1-I)!D^{(I-1)}}\right) \tag{10}$$

$$r^{Gen} - r^{Con} = \sum_{J=0}^{n} \Delta a_J\, t^J \quad with \quad \Delta a_J = a_J^{Gen} - a_J^{Con} \tag{11}$$

Φ^0– concentration at time zero is Φ^0(M).

However, assuming a steady (chemostat) state at time zero, characterized by constant rates of $r^{Gen,SS}$–$r^{Con,SS} = \Delta a_0{}^{SS}$, a simpler solution is obtained.

$$\Phi = \left(\tfrac{\Delta a_0^{SS} - \Delta a_0}{D}\right) + \tfrac{\Delta a_0}{D} + \Phi_0 + \sum_{J=1}^{n} \tfrac{\Delta a_J}{D}\left((-1)^{(J+1)}\tfrac{J!}{D^J}e^{-Dt} + \sum_{I=1}^{J+1}(-1)^{(I+1)}\tfrac{J!\;\,t^{(J+1-I)}}{(J+1-I)!D^{(I-1)}}\right) \tag{12}$$

In all cases considered in this study, a polynomial of degree 1 was sufficient to describe the evolution of the respective rate.

Continuous growth of *Halomonas* sp. EF11 on phenol at a C/N ratio of 17.6

To test the proposed method for evaluating changes induced by NaCl during productive degradation of harmful agents, *Halomonas* sp. EF11 growing on phenol provides an excellent model, because the strain is able to use phenol as a sole source of carbon and energy, and it maintains osmotic equilibrium by adjusting the intracellular level of just one major compatible solute, i.e., ectoine. Therefore, it is an ideal organism for thermodynamic analysis. In these studies, *Halomonas* sp. EF11 was first grown chemostatically in the presence of 3% NaCl, with a phenol input concentration of 4.7 g/l. The attainment of steady state at a dilution rate of 0.062/h was indicated by the constant rates of heat flow (−1.90 W/l), phenol consumption (3.08 mM/h), formation of ectoine (0.039 mM/h), and biomass synthesis [0.095 g/(l h)]. Calculations derived

using the Y_{ATP}-concept and assuming P/O = 2 predict heat flow rates of −1.77 W/l for *ortho* assimilation and −1.94 W/l for *meta* assimilation, accompanied with biomass formation rates of 0.221 g/(l h) and 0.195 g/(l h), respectively, under the experimental conditions. The first prediction is consistent with the experimental results, but the predicted biomass is twice as high as the amount found. Therefore, the calculated enthalpy balance, which took into consideration just phenol, biomass, ectoine, carbon dioxide, and water, suggests that the heat flow rate should have been much higher (−2.62 W/l) than that found empirically, indicating the occurrence of unexpected carbon fluxes.

Figure 2 shows possible side products and their dependence on the respective phenol assimilation pathways. The culture broth was screened for all of these compounds, and both formate and acetate formation were found, indicating that the phenol assimilation occurred via the *meta* pathway.

However, the activities of the key enzymes (Fig. 3c) revealed the potential for both pathways, and that the catechol 1,2-dioxygenase activity prevailed. In accordance with the postulated hypotheses, two transient growth phases can be distinguished from the evolution of the heat flow rate and the growth patterns observed during linear increases in the sodium chloride concentration from 3% to 21.8% (Fig. 3). As soon as the salt gradient started, the heat flow rate increased linearly, implying that less exothermic growth reactions were involved, until the NaCl concentration reached 14.3%. This phase may be called the adaptation phase because the ectoine content increased, but the ectoine-free biomass concentration decreased according to the previously postulated hypotheses. Phenol was not detectable in the calorimeter and neither the formate excretion rate (0.62 mM/h) nor the acetate formation rate (0.25 mM/h) were influenced by salinity. The best description of the concentration profile during this haloadaptation phase was obtained by assuming a linear enhancement of the ectoine formation rate (Eq. 13) and a linear reduction of the biomass formation rate (Eq. 14) (Fig. 3c).

$$r_E = \left(0.039 + 0.0030\, t\, h^{-1}\right) mM\, h^{-1} \quad (13)$$

$$r_X = \left(86.9 - 1.48\, t\, h^{-1}\right) mg/(L\, h) \quad (14)$$

At a certain salinity (14.3%, t = 30.5 h) the capacity of the cells to withstand further increases in salinity was exhausted. Consequently, the residual phenol concentration increased exponentially (data not shown) and the heat flow rate decreased rapidly. Therefore, this transient growth phase could be called wash-out. Further characteristic phenomena observed during the wash-out phase included rapid reductions in biomass and ectoine content. A minor amount of hydroxyectoine (0.2 mM) appeared during this phase (not shown in Fig. 3). Note, the maximum ectoine formation rate clearly coincided with a change in the slope in the heat flow rate.

Continuous growth of *Halomonas* sp. EF11 on phenol at a C/N ratio of 23.6

The results of chemostatic growth of *Halomonas* sp. EF11 on phenol at a higher C/N ratio during adaptation on a linear salt concentration gradient between 3% and 18% NaCl is shown in Fig. 4.

In general, the results obtained were similar to those from the experiments with the lower C/N ratio. The steady state at a dilution rate of 0.07/h (not shown) was characterized by constant rates of heat flow (−2.02 W/l), phenol consumption (4.69 mM/h), formation of ectoine (0.031 mM/h), and ectoine-free biomass synthesis [0.133 g/(l h)]. As in the experiment described above, there was a discrepancy between the heat flow rate

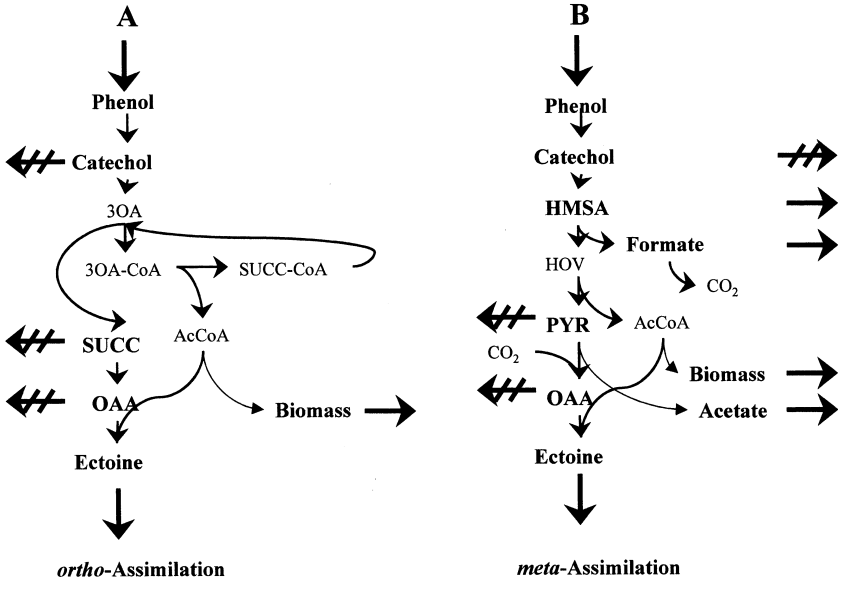

Fig. 2a, b Main substrate-carbon distribution patterns during growth on phenol via **a** *ortho* and **b** *meta* (with pyruvate carboxylation) assimilation pathways. *Thick arrows* indicate empirically detected mass transfer routes between the cells and their environment. *Crossed-out arrows* indicate undetectable mass transfer paths. *AcCoA* Acetyl coenzyme A, *HMSA* 2-hydroxymuconic acid semialdehyde, *HOV* 4-hydroxy-2-oxovalerate, *OA* 3-oxoadipate, *OAA* oxalacetate, *PYR* pyruvate, *SUCC* succinate

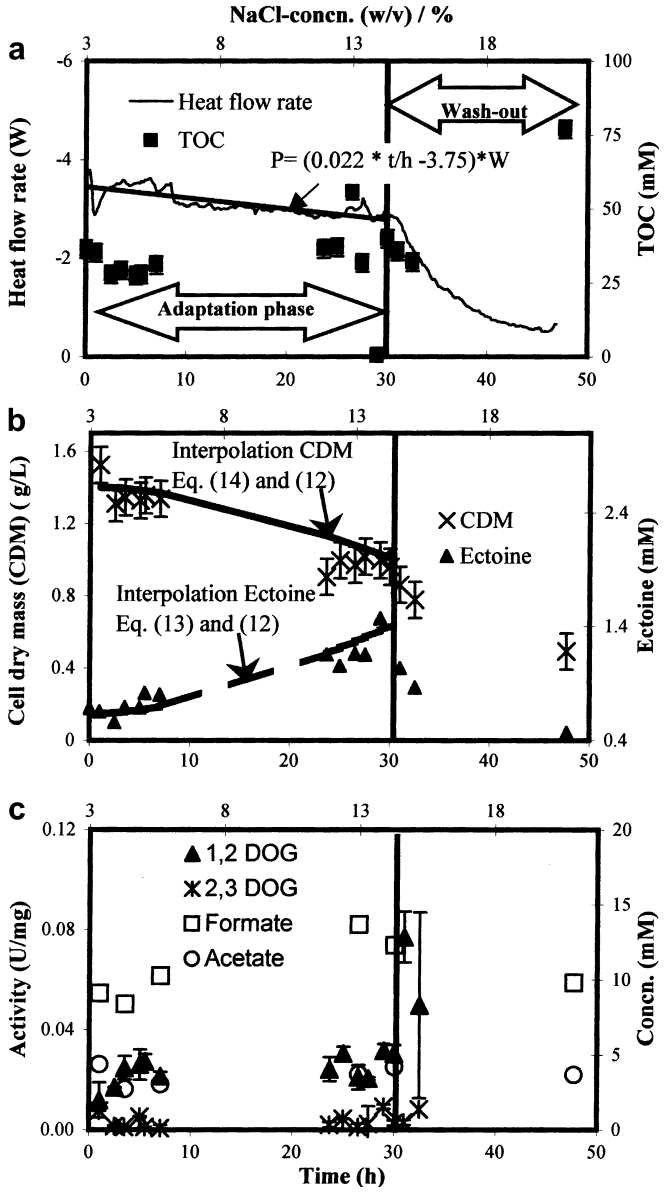

Fig. 3a–c Growth pattern during continuous growth of *Halomonas* sp. EF11 on phenol at a C/N ratio of 17.6 as a function of salinity. The strain was continuously grown at a dilution rate of 0.062/h on a phenol input concentration of 50 mM. *TOC* Total organic carbon, *DOG* dioxygenase

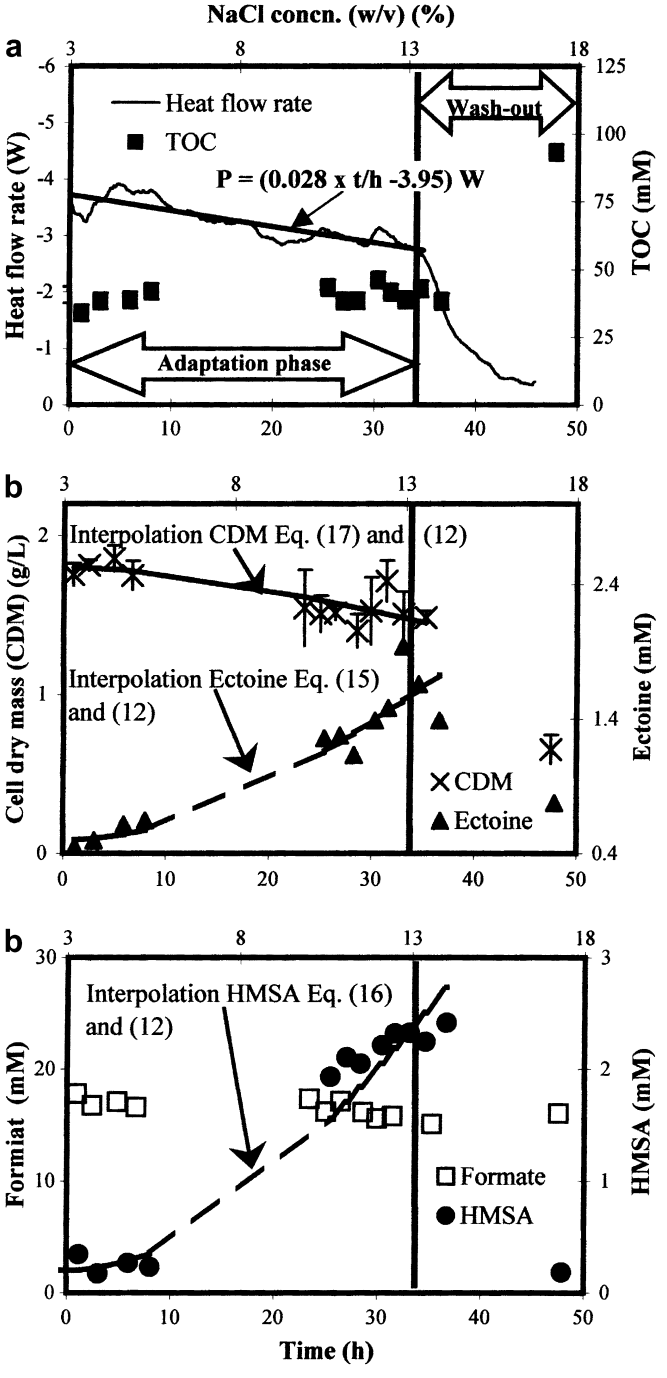

Fig. 4a–c Growth pattern during continuous growth of *Halomonas* sp. EF11 on phenol at a C/N ratio of 23.6 as a function of salinity. The strain was continuously grown at a dilution rate of 0.07/h on a phenol input concentration of 67 mM

estimated from the enthalpy balance (−3.55 W/l) and the experimentally measured rate, indicating once again the occurrence of unexpected carbon fluxes. The end-products of these carbon fluxes were found to be excreted formate and 2-hydroxymuconic acid semialdehyde. The side product spectrum is consistent with the solely measured activity of catechol 2,3-dioxygenase (the key enzyme of the *meta* assimilation pathway). Again, the concentration profile during the haloadaptation phase may be described by linear enhancement of ectoine (Eq. 15) and a linear reduction in the biomass formation rate (Eq. 16). Surprisingly, the adaptation process included here a sizable linear enhancement of the rate of formation of 2-hydroxymuconic acid semialdehyde (Eq. 17).

$$r_E = \left(0.035 + 0.0044\, t\, h^{-1}\right) mM\, h^{-1} \quad (15)$$

$$r_{HMSA} = \left(0.014 + 0.0088\, t\, h^{-1}\right) mM\, h^{-1} \quad (16)$$

$$r_X = \left(126 - 1.05\, t\, h^{-1}\right) mg/(L\, h) \quad (17)$$

The wash-out occurred at lower salinity (13.2%, t = 34.2 h) than with the lower C/N ratio, perhaps because of losses of carbon and energy due to 2-hydroxymuconic acid semialdehyde formation. Again, the maximum ectoine formation rate coincided with a change in the heat flow rate. If this finding reflects a general rule rather than an exception, as our knowledge of the function of compatible solutes implies, then calorimetry can be used to control, stabilize, and maximize the microbial conversion of toxic substrates into compatible solutes. The method ought to be independent of the strain and toxic substrate used. The successful examples presented here support this hypothesis. The heat control approach seems to be promising, despite the high price of calorimeters, for several reasons. Firstly, it does not require any sophisticated probes or programs. Secondly, more sensitive bench-scale calorimeters have been developed recently (Marison et al. 1998; Tietze 1998; Bou-Diab et al. 2001; García-Payo et al. 2002). Thirdly, large-scale bioreactors may be considered a type of calorimeter since the ratio of the heat-producing volume to the heat-exchanging surface increases and improves the sensitivity of heat measurements as the scale increases (von Stockar and Marison 1991; Anderson et al. 2002). Finally, cheap, miniaturized "nanocalorimeters" and integrated circuit calorimeters (Lerchner 1999) are being developed.

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